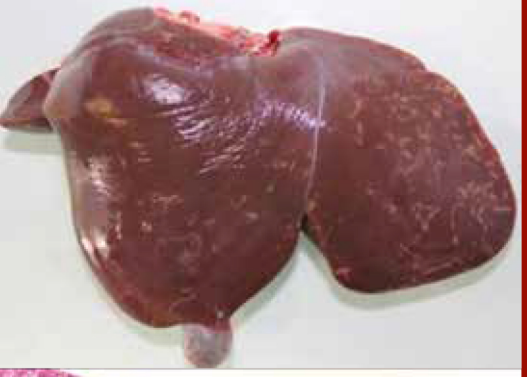

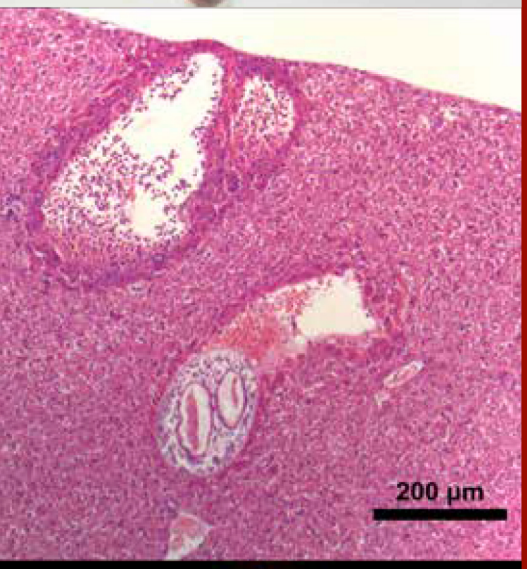

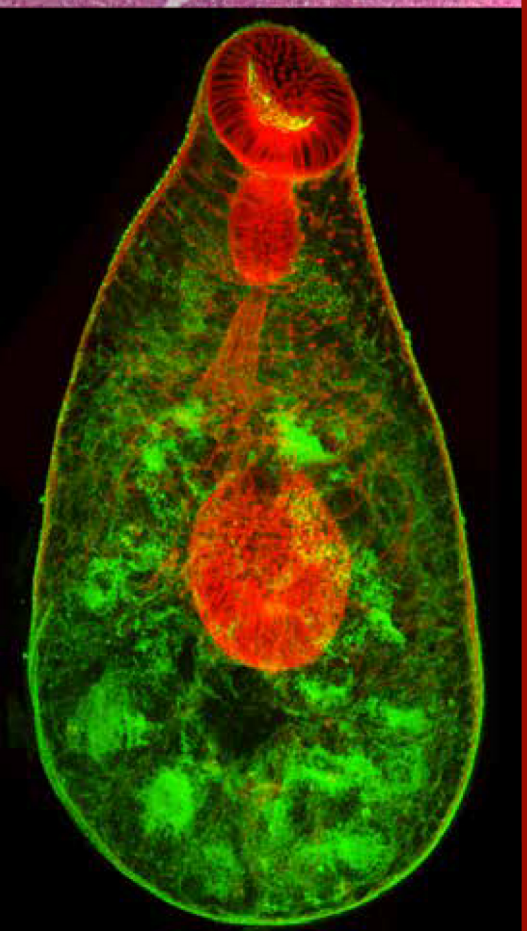

FASCIOLOSIS

2nd Edition

Edited by John Pius Dalton

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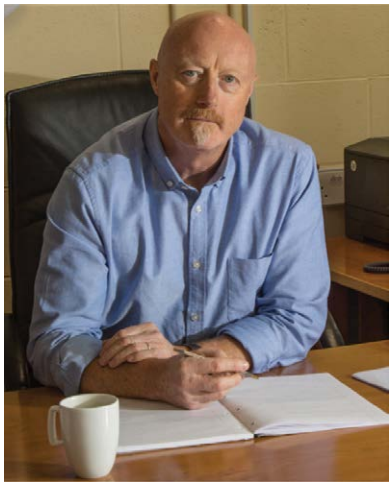
Preface



Over the past 22 years since the first edition of *Fasciolosis* was published, we have seen a tremendous acceleration in research output concerning the diseases caused by both *Fasciola hepatica* and *F. gigantica*. In keeping with advances in the general field of biology we have applied modern technologies to learn much more about liver fluke molecular biology, biochemistry, metabolism, immunology, immunopathology and genetics. In addition, we have developed a greater understanding of the global importance of liver fluke disease, its costs to the agricultural community, and its distribution and epidemiology. In many ways, fasciolosis research has come-of-age over the past two decades, particularly since the publication of draft genomes for both parasites and the availability of extensive '-omics' databases. With many other trematode/helminth genomes readily accessible, comparative genomic studies are providing broader insights about these parasites.

Fasciolosis is now fully recognized as a major globally important food-borne zoonotic disease. Both *F. hepatica* and *F. gigantica* are culprits for the concerning increase in human infections, and where they are sympatric their hybridization is resulting in the spread of parasites of unknown transmission capabilities and pathogenicity to both humans and their livestock.

We know very well the challenges we face to control infection, especially considering the widespread emergence of drug-resistant parasites and the prospects of changing disease dynamics due to climate change. On the other hand, we must be optimistic considering the advances that have been made in diagnostics and vaccine development, although the latter remains a Holy Grail.

In *Fasciolosis, 2nd Edition*, we have captured all these recent advances under one roof. A combination of prominent and emerging scientists in the field have collaborated to produce a comprehensive volume that is a treasure-trove of information for researchers and students alike. I felt it important not to discard the old for the new and, therefore, have retained important fundamental research spanning back to the first edition and further to ensure a seamless transition between the two volumes.

I hope readers will find how exciting research on Fasciolosis has been and can be. Indeed, maybe they will see the gaps that they may wish to fill themselves.

John P. Dalton

Acknowledgements

I wish to express my sincere thanks to all my colleagues in *Fasciolosis I* and *II* who have been a pleasure to compete and collaborate with over the past few decades. This book is a credit to their hard work, dedication and pursuit of excellence.

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Lastly, I'd like to thank my family, Georgina, Matthew and Fintan, for putting up with all my antics since *Fasciolosis I*!

1 The Discovery of *Fasciola hepatica* and its Life Cycle

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1.1 Introduction

Fasciolosis is a parasitic disease of humans and their livestock caused by digenean trematodes of the genus *Fasciola*, referred to as liver flukes. The two species most commonly implicated as the aetiological agents of fasciolosis are *F. hepatica* and *F. gigantica* (subfamily Fasciolinae, family Fasciolidae). *Fasciola* species are located within the Digenea subclass of the class Trematoda, phylum Platyhelminthes.

The Digenea are characterized by a complex life cycle involving one or more intermediate hosts. Many variations on the life cycle exist, but each typically includes a molluscan primary or intermediate host in which larval asexual multiplication occurs, and a vertebrate final or definitive host in which sexual reproduction occurs. Members of the family Fasciolidae are hermaphroditic and self-fertilization can occur, although preferential cross-fertilization is usual.

Fasciolosis has the widest longitudinal, latitudinal and altitudinal distribution of any helminth disease; it is present on every continent excluding Antarctica (Mas-Coma *et al.*, 2019). *F. hepatica* has a worldwide distribution but predominates in temperate zones, while *F. gigantica* is found on most continents but primarily in tropical regions, in particular Asia and Africa. However, where climatic conditions favour both parasites, they can coexist and hybridize. Recent animal trade has also influenced their distribution, especially in Asia and Africa (Mas-Coma *et al.*, 2005, 2019). The two species are believed to have diverged 5 million years ago close to the Miocene–Pliocene boundary (Choi *et al.*, 2020).

Fasciolosis is an economically important disease of domestic livestock, particularly cattle, sheep and water buffalo, and the cost of the disease to the global farming community is conservatively estimated at more than US$3 billion (Spithill *et al.*, 1999; Beesley *et al.*, 2018). The parasite also infects many wild animals, such as rabbits, hares, deer, coypu, rats, horses and camels, some of which can be regarded as important reservoir hosts. The potent immunomodulatory effects of *Fasciola* spp. on the host immune system may compromise the host's ability to resist other microbial infections, such as tuberculosis, salmonella and clostridiosis (Claridge *et al.*, 2012; Cwiklinski *et al.*, 2016), adding further impairment to animal health, welfare and productivity.

Since the 1990s, fasciolosis has emerged as an important food-borne disease of humans and a major public health issue in a growing number of countries. Estimates of human infections are still very approximate and range from 2.4 to 17 million people (Mas-Coma *et al.*, 2019), with 91–180 million people at risk of infection (Mas-Coma, 2005; Keiser and Utzinger, 2009).

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Countries where human infections are highest are Bolivia, Peru, Ecuador, Egypt, Iran, China, Vietnam and, less so, Portugal and Spain. Recently, the World Health Organization has classified fasciolosis as a 'Neglected Tropical Disease' (WHO, 2020).

The disease caused by *F. hepatica* and *F. gigantica* is known within the literature under various synonymous names, namely fasciolosis and fascioliasis. In keeping with the World Association for the Advancement of Veterinary Parasitology standardized nomenclature of parasitic diseases, the term fasciolosis will be used within this text (Kassai, 2006).

1.2 The Discovery of the Life Cycle

An overview of the historical discovery of the life cycle of *F. hepatica* is given below, together with the main biological factors relevant to the successful completion of the cycle. Further information and perspectives can be found in past literature, including Taylor (1937, 1964), Reinhard (1957), Jefferies and Dawes (1960), Pantelouris (1965), Dawes (1968), Smyth and Halton (1983), Andrews (1999) and Wilson (2020).

A list of the principal discoveries in the life cycle of *Fasciola* is shown in Table 1.1. The earliest description of fasciolosis in the literature is contained in a book entitled *Black Book of Chirk*, published circa 1200, in which reference is made to liver fluke in sheep (Froyd, 1969). Within this book, a reference is drawn from an earlier text that may describe fasciolosis in the Gwentian Code of Wales, describing the laws enacted by Howel the Good in the 10th century (Froyd, 1969).

The first observation of liver fluke was made by Jean de Brie in 1379. While he was preparing a treatise on wool production and sheep management for Charles V of France, de Brie made mention of the disease 'liver rot' in sheep, though he did not actually describe the appearance of the worm in this treatise (Huber, 1890). Unfortunately, the original account of his work has been lost and his observations are only known from various editions published between 1542 and 1594. Like many other early writers, de Brie did not associate the liver fluke with the disease 'liver rot', but thought that this was a consequence of the liver being affected by toxic substances produced by certain plants eaten by the sheep.

According to Cole (1944) another recognizable description of liver fluke appeared in a book entitled A newe Tracte or Treatyse moost profytable for all Husbandemen which was published in 1523 by Sir Anthony Fitzherbert. Twenty-six years later, in 1549, reference was

Table 1.1. Milestones in the discovery of the life cycle of *Fasciola hepatica*.

Person/year	Milestone
De Brie, 1379	First to observe the liver fluke *F. hepatica*
Redi, 1688	Disproved the theory of spontaneous generation
Swammerdam, 1737	First to see cercariae dissected from a snail (see Swammerdam, 1758)
Müller, 1773	Observed cercariae swimming in water
Zeder, 1803	Described a miracidium hatching from fluke egg
Nitzsch, 1807	Observed cercariae encysting
Bojanus, 1818	Described redia and development of cercariae
Steenstrup, 1842	Published theory of alternation of generations (see Steenstrup, 1845)
La Valette St George, 1855	Observed infection of a snail by a miracidium
Wagener, 1857	Observed penetration of a snail by miracidium and subsequent development of redia
Weinland, 1875	Suggested that the larval stages of the liver fluke occur in *Lymnaea truncatula*
Leuckart, Thomas, 1882	Confirmed *L. truncatula* as intermediate host and worked out life cycle of *F. hepatica*
Lutz, 1892; Lutz, 1893	Confirmed that herbivorous animals acquire adult fluke by ingestion of metacercariae
Sinitsin, 1914	Confirmed route of migration of *F. hepatica* to the liver

made in a book entitled *De lumbricis alvum occupantibus* to an observation made by an Italian physician by the name of Fanensi Gabucinus who described worms resembling pumpkin seeds in the blood vessels of the liver of sheep and goats. Additional observations on liver fluke disease were recorded during the second half of the 16th century, namely those of Conrad Gesner (1551) and Cornel Gemma (1575). Nevertheless, it was still commonly thought at the time that the feeding of particular plants to sheep was responsible for the disease.

It was not until 1688 that this theory was challenged by Francesco Redi, after whom the redia stage in the digenetic life cycle was named. He was a physician in Italy, who, by showing that parasites lay eggs, destroyed the false doctrine of spontaneous generation, i.e. the hypothetical process by which living organisms arise from inanimate matter. It is uncertain in which parasite this observation was first made, as Redi described a number of different types of worms from many kinds of animals, but he was the first to publish a picture of the liver fluke – a sketch from a specimen removed from the liver of a castrated ram.

The rejection of the theory of spontaneous generation was a significant breakthrough and was to stimulate a new wave of research. Prominent in this new research was Govert Bidloo, a professor of anatomy at The Hague and physician to William III. Bidloo (1698) observed worms in the bile ducts of sheep, stags and calves and recalled having seen similar worms in the livers of humans. This followed the observation by John Faber in 1670, who was the first to state that the liver fluke lives in the bile ducts, not in the blood vessels (Reinhard, 1957). Bidloo also observed eggs inside the living worm and thought that sheep probably became infected by swallowing the worms or their eggs and that the worms got to the liver in the blood rather than by passing via the small intestine. His results were reported in the form of a memoir to Antony van Leeuwenhoek, which stimulated Leeuwenhoek to investigate further. Leeuwenhoek thought that the worms lived in water and that sheep became ill by drinking this water, but he could not explain why he was unable to find such creatures in water samples taken from ditches in fields near the city of Delft. His observations were recorded in letters sent to the Royal Society that were subsequently published in the Philosophical Transactions (Leeuwenhoek, 1700, 1704).

It was not until the end of the 18th century that reference was made in the scientific literature to the intermediate stages of the life cycle. It was a chance observation by a Dutchman named Johann Swammerdam (1758) who, while dissecting a snail (*Paludina vivipara*) in order to examine its internal structure, saw living things that he thought were not of snail origin. Examination of his illustrations of these 'worms' clearly shows them to be the cercariae of some trematode. Later, in 1755, Frank Nicholls, a physician and prominent anatomist in England, presented a report to the Royal Society in which he remarked that the bile ducts in the livers of bullocks infected with 'liver rot' were blocked by 'a wall of stone' around the flukes – this was the first reference to calcification of the bile ducts and thus the earliest insight into the pathology of the disease.

The next contribution towards an understanding of the life cycle was made almost 20 years later by Otto Müller. In 1773, he wrote of finding microscopic tadpole-like creatures swimming in the water of ponds and called them cercariae, a generic name which he gave to all of these creatures characterized by having a tail. He mistakenly thought that these cercariae were Infusoria (a term applied to microscopic organisms, including various Protozoa and Rotifera, found in infusions of organic substances), as did many others who subsequently followed in his footsteps. Several different kinds of cercariae were observed and described, including those depicted by Johann Eichhorn (1781) and by Johann Hermann (1783). However, at this time the life cycle of flukes was still a mystery and no one even suspected that more than one animal host was required for its completion. The first person apparently to have an inkling was Peter Abildgaard of Copenhagen (1790). Ahead of his time, the results of his experiments (which provided evidence that the development of the tapeworm *Diphyllobothrium* sp., from larval to adult stage, required the larval stage to pass from a fish host to a bird) were soon forgotten and the idea that an immature form of a parasitic worm can require a different host from that of the adult had to wait another 60 years (Küchenmeister, 1852) before being accepted.

In 1803, Johann Zeder reported observing the hatching of eggs from a number of different species of trematode, although not of *F. hepatica*, and the subsequent escape of a ciliated embryo (miracidium) into the water. After Zeder's observation, Christian Nitzsch (1807) followed with the first account of cercariae encysting. He had been watching some cercariae swimming in water and had noticed that after a while they attached themselves to a substrate, then lost their tails prior to becoming covered by a gelatinous substance. Having lost all means of movement he assumed that he had witnessed the cercariae dying. Nitzsch continued working with cercariae and nine years later he had described several new cercariae (in Dawes, 1968). In doing so, he noticed the similarity between the anterior end of a cercaria and a distome fluke (Nitzsch, 1817), although he still thought that cercariae were different from flukes and from all other known forms of pond life.

During this period, despite such advances, the only thing that was definitely known about the life history of the liver fluke, or any other fluke, was that a ciliated larval form emerged from the eggs. Although many observations were recorded on other stages of the cycle, such as cercariae and rediae, a connection between them remained elusive. The next observation of relevance was made by Ludwig Bojanus who, in 1818, unaware of Swammerdam's work published in 1737 (see Swammerdam, 1758), rediscovered the redial stage of a trematode. Unlike Swammerdam, who failed to grasp the significance of his finding, Bojanus noted the resemblance between rediae, cercariae and adult flukes. By observing the birth of cercariae from the rediae, Bojanus put forward the idea that there may well be a connection between cercariae and flukes. However, like Swammerdam, Bojanus did not work on the snail *Lymnaea truncatula* and therefore did not observe the larval stages of *F. hepatica*. Despite these publications, the consensus at this time remained that cercariae were independent forms of life.

E. Mehlis, a German medical practitioner, made the next significant contribution in 1831 by describing the hatching of 'ciliated embryos' from the eggs of trematodes, although it was Friedrich Creplin (1837), six years later, who observed such forms (miracidia) hatching from the eggs of *F. hepatica*. Not only did Mehlis observe the hatching of miracidia but he also noted their energetic swimming in water and suggested that this behaviour might be associated with the need to find something that would enable them to develop to a stage that could eventually infect the final host. Such a theory went against the more popularly accepted theory that the final host became infected by the ingestion of the egg stage. However, in 1852 the latter theory was finally disproved by Professor James Simonds of the Royal Veterinary College, London. Simonds (1880) reported that he failed to find a single fluke or any signs of liver disease in an experimental sheep infected six months earlier with thousands of fluke eggs.

By the middle of the 19th century most of the individual parts of the life history of many species of trematode had been noted. Johannes Steenstrup was the first person to start to bring together the various pieces of the story. In 1842, he published his work *On the Alternation of Generations* in Danish; the same year saw the publication of the German edition, which was translated into English (Steenstrup, 1845). He fitted the theory to various forms of life, including trematodes; most of his work on trematodes described echinostome and stylet cercariae with only a brief mention of *F. hepatica*. However, the term 'alternation of generations' was not novel, having been previously used to describe the life cycle of tunicates (benthic invertebrates) in 1819 by the Franco-German poet and naturalist, Louis Charles Adelaide de Chamisso (in Jefferies and Dawes, 1960).

Carl von Siebold (1854) provided more evidence to support the theory. He found rudimentary sex organs in some encysted cercariae and suggested that it was likely that this stage, which occurred in invertebrates, was the infective stage for vertebrates, in which the sexually mature fluke occurred. The first suggestion of a definite connection between a specific cercaria and an adult fluke was probably made by Rudolph Leuckart in 1852 (in Taylor, 1937). He observed the similarity between a certain fluke found in the intestine of a predacious fish and of encapsulated cercariae found in the gills of its prey. Adolphus von La Valette St George (1855) demonstrated, by feeding experiments, that certain encysted cercariae from water snails developed into sexually mature flukes in birds, and that cercariae which had not encysted were not infectious.

Further pieces of the jigsaw were put in place by Guido Wagener (1857) who observed the penetration of miracidia into snails and the subsequent development of rediae. German helminthologist David Weinland (1875), according to Reinhard (1957), was the first person to suspect that larval stages of liver fluke occurred in *Lymnaea truncatula*. In 1875, Weinland found 'cercaria sacs' in the digestive gland of *L. truncatula* snails and also noted that cercariae showed a strong inclination to leave water and climb on to foreign objects. He conjectured that cercariae encysted on grass in order to be eaten by sheep and that these cercariae were in fact young liver flukes. Twenty years later *L. truncatula* was confirmed as the common intermediate host of *F. hepatica* (other species of snail may also be infected, see for example Boray, 1969). The discovery was made independently by Algeron Thomas (1881, 1882a,b, 1883a,b) in the UK and by Leuckart (1881, 1882) in Germany. However, it was Thomas who established the right to be acknowledged as the first person to make the discovery, details of which were first published in Nature (Thomas, 1882b). The work of Thomas and Leuckart was summarized by Reinhard (1957).

Despite the work of Thomas and Leuckart, certain parts of the life history were still uncertain and required experimental evidence. For example, proof was still required that herbivores acquired the parasite by swallowing metacercariae. The discovery of the exact migration route by which young flukes reached the liver of the final host was also still to be elucidated. Experimental data confirming the first issue were generated by Adolpho Lutz (1892, 1893) who successfully infected guinea pigs, a rabbit, a goat and a brown rat by adding metacercariae to their food. However, according to Joseph Alicata (1938), the species of liver fluke with which Lutz was working was *F. gigantica* rather than *F. hepatica*. The final piece of the jigsaw was added by the Russian helminthologist Dimitry Sinitsin in 1914. Sinitsin proved that young flukes in the rabbit, after liberation from their cysts in the small intestine, penetrated the wall of the gut and migrated to the liver via the peritoneal cavity. This observation was supported by subsequent investigations by Shirai (1927), Susuki (1931), Shaw (1932), Schumacher (1939) and Krull and Jackson (1943).

1.3 The Life Cycle

The life cycle of *F. hepatica* consists of five phases as shown in Fig. 1.1. They are: (i) passage of eggs from the host to the outside environment and their subsequent development; (ii) hatching of miracidia, their search for and penetration of the intermediate snail host, usually *Galba (Lymnaea) truncatula*; (iii) development and multiplication of the parasites inside the snail; (iv) emergence of the cercariae from the snails and their encystment; and (v) ingestion of infective metacercariae by the final hosts and development to adult worms.

1.3.1 Development and survival of the fluke egg

Liver fluke eggs are passed from the common bile duct into the duodenum and subsequently are passed with the faeces. The eggs consist of a fertilized ovum surrounded by a large number of yolk granules. They are yellowish brown in colour, oval in shape, 130–145 μm long by 70–90 μm wide and have an indistinct operculum (Fig. 1.2A–C). The eggs that are passed out in the faeces on to pasture are undeveloped and, therefore, undergo embryonation outside the host.

Although partial development of the egg can occur while still inside moist or wet faeces, complete development and hatching will only occur after the egg has been liberated from the faeces, a requirement that is normally facilitated by factors such as the action of heavy rain, the deposition of faeces in water and the trampling action of animals. The inhibitory effect of faeces is likely to be caused by a number of factors, for example competition for oxygen by micro-organisms or the presence of toxic substances. Eggs, however, can remain viable in faeces from 3 weeks to several months, according to various conditions and the time of the year. Egg viability persists for a longer period during the winter than in the summer months and tends to decrease with increasing dryness (Rowcliffe and Ollerenshaw, 1960). Several physico-chemical factors, especially temperature, humidity and oxygen tension, are known to influence embryonation.

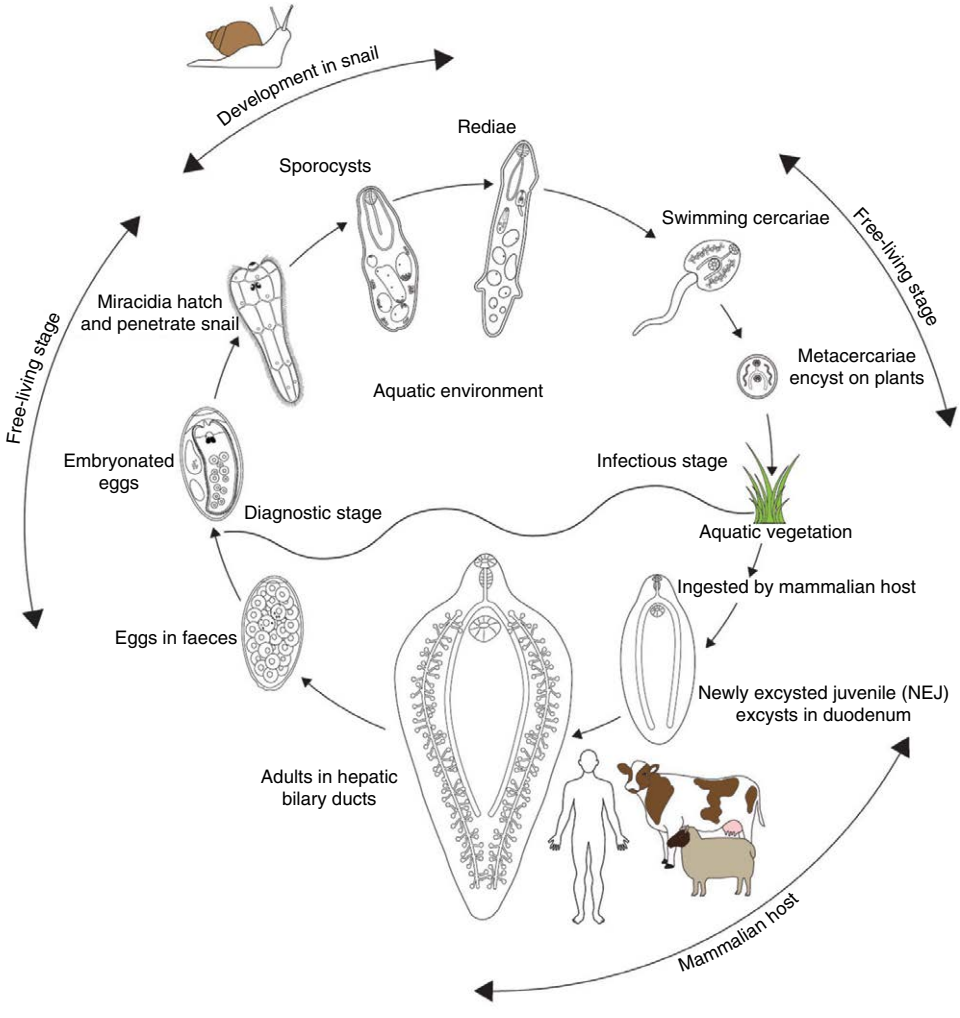


Fig. 1.1. The life cycle of *Fasciola hepatica*. (Drawn by Fiveprime Design.)

1.3.1.1 Temperature

A temperature of at least 10°C is necessary for embryonation (Ross and McKay, 1929). Under laboratory conditions, the rate of development of the egg increases with temperature within the range 10–30°C. Thus, at 10°C development of the egg takes about 6 months but at 30°C it is completed in 8 days. Above 30°C, development is increasingly inhibited and at 37°C it does not occur at all. Mortality increases the longer the eggs remain at 37°C, with 100% mortality being reached after about 24 days (Rowcliffe and Ollerenshaw, 1960).

At temperatures below 5°C, development of the egg stops but can be resumed if the temperature is increased to 13°C. Eggs refrigerated (2–10°C) for 2.5 years remain viable, although undeveloped, and, after being kept at room temperature for 18 days following refrigeration, they will hatch over a period of 14 days (Krull, 1934). Similar findings were recorded by Boray (1969) who kept eggs of *F. hepatica* in the refrigerator at 4°C for at least 2 years that, after

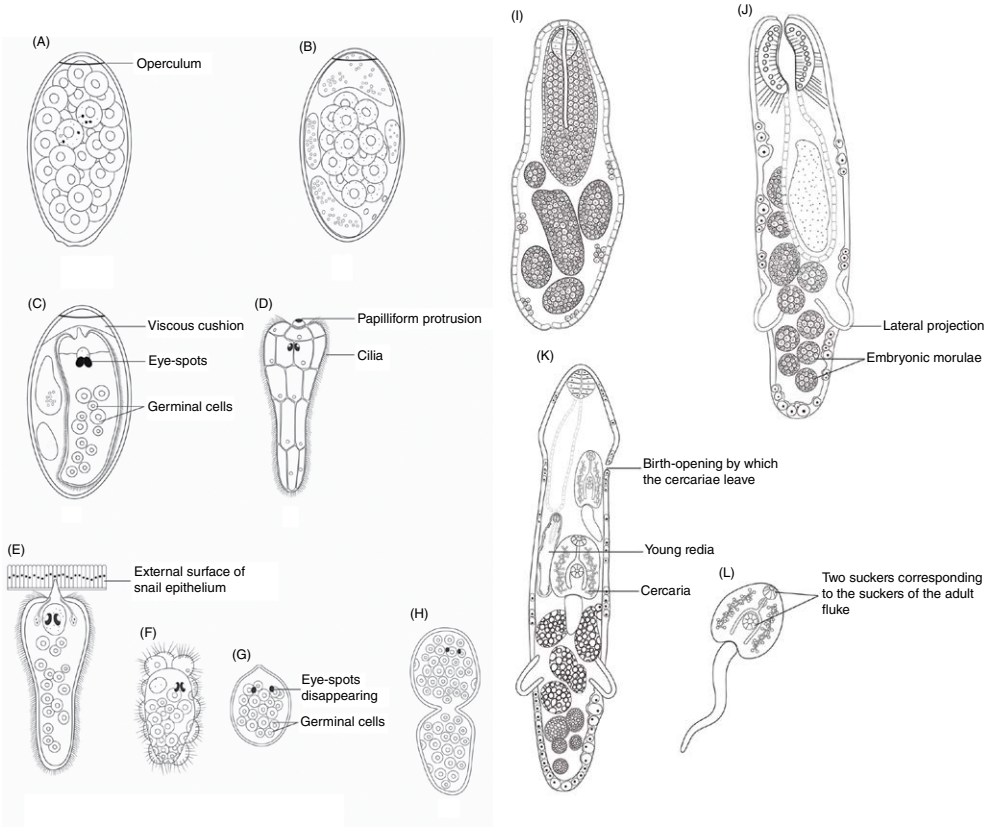


Fig. 1.2. Stages in the life cycle of *Fasciola hepatica*. (A) Undeveloped egg – note operculum (cap) and difference between embryonic cells (small mass in upper central part of egg) and yolk-bearing cells. (B) Morula – embryonic cells have grown at the expense of the yolk cells. (C) Fully developed egg ready to hatch – miracidium – note cilia and eye-spots. (D) A free-swimming miracidium, external view. (E) A miracidium penetrating a snail, internal view. (F) After penetrating the snail, the miracidium loses its cilia and becomes a sporocyst. (G) Sporocyst. (H) The sporocyst dividing. (I) The sporocyst forming redia (form with sucker and primitive gut). (J) A more mature redia – note other embryonic morulae in the body cavity. The two lateral projections are characteristic of this stage. (K) A fully mature redia showing developing redia and cercariae (the forms with the tail). (L) The cercaria; the free-swimming form which encysts on vegetation. (Drawn by Fiveprime Design.)

incubation, hatched miracidia that were infective to snails (*Galba tomentosa*).

1.3.1.2 Moisture

Water is required for embryonation. Maintenance of a surface film of moisture around the egg for at least 3 weeks is essential. Eggs on soil will develop without the presence of free surface water, provided that the soil is saturated (Ollerenshaw, 1959). Eggs in moist faeces can survive for at least 10 weeks in the summer and 6 months in the winter in the UK. However, if the faecal mass dries out, there is rapid mortality of the eggs (Ollerenshaw, 1971).

1.3.1.3 Oxygen tension and pH

Eggs do not develop in a concentrated faecal suspension, although eggs will survive twice as long in aerobic conditions compared with anaerobic conditions. Eggs kept in cultures without faeces show little variation in mortality, but those in aerobic conditions hatch in one-fifth of the time taken for those at a lower oxygen tension (Rowcliffe and Ollerenshaw, 1960).

Eggs incubated at 27°C will develop and hatch within a pH range of 4.2–9.0, but development is prolonged above pH 8.0 (Rowcliffe and Ollerenshaw, 1960). The optimum pH for embryonation appears to be 7.0 (Al-Habbib, 1974).

1.3.2 Hatching of the miracidium from the egg and penetration of the intermediate snail host

1.3.2.1 Hatching of the egg

As originally noted by Thomas (1883a,b), fully embryonated eggs exposed to the same conditions do not always hatch together, with hatching occurring on successive days for some weeks, even months. Such a strategy is obviously of practical importance to the parasite but renders a suitable habitat dangerous for grazing animals for a long period of time. It is generally accepted that light and temperature affect the hatching of eggs of *F. hepatica*. Experiments carried out by Roberts (1950) showed that eggs of *F. hepatica* incubated for 14 days in the dark hatched only on exposure to light. It is common laboratory practice to induce mass hatching of miracidia by placing incubated eggs in strong light. A method for hatching eggs for teaching and research purposes is shown in Fig. 1.3.

1. Add eggs to a 12-well plate to give approx. 50 eggs/well then add sterile water to a total volume of 1 ml (all wells in duplicate).
2. Incubate eggs for 14 days at 26°C, wrapped in foil.
3. For EMBRYONATION ASSESSMENT:
    a. Expose plate to bright light for 30 min at room temperature.
    b. Using an inverted microscope assess egg embryonation.
    c. To assess egg hatch follow hatch protocol from (4b) onwards.
4. For EGG HATCH ASSESSMENT:
    a. On day 14 put plate at 4°C overnight.
    b. Day 15 remove plate from fridge and incubate at 26°C for 30 min, followed by 1 h incubation at room temperature under a bright light.
    c. Using an inverted microscope assess egg embryonation.

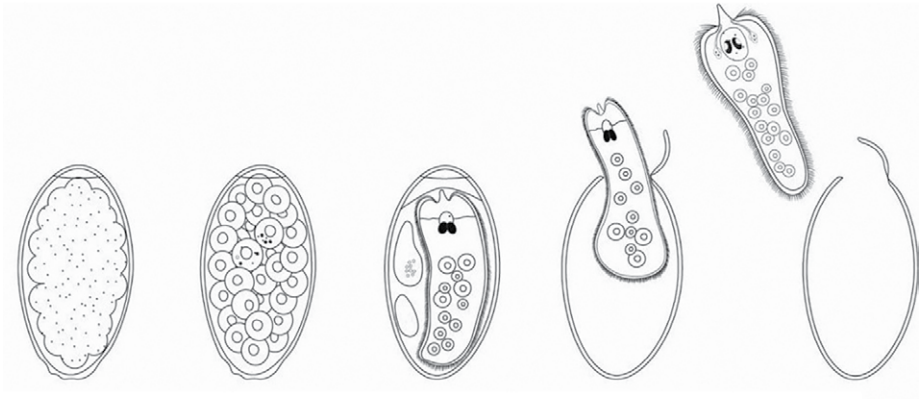


Fig. 1.3. *Fasciola hepatica* egg hatch assay and schematic detailing the stages of egg development. (Schematic drawn by Fiveprime Design.)

1.3.2.2 Survival of miracidia

The miracidium is about 130 μm in length, broad anteriorly and tapering posteriorly to a blunt end. The cuticle is ciliated, and there is an anterior papilliform protrusion and a pair of darkly staining eye-spots visible near the anterior end of the body (Fig. 1.2D,E). Once hatched from the egg the miracidium becomes active, immediately starting to swim at great speed (on average, 1 mm/s; Wilson and Denison, 1970). Characteristic swimming movements have been used as a means of determining the infectivity of miracidia. Those swimming in circles are not usually infective. Boray (1969) suggested that speed of movement of the miracidia after hatching has also been found to be a good indicator of their infectivity; those taking about 30 s to move 1 cm do not infect snails, while those requiring 4–12 s to travel 1 cm are generally infective.

The need to find a suitable host to penetrate is an urgent one, as miracidia failing to do so generally die within 24 h (Thomas, 1883a,b; Hope Cawdery *et al.*, 1978), at a rate which is age dependent (Smith and Grenfell, 1984). Such age-dependent mortality is a common feature of short-lived, non-feeding larval stages in the Digenea subclass and is associated with the depletion of finite energy reserves (Anderson *et al.*, 1982). It has been shown, using histochemical techniques, that glycogen levels in the miracidia of *F. hepatica* decline with age (Wagner, 1965). Although miracidia are capable of covering long distances during their short lifetime (over 50 m at temperatures of 10–15°C), in practice they are often confined to small bodies of water just a few centimetres across, most of which do not contain snails (Smith, 1978).

There appears to be no evidence that miracidial mortality varies with pH of the medium, at least in the range 6–8 (Smith and Grenfell, 1984). On the other hand, these authors showed that mortality does vary both with the temperature of the medium and with the age of the miracidia. The mean expected lifespan of miracidia decreases from about 36 h at 6°C to about 6 h at 25°C. At 10°C the mean lifespan is about 1 day (Al-Habbib, 1974). However, in his early reports Thomas (1883b) kept miracidia alive for 3 days in a slightly alkaline solution of peptone.

1.3.2.3 Location and penetration of the snail host

The behaviour patterns of miracidia have been extensively studied, especially with regard to their phototactic, thermotactic, geotactic and chemotactic responses. Three main areas have received special attention: (i) the extent to which a miracidium is 'attracted' towards a snail; (ii) whether such responses (if they occur) are specific for a particular species of snail; and (iii) whether the general responses of a miracidium are merely responsible for bringing a miracidium into an ecological niche similar to that of the snail so that contact is readily established (Smyth and Halton, 1983; Haas and Haberl, 1997).

The sequence by which a miracidium finds its snail host can be divided into three main steps (Wright, 1959; Ulmer, 1971; Saladin, 1979; Christensen, 1980): (i) host habitat selection – the newly emerged miracidium makes its way to the environment of its specific snail host; (ii) a period of random movement in the host habitat; and (iii) host finding – the miracidium orientates towards the host using tactic or kinetic mechanisms. The miracidium of *F. hepatica* is strongly phototropic, which is generally believed to be an adaptation for locating the snail host.

The intermediate snail hosts are amphibious. In Europe and some parts of Asia, the intermediate snail host is usually *Galba truncatula*, though elsewhere several other species are implicated (see Chapter 2). Their habitat is typically along the edge of small ponds, ditches and marshy land in areas that are subject to alternate flooding and desiccation. The ability of the miracidium to move towards the light ensures that it will not waste vital energy reserves by exploring the bottom of ponds where the snails are not to be found. An interesting comparison can be made with the miracidium of *F. gigantica*, which infects different species of snail. Studies on the infection of snails show this species of *Fasciola* to be less host-specific than *F. hepatica* (Kendall, 1954; Boray, 1966). In Africa, where *F. gigantica* primarily infects *Lymnaea natalensis* (a species that lives in deeper water), the miracidium is actively repelled by light and therefore dives further down into the water, where the snail is more likely to be found (Taylor, 1964).

The presence of a wide range of potential stimulant molecules in the mucus of snails is

well documented (see for example, Wilson, 1968b; Wilson and Denison, 1970; Wilson *et al.*, 1971). Concentrations of glucose, 16 amino acids and various lipids have been identified from the mucus of *G. truncatula* (Wilson, 1968). Short-chain fatty acids C7–C9 (> 0.1 mM) will stimulate miracidia of *F. hepatica* to attach to *G. truncatula* but such treatment appears to have a detrimental effect on the miracidia after about 10 min; chain lengths of between C6 and C9 (> 0.01 mM) stimulate a turning response by the miracidium (Wilson *et al.*, 1971). Experiments by Nansen *et al.* (1976) and Christensen *et al.* (1976b) confirmed the existence of a special chemical attraction of miracidia of *F. hepatica* not only towards *G. truncatula* but also towards other snail species, which is not affected by an existing infection. These chemoattractant molecules allow the *Fasciola* spp. to distinguish between different snail species, enhancing host-finding of susceptible snails (Bargues *et al.*, 1997; Kalbe *et al.*, 1997). Recent studies have shown that glycoproteins/glycoconjugates within the snail mucus play a role in the attraction and attachment of *Fasciola* miracidia with their snail host (Kalbe *et al.*, 2000; Haas, 2003; Georgieva *et al.*, 2019). These glycoproteins also play a role for the stages within the snail, as they are used in a host-mimicry fashion to protect the parasite stages from immune recognition (Georgieva *et al.*, 2012, 2014, 2016).

Although light is known to stimulate the hatching process, and the miracidium is positively phototropic, light does not appear to be a stimulus for the infectivity of the miracidium (Christensen, 1975). Infectivity of the miracidium is independent of pH (in the range 5.4–8.4), but dependent on water temperature. At or below 5°C, the miracidium is not infective, regardless of the exposure time. Optimum temperature is in the range of 15–26°C and a clear inverse relationship has been demonstrated between the environmental temperature and the duration of the host-finding capacity of the miracidia. At 8°C, 16°C and 24°C the host-finding capacity ceased after 24–30, 20–24 and 13–20 h, respectively (Christensen *et al.*, 1976a).

Penetration of the miracidium of *F. hepatica* into *G. truncatula*, and its transformation into a sporocyst, has been described by a number of works, including, at the light microscope level, by Thomas (1883a,b), Faust (1920), Eales (1930), Mattes (1949), Roberts (1950), Dawes (1959), Jefferies and Dawes (1960), and Southgate (1970); and at the transmission and scanning electron microscope level, by Wilson *et al.* (1971), Blankespoor and van der Schalie (1976), Køie *et al.* (1976) and Coil (1977). The penetration process involves a mechanical boring action by the miracidial anterior papilla and is also likely to be facilitated by the secretion of proteolytic enzymes (Smyth and Halton, 1983). Tissue at the point of penetration, generally near the branchial aperture, is observed to be degraded (Wilson *et al.*, 1971).

1.3.3 Development and multiplication inside the snail

One of the most favourable factors contributing to the successful completion of the life cycle, and thus the propagation of the liver fluke, is parthenogenetic multiplication within the snail. It was first demonstrated by Krull (1941) that a snail infected with a single miracidium can produce about 4000 metacercariae. Similar experiments performed by Hodgkinson *et al.* (2018) revealed a range of about 500–3200 metacercariae recovered following single snail/miracidia infections for six different *F. hepatica* isolates. Molecular analysis of the resulting metacercariae of the six isolates using microsatellite markers confirmed that genetic clonal expansion occurs within the snail host (Hodgkinson *et al.*, 2018).

Although a number of snail species can act as intermediate host for *Fasciola* spp. (Váquez *et al.*, 2018) (see also Chapter 2), the snail species can impact the dynamics of *Fasciola*–snail infection, with varying numbers of rediae and metacercariae being produced. For example, *Pseudosuccinea columella*, an important vector for fasciolosis in South America, has been shown to generate larger numbers of *F. hepatica* metacercariae when compared with *G. truncatula* (Dar *et al.*, 2014; Vignoles *et al.*, 2015). For details of some of the factors affecting the development of the fluke within the snail, see for example Faust (1920), Ross (1930), Rees (1931), Schumacher (1939), Kendall (1949, 1953), Kendall and McCullough (1951), Kendall and Parfitt (1959), Boray (1963, 1966, 1967a,b), Kendall and

Ollerenshaw (1963), Hodasi (1972), Rondelaud and Barthe (1987), and Graczyk and Fried (1999).

Once inside the snail, the young sporocyst (Fig. 1.2F) migrates via the blood vessels or lymph channels primarily to the digestive gland (often referred to as the liver), which is situated in the upper spirals of the shell. Here the sporocyst (Fig. 1.2G,H) begins to grow. Consisting initially of a minute ball of tightly packed germinal cells in which remnants of the eyespots can be seen, each germinal cell gives rise to a ball of new germinal cells from which the next larval stages, the rediae, develop. Ultimately, the sporocyst, distended by rediae (Fig. 1.2I), ruptures, liberating the rediae into the digestive gland. The rediae move about more actively than the parent sporocysts and cause considerable damage to this gland.

The redia is roughly cylindrical in shape and possesses a pair of marginal lappets (bulging projections) at the posterior end and a raised collar-like structure just behind the anterior end of the body (Fig. 1.2J,K). There is a mouth which leads into a muscular pharynx and posteriorly to a simple unbranched intestine. The body of the redia contains numerous germinal cells which, like those in the sporocyst, multiply to form germinal balls from which the final larval stage, the cercaria, is produced. Between 16 and 20 of these germinal balls are produced within each redia. Under adverse conditions, such as high or low temperatures or drought, two redial generations may occur from which the cercariae arise but the reasons for this to occur are not clearly understood. This unusual further multiplication phase was first observed by Thomas (1883a,b) (see also Fig. 1.2K). However, comparable to *F. gigantica*-infected snails where five redial generations have been observed (Dinnik and Dinnik, 1956, 1964), up to four generations of *F. hepatica* may arise during snail infections (Rondelaud *et al.*, 2009). The differences between *F. gigantica* and *F. hepatica* result in a larger number of rediae being generated for *F. gigantica* in comparison with *F. hepatica* (Dreyfuss and Rondelaud, 1995) (see Chapter 2).

The mature rediae measure 1–3 mm in length and are capable of considerable movement. Their migrations can cause serious damage and, in heavy infections, death of the snail. Normally, however, the snail shows a remarkable power of regeneration. When the cercariae are fully developed, they escape from the redia by way of the birth pore, which is situated laterally behind the anterior collar. Snails larger than 5 mm are more likely to shed cercariae than smaller snails (Olsen, 1944) (see also Chapter 2). Consistent with other parts of the *Fasciola* spp. life cycle, temperature plays a role in the maturation and development of the stages within the snail. Experiments by Dinnik and Dinnik (1964) revealed that *F. gigantica* redial development to cercariae did not occur at temperatures of less than 16°C but could be reverted when the temperatures rose to 20°C.

The cercariae are tadpole-like with a discoidal body and a long tail. The body measures 250–350 μm and the tail is twice as long. An oral sucker and a ventral sucker are in the centre of the body (as in the adult fluke). Leading from the oral sucker there is a pharynx, on either side of which are very conspicuous cystogenous glands, an oesophagus and a forked intestine (Fig. 1.2L). The mobile cercariae generally leave the snail 4–7 weeks after infection by migrating through the tissues. Different larval stages may coexist in a single snail (Agersborg, 1924). It follows, therefore, that the cercariae do not mature at the same time and, as demonstrated by Faust and Hoffman (1934), leave the snail over a period of time.

1.3.4 Emergence of cercariae from snails and their encystment

For a short time after they have emerged from the snail the cercariae swim freely in the water. They are very active and frequently change direction, both horizontally and vertically, although tending to keep near to the surface rather than going down into deeper water. The process of encystment and the structure of the cyst wall are complex and have been described by Wright (1927), Stirewalt (1963), Dixon and Mercer (1964), Dixon (1965), Køie *et al.* (1977) and Smyth and Halton (1983). Alicata (1938) described the structure of the cyst wall of *F. gigantica*.

1.3.4.1 Encystment

During a few minutes to 2 h after emergence, the cercaria settles on various objects, including blades of grass, and attaches by means of the

ventral sucker. Encystment may also take place upon the surface of the water (Wright, 1927) (see Chapter 2). Once attached, the body contracts inwards, releasing the outer layer of the cyst. Simultaneously, as the embryonic 'epithelium' is shed and the outer layer is laid down, the tail separates from the body. The tail is sometimes shaken off before the encystation begins but, as a rule, the tail remains in connection with the body during the process (Thomas, 1883a,b). The cyst is white when laid and is almost immediately infective to the definitive host. After a day or two the cyst gradually becomes yellow in colour, due to the presence of quinine, and darkens as it hardens.

1.3.4.2 Structure of the metacercarial cyst

The structure of the cyst wall consists of an outer cyst and an inner cyst. The outer cyst is composed of an external layer of tanned protein and an underlying fibrous layer of mucoprotein. The inner cyst has a complex mucopolysaccharide layer subdivided into three, and an additional layer (layer IV) of laminated or keratinized protein. A region of layer IV is specialized to form the ventral plug. The outer cyst wall probably acts as a barrier against bacterial and fungal infections and is also important for attachment to the substrate, normally grass (Dixon, 1965). Strong adhesion to grass for long periods is important for the survival of metacercariae and the infection of the final host. As the cysts may survive for long periods and remain infective if the outer wall is removed, the inner cyst walls must play a more important part in the survival of the metacercariae (Boray, 1963).

1.3.4.3 Longevity of metacercariae

Metacercariae may survive for more than 1 year on pasture (Soulsby, 1965). Survival is mainly dependent on sufficient moisture and moderate temperatures. Metacercariae are resistant to freezing between −2°C and −10°C but lose their infectivity at −20°C (Boray and Enigk, 1964). They can retain their infectivity after being maintained at −2°C for 8 weeks (Taylor, 1949) and may survive for as long as 11 months at temperatures varying from −3°C to 2°C if the average temperature remains above freezing (Shaw, 1932). Approximately 50% of metacercariae encysting on pasture in September can survive winter conditions in the UK to infect animals in the spring (Ollerenshaw, 1967). Survival rates and subsequent viability for parasites in silage and hay are less well known, given the varying practices of ensiling and storage as recently reviewed by John *et al.* (2019). Temperature of stored silage, bacterial proliferation during the fermentation processes, presence of oxygen and moisture content of stored hay all play a role in the viability of metacercariae present (John *et al.*, 2019).

Metacercariae produced by *L. tomentosa* can survive for 6 months at temperatures of 12–14°C, with 25% surviving for 8 months. When stored at between 2°C and 5°C, only 10% will survive for 1 year; others will survive for 8 weeks at 20°C, but none will survive for more than 6 weeks at 25°C (Boray, 1963). Under natural conditions in the USA, it has been shown that metacercariae are destroyed by heat and drought during the four summer months (Olsen, 1947). Similarly, metacercariae will not survive a typical Australian summer (Boray and Enigk, 1964). The infectivity of metacercariae depends not only on various climatic conditions but also on the temperature during their development through the larval stages in the snail (Davtyan, 1956; Boray, 1963).

1.3.5 Ingestion of infective metacercariae

Within an hour of ingestion by the definitive host, metacercariae begin to excyst in the small intestine. Within 2 h following infection these have bored through the wall of the intestine and can be found in the abdominal cavity en route to the liver.

1.3.5.1 Excystation

The process of excystation is likely to involve extrinsic factors (such as elevated temperatures, reducing conditions, pH, pCO_2 and the presence of bile salts) and intrinsic factors such as secretions by the fluke. There are basically two phases of excystation: a passive activation

phase followed by an active emergence phase. These have been described by Dixon (1966) and Smith and Clegg (1981) and reviewed by Sukhdeo and Mettrick (1987). Activation is believed to occur in the stomach or rumen and is a prerequisite to emergence. Conditions that stimulate activation *in vitro* are high pCO_2, temperature about 39°C and reducing conditions. During activation, the metacercariae rotate vigorously for a while before the quiescent phase (Dixon, 1966), during which time the predominant activity appears to be the emptying of their caecal contents (Sukhdeo and Mettrick, 1986). During excystment the parasites secrete cathepsin L and B-like cysteine proteinases which likely assist their emergence, as inhibition of these enzymes prevents this event (Robinson *et al.*, 2009; Cwiklinski *et al.*, 2018).

Excystation occurs in the small intestine of the host below the opening of the *ductus choledochus* or common bile duct. However, Hughes (1959) and Dawes (1961) showed that young flukes can emerge from cysts injected into the peritoneal cavity of hosts, suggesting that, apart from a temperature of about 39°C and possibly the presence of a low concentration of carbon dioxide, excystation may require very little additional stimulus. However, the emergence phase is probably triggered by bile and its presence may activate an enzyme secreted by the parasite, inducing muscular movements of the young fluke (Dixon, 1966). A method for the *in vitro* excystment of metacercariae for teaching and research purposes is shown in Fig. 1.4.

1.3.5.2 Migration to the liver

Details of the process of penetration of the intestinal wall, movement through the abdominal cavity and penetration of the liver have been described in great detail by Schumacher (1956), Dawes (1961, 1962, 1963), Dawes and Hughes (1964), Boray (1969) and Fairweather *et al.* (1999) and will be covered in more depth in Chapter 3.

After the metacercariae have excysted in the small intestine, the newly excysted juvenile flukes rapidly penetrate the intestinal mucosa, in doing so breaking down epithelial cells, connective tissue and unstriped muscle fibres, and move into the peritoneal cavity. Once in the peritoneal cavity, the flukes apparently browse on whatever tissue is available, occasionally penetrating organs, including the local lymph nodes. The liver appears to be reached by random wanderings, although evidence suggests that newly excysted juveniles may migrate towards the liver in response to some stimulus, an orthokinesis (Sukhdeo and Mettrick, 1987). Once through the liver Glisson's capsule (a process that typically takes place between 4 and 6 days after infection), the young flukes burrow through the liver for between 5 and 6 weeks, causing extensive haemorrhage and fibrosis. Significant growth of the fluke occurs during this period (see Chapter 3).

There is evidence that young flukes prefer to feed on hepatic cells rather than blood, although some blood is inevitably ingested. The flukes eventually reach the bile ducts, beginning about 7 weeks after infection, in which they grow to adults and become permanently established. Occasionally, immature flukes may be found in unusual sites, e.g. lungs, pancreas, lymph nodes and thymus, and some may infect the fetus in pregnant animals. From 8 weeks after infection, eggs are found in the bile, and later in the faeces, thus completing the life cycle.

Some adult flukes may live for a considerable time in the liver (Fig. 1.5 illustrates the adult fluke). For instance, flukes 11 years of age have been recorded in sheep by Durbin (1952), each producing up to 20,000 eggs per day. It is noteworthy that fertile eggs can be obtained from an animal infected with a solitary fluke (Hughes, 1959). Recent genomics, transcriptomics and proteomic studies have shed light on the molecular changes that take place when the parasites migrate through the host tissues and have identified the major molecules (proteinases, proteinase-inhibitors and antioxidants) that they secrete to affect this migration (Robinson *et al.*, 2009; Cwiklinski *et al.*, 2015, 2018; Zhang *et al.*, 2019).

Finally, many methods and procedures for the study of the biology and molecular biology of various stages of *F. hepatica* can be found in the book *Fasciola hepatica*: *Methods and Protocols*, edited by Cancela and Maggioli (2020).

Removing the outer cyst wall

1. Fill the lid of a Petri dish with 2% sodium hypochlorite solution. Scrape the encysted metacercariae from the visking tubing into the sodium hypochlorite solution.
2. Remove the outer cyst wall with agitation by pipetting the metacercariae up and down in the glass watch glass using a 1 ml pipette for no more than 10 min.
3. Once the outer walls have been removed, transfer the cleaned metacercariae to a watch glass with distilled water. Wash the metacercariae in distilled water by sedimentation several times to remove all traces of sodium hypochlorite.
4. Do not vortex or centrifuge the metacercariae as the parasites will burst out of the cysts.
5. The metacercariae can be stored at 4°C in water until required.

Excystment protocol

1. Prepare the excystment media solutions in separate tubes.
   a. 5 ml of a pre-made stock of 0.9% NaCl and 1.2% $NaHCO_3$ (for 100 ml: 0.9 g NaCl + 1.2 g $NaHCO_3$ in 100 ml dH_2O/RO) + 40 mg sodium tauroglycocholate
   b. 5 ml of N/20 HCl (4.75 ml of dH_2O/RO + 0.25 ml 1N HCl of pre-made stock; 1N solution – 50 ml: 4.9 ml 37% HCl + 45.1 ml H_2O) + 40 mg L-cysteine
2. Warm the excystment solutions at 37°C.
3. Transfer the metacercariae to a glass watch. Remove as much of the water as possible.
4. Mix the excystment media solutions together by inversion. The solution should effervesce when combined, but remain a translucent yellow (with bubbles on top). If the media does not effervesce or turns cloudy the metacercariae will not excyst (likely that too much HCl has been added).
5. Add the excystment media immediately to the watch glass, to completely cover the cysts. Place another watch glass on top and place the watch glasses into a humidity box, to prevent the excystment solution evaporating.
6. Incubate at 37°C for no more than 3 h. At approximately 1 h 15 min check for movement of NEJ inside the cysts and any parasites that have emerged. Check at 10/15 min intervals for excystment.
7. Once the NEJ emerge, collect using a 10 µl pipette and place in another watch glass with pre-warmed RPMI media.
8. Wash the recovered NEJ with RPMI media to remove traces of excystment media.

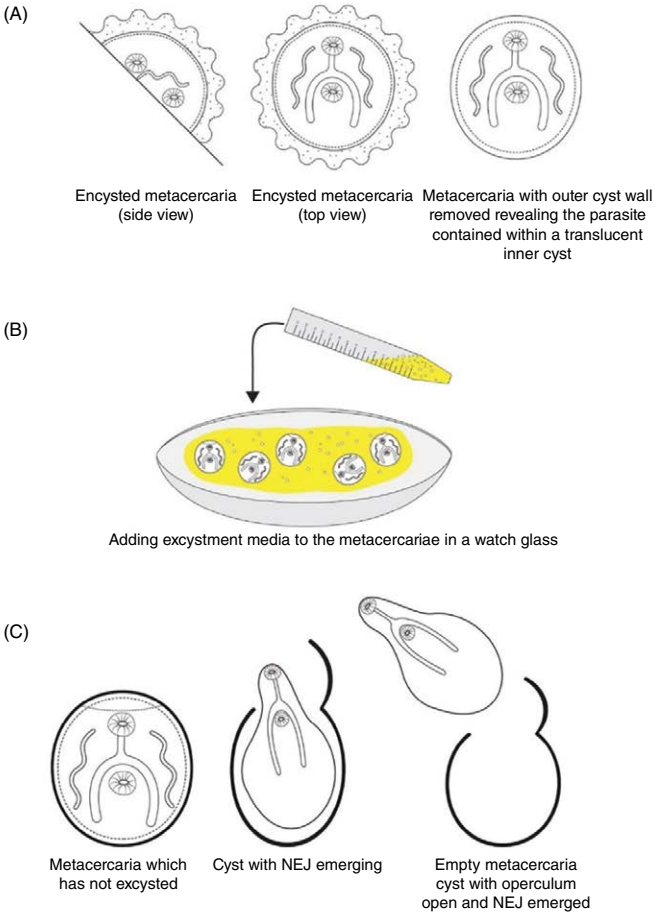
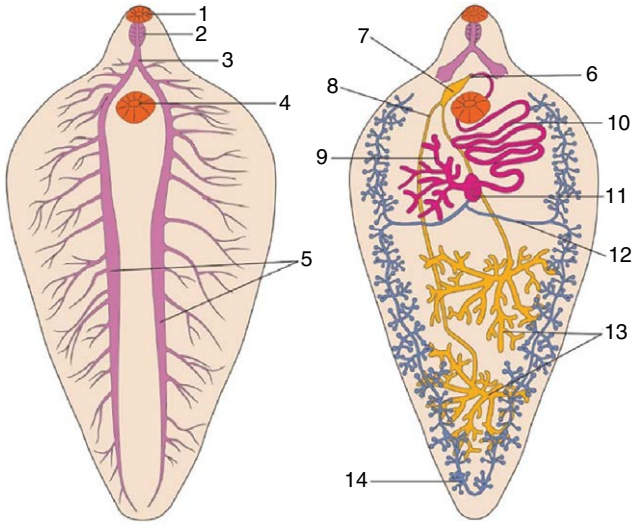


Fig. 1.4. *In vitro* excystment of *Fasciola hepatica* metacercariae and schematic detailing the stages of the *in vitro* excystment protocol. (A) Metacercaria with and without the outer cyst wall. (B) The addition of the effervescent excystment media to the metacercariae within a watch glass. (C) The excystment process with the NEJ emerging from the metacercaria cyst. (Schematic drawn by Fiveprime Design.)



Internal structures of *Fasciola hepatica*

1. Oral sucker
2. Pharynx
3. Oesophagus
4. Ventral sucker
5. Caeca
6. Genital pore
7. Cirrus sac
8. Vas deferens
9. Ovary
10. Uterus
11. Ootype
12. Vitelline duct
13. Testes
14. Vitelline gland

Fig. 1.5. Diagram of an adult *Fasciola hepatica* showing major structures. (Drawn by Fiveprime Design.)

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2 *Fasciola hepatica* Larval Development Within the Intermediate Host

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2.1 Introduction

The life cycle of *Fasciola hepatica* requires the intervention of a snail host that ensures the development of larval forms of the parasite. This role of intermediate host is generally ensured by a pulmonate gastropod of the family Lymnaeidae. Within its intermediate host, three larval stages, i.e. sporocyst, redia and cercaria, follow one another after the penetration of the miracidium. These cercariae finally exit from the snail and usually transform into metacercariae, which cause a new infection in the definitive host if they are ingested with wild plants (most often watercress). The role of the intermediate host is therefore essential in the transmission of fasciolosis in a given area.

The development of larval forms of the parasite in this snail depends on a number of environmental and biotic factors. Although most of them are known, the actions of several factors remain unknown and only the study of natural or experimental infections of the snail with *F. hepatica* can determine them. The quantity of cercariae provided during shedding depends largely on the action of these factors. The growth of these larval forms is not without danger for the snail, because it induces the development of tissue lesions in several viscera in the form of epithelial necrosis, followed by reconstitution of this tissue.

Sections 2.2 and 2.3 of this chapter focus on host snail species and dynamics of larval forms in the snail. Sections 2.4 to 2.6 deal with the characteristics of parasitic infection in naturally or experimentally infected snails, or in co-infected snails. The consequences of parasitism for the host snail are reviewed in section 2.7.

2.2 Snail Hosts of *Fasciola hepatica*

As the nomenclature in the Lymnaeidae family is still very controversial, the species whose names have changed are indicated here by their old name, followed by the current name in parentheses. The manual used for European species is that of Welter-Schultes (2012). For other species, we use the names referenced in the IUCN Red List of Threatened Species.

2.2.1 Snail species known to be intermediate hosts

At least 20 species of Lymnaeidae have been reported as potential intermediate hosts in the life cycle of *F. hepatica* (Torgerson and Claxton, 1999; Correa *et al.*, 2010). This number is an underestimation, because several species are

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able to ensure complete larval development of the parasite when subjected to experimental infections. In Europe, *Galba truncatula* is considered the main host snail of *F. hepatica* (Taylor, 1965). Four other species have been reported at a regional level: *Galba occulta* (= *Catascopia occulta*; = *Stagnicola terebra*) in Poland (Czapski, 1962, 1977), *Omphiscola glabra* (= *Lymnaea glabra*) in France (Abrous *et al.*, 1998) and *Radix balthica* and *R. labiata* in Belgium and Luxembourg (Caron *et al.*, 2007, 2014). Most other lymnaeid species living in Europe can be successfully infected under laboratory conditions when their juveniles (shell height < 2 mm) are exposed to miracidia of *F. hepatica* (Boray, 1978). Another example is South America, where 12 species of Lymnaeidae, sometimes very close morphologically, have been reported as natural intermediate hosts (Correa *et al.*, 2011). The problem posed by this family is the great diversity that Lymnaeidae present in the morphology of their shells, which is related to their important eco-phenotypic plasticity (Samadi *et al.*, 2000; Hurtrez-Boussès *et al.*, 2005). The use of molecular markers is currently required to identify the various species of this family, particularly in the genera *Galba*, *Radix* and *Stagnicola* (Bargues *et al.*, 2001, 2003, 2011). These markers have been largely used to clarify the taxonomic status of closely related taxa (cryptic species) within the genus *Galba*, particularly in Africa (Mahulu *et al.*, 2019) and South America (Correa *et al.*, 2011; Standley *et al.*, 2013; Pujadas *et al.*, 2015; Alda *et al.*, 2019).

As a result, the taxonomic structure of the Lymnaeidae is rather complicated and still very controversial, with several subfamilies, genera and subgenera within it (Vinarski, 2013).

Species belonging to families other than Lymnaeidae have also been reported as intermediate hosts of the parasite. A natural infection of *Bulinus truncatus* with *F. hepatica* has been reported in northern Tunisia (Hamed *et al.*, 2009), confirming the positive results of experimental infections that Barthe *et al.* (1986) obtained with this species. Successful infection of *Planorbis leucostoma* (= *Anisus leucostoma*) has also been noted in the laboratory when this snail is co-infected with *F. hepatica* and another digenean, *Paramphistomum daubneyi* (= *Calicophoron daubneyi*) (Abrous *et al.*, 1998).

Using molecular markers, *F. hepatica* DNA was detected in two field-collected succineids, *Omalonyx matheroni* in Ireland (Relf *et al.*, 2009) and *Succinea putris* in Sweden (Novobilsky *et al.*, 2014). Similarly, parasite DNA was found in *Lymnaea palustris* (= *Stagnicola palustris*) in Sweden (Novobilsky *et al.*, 2013). However, these studies do not indicate whether these species can ensure the complete development of *F. hepatica* or if the DNA comes from larval forms that would have degenerated after penetration of the miracidium into the snail.

2.2.2 Susceptibility of lymnaeid species to *Fasciola hepatica*

Snail species do not all have the same susceptibility to *F. hepatica* miracidia. Four groups of species were defined by Boray (1978) according to the results of numerous experimental infections that this author has carried out. The first group includes species with low mortality during experimental infection, high infection rate and high cercarial production. This group includes *Galba truncatula*, regardless of the *F. hepatica* strain, and other species such as *Galba bulimoides*, *G. cubensis* or *G. viatrix* when the parasite strain is adapted to the snail. In the second group, the characteristics of the infection show a relative disparity with low or high mortality, prevalence and cercarial production. Several species, such as *Pseudosuccinea columella* or *Radix viridis*, belong to this group. The third group of species shows resistance to parasite infection related to snail age. Only juveniles less than 2 mm in height when exposed to miracidia can ensure complete parasite development, but mortality is high while prevalence and cercarial production are low. This group includes, for example, *Lymnaea stagnalis*, *Omphiscola glabra* and *Stagnicola palustris*. The fourth group shows complete parasite resistance, with non-penetration of miracidia or with limited development to the sporocyst stage if miracidial penetration occurs. This group includes *Austropeplea lessoni* and some populations of *Radix auricularia*.

The classification of species according to Boray's criteria is more complex, because interpopulation and intrapopulation variability in susceptibility to parasite infection occurs within each snail species, as in *G. truncatula* (Rondelaud, 1993; Vignoles *et al.*, 2002a).

2.3 Dynamics of Larval Forms Within the Snail

2.3.1 Miracidial penetration and outcome of the sporocyst

Penetration of the miracidium into the host snail has been described by many authors at the level of the light microscope and of the transmission and scanning electron microscope (see reviews by Andrews, 1999; Graczyk and Fried, 1999). This larva is attracted by short-chain fatty acids present in the snail mucus (Haas and Haberl, 1997). Macromolecular glycoconjugates in snail mucus have been reported as substances capable of stimulating *F. hepatica* miracidia to penetrate (Haas and Haberl, 1997; Kalbe *et al.*, 1997, 2000). The epithelial cells of the snail are first lysed by the proteolytic secretions of the apical and unilateral glands of the miracidium (Buzzell, 1983), while the apical papilla of this larva attaches to the snail as a sucker. As the miracidium penetrates the snail, its ciliated epithelial cells are shed in sequence from anterior to posterior. Contraction of the miracidium is followed by destruction of the underlying connective tissue of the snail and its body penetrates the snail (Saint-Guillain, 1968; Wilson *et al.*, 1971).

After penetration, the miracidium turns into a sporocyst. The digestive system, the nervous ganglia and the eye-spots of the larva disappear, while the germinal cells actively multiply to form rounded morulae at the origin of the future rediae. A fibrous basal lamella and an underlying connective muscular layer cover the body of the sporocyst. This larva migrates into the snail to a favourable site for its development and then secretes a hyaluronidase that acts on the snail tissues (Wilson *et al.*, 1971). At the same time, it grows and forms an elongated bag up to 700 μm in length. Inside the sporocyst, rediae differentiate and exit alternately. Their germinal cells actively multiply to form morulae that will produce other rediae or cercariae (Thomas, 1883a,b).

During its migration into the host snail, the sporocyst leaves empty tunnels (Fig. 2.1) and it is easy to follow the movements of this larva on histological sections of the host snail. These tunnels have been observed in *F. hepatica*-exposed snails belonging to three lymnaeids (*G. truncatula*, *Omphiscola glabra*, *Radix balthica*), two physids (*Aplexa hypnorum*, *Physella acuta*) and two planorbids (*Anisus rotundatus*, *Bulinus truncatus*), regardless of the snail's stage of life and the type of infection (active or abortive). On the other hand, they have never been observed in uninfected controls (Préveraud-Sindou *et al.*, 1989). Tunnels were present in 55–95% of mantles, 10–60% of tentacles and 10–40% of feet of infected snails. They have also been found in the internal organs of snails (Préveraud-Sindou *et al.*, 1990). Their distribution was larger in *G. truncatula* than in the other two lymnaeids studied. Similarly, the density of these tunnels was lower in the physids and planorbids than in the lymnaeids (Préveraud-Sindou and Rondelaud, 1995). The location of tunnels and that of the sporocyst are also related to the mode used by the miracidium to enter the snail. When penetration occurred through the snail's mantle, the tunnels were found mainly in the mantle and kidney of adult lymnaeids, whereas they extended to the foot in juveniles of the seven species and in many adult physids. The sporocysts preferentially localized in the zone surrounding the kidney and the heart in lymnaeids from the fourth day after exposure, while they disappeared in *Aplexa* and *Physella* snails from the third day. When penetration occurred through the foot or the tentacles, the tunnels were found in the foot and extended inconstantly to the mantle, according to the snail species involved. Sporocysts have been found in the foot, mantle, or in the zone surrounding the kidney and heart (Préveraud-Sindou *et al.*, 1994).

2.3.2 Redial generations of *Fasciola hepatica*

Among digenean species that have a redia stage in their life cycle, the pattern of two redial generations developing one after the other within the intermediate host is frequent. After their emergence from the sporocyst, the mother rediae constitute the first generation and produce the daughter rediae of the second generation. According to parasite species, the mother rediae produce either daughter rediae, or daughter rediae and cercariae, while daughter rediae form cercariae. In the case of the *Fasciola* genus, the

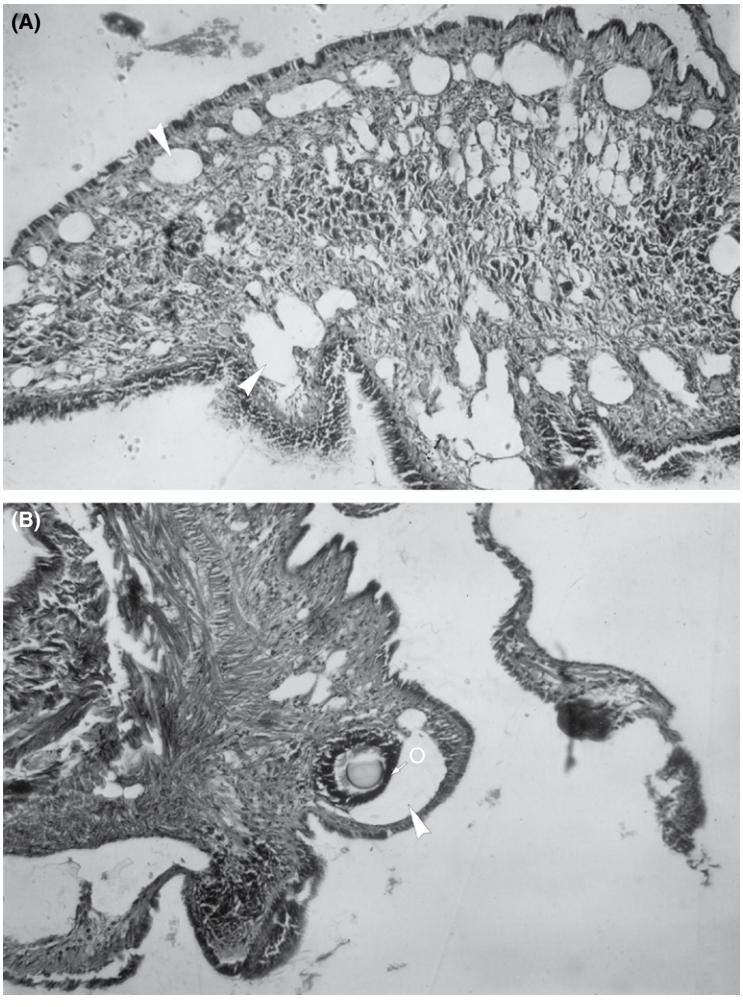

Fig. 2.1. Tunnels resulting from the migration of *Fasciola hepatica* sporocysts in *Galba truncatula*: general view of tunnels (arrows) in the foot of the snail **(A)** and in a tentacle (arrow) near the eye **(B)**. Magnification: **(A)** ×150; **(B)** ×580. From Préveraud-Sindou (1991).

existence of two redial generations is still open to question. Indeed, in the case of *Fasciola gigantica*, up to five redial generations can develop within the snail host (Dinnik and Dinnik, 1956, 1964). In contrast, the life cycle of *F. hepatica* is more complex, as it comprises either a single redial generation (mother rediae), or two generations with the second generation (daughter rediae) only produced when snails are under adverse conditions, e.g. summer months (Thomas, 1883a,b), winter (Leuckart, 1879–1886), cold (Kendall, 1964, 1965) or drought (Styczynska-Jurewicz, 1965; Wilson and Draskau, 1976). In spite of this contradiction (Andrews, 1999), the concept of two redial generations in the life cycle of *F. hepatica* is still widely accepted by parasitologists. In reality, the life cycle of *F. hepatica* is closely similar to that of *F. gigantica*, as up to four generations of *F. hepatica* rediae may succeed within the intermediate host.

2.3.2.1 Identification of redial generations

According to Thomas (1883a,b) and Kendall (1965), mother rediae cannot be distinguished morphologically from daughter rediae. However,

the redial generations can be identified according to the form and structure of their pharynx (Rondelaud and Barthe, 1978a,b). Using serial sections of infected snails, these authors described three types of pharynxes (Fig. 2.2). The first is that of first-generation rediae where the pharynx is ovoid in young rediae and progressively becomes spherical in older larvae after day 35 post-exposure (pe) at 20°C. Like the pharynx, the lumen is ovoid or spherical and its width grows proportionately with increasing length of the body (Fig. 2.2A). The second type comprises the first cohort of second-generation rediae (Fig. 2.2B). The pharynx is smaller and ovoid and its lumen is clearly larger in the pre-intestinal zone than in the other zone. The third type is not distinct from a given redial generation, as it is observed in the second cohort of the second generation and in subsequent generations. The pharynx is small and ovoid and its long lumen is very narrow (Fig. 2.2C).

At day 49 pe at 20°C, the length of the pharynx and the width could reach 105 µm and 100 µm, respectively, for the first redia of the first generation, while the width of the lumen does not exceed 60 µm. The averages are lower for the other first-generation rediae, because they do not exceed 70 µm for the length of the pharynx and for the width, and 35 µm for the width of the lumen. This decrease also affects the first cohort of the second generation, because the values do not exceed 65 µm, 50 µm and 12 µm, respectively. The lowest values were those of the second cohort of the second generation and subsequent generations: not more than 35 µm for pharyngeal length, 25 µm for pharyngeal width and 4 µm for width of the lumen (Augot *et al.*, 1998, 1999). These values allow the first redia of the first generation to be distinguished from the other redial categories, even when this larva is old. The determination of generations is not easy when the rediae are alive, because of continuous movements of their pharynx under the microscope. If these larvae are fixed in 10% (v/v) formalin, i.e. 4% (w/v) formaldehyde, the pharynx

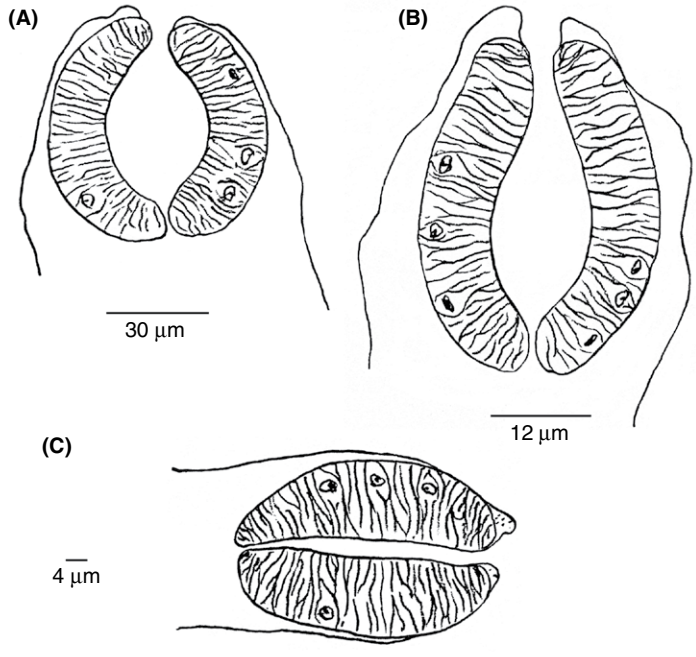

Fig. 2.2. Schematic drawings of pharynxes belonging to **(A)** the first redial generation of *Fasciola hepatica*, **(B)** the first cohort of the second generation and **(C)** the other cohorts and generations. From: Rondelaud, Belfaiza, Vignoles, Moncef and Dreyfuss (2009a): Redial generations of *Fasciola hepatica*: a review. *Journal of Helminthology*, 83, 245–254 (Fig. 1, page 247). RightsLink Printable License no. 4666941365625. With permission from Cambridge University Press.

walls are often distorted, thus making redial identification difficult.

The other structures of the redia do not allow differentiation of the different generations from each other. Each redia has a body with a roughly cylindrical shape, a raised collar-like structure just behind the anterior end of the body and a pair of bulging projections in the posterior end (Andrews, 1999). It contains an unbranched intestine and germ cells that actively multiply to form morulae at the origin of pro-redial embryos or pro-cercarial embryos. Apart from the first redia of the first generation which only produces daughter rediae, each of the other rediae forms one or two daughter rediae before the production of cercariae. Two indexes allow differentiation of the rediae of *F. hepatica* from those of *F. gigantic* (Dar *et al.*, 2003):

1. The distance from the anterior end of the body to the collar/body length was an effective way of distinguishing the rediae of *F. hepatica* from those of *F. gigantica*: second-appearing mother rediae (R1b) of the first generation, 0.14 instead of 0.22; daughter rediae (R2a) produced by the first mother redia, 0.19 instead of 0.24.
2. The distance between the anterior end of the body and the collar/collar diameter could also be used to distinguish these rediae: R1b, 0.80 for *F. hepatica* instead of 1.09 for *F. gigantica*; R2a, 0.90 instead of 1.26, respectively.

2.3.2.2 *Developmental patterns of redial generations*

The pattern depends on the behaviour of the first redia of the first generation after its exit from the sporocyst. If this larva remains alive throughout snail infection, at least up to day 49 pe, the development is normal. In contrast, if the first redia dies during week 2 or week 3 of snail infection, the development is abnormal and two variants have been described (Rondelaud and Barthe, 1982b; Belfaiza *et al.*, 2005).

Figure 2.3 shows the four successive generations in the case of normal development. The numbers of live rediae for each generation and the dates of exit for the first rediae are also indicated. Two points are important. The first is the rapid emergence of the first mother redia from the sporocyst on day 7 pe (at 20°C), its rapid growth within the snail, and its production of only daughter rediae. The second point is the time of this production. Indeed, the daughter rediae produced by the first mother redia become free during the same period as the other mother rediae that emerge later from the sporocyst. As a consequence, the free rediae, other than the first mother redia, observed between day 15 and day 35 pe, are composed of two different cohorts, the first belonging to the first generation and the other to the second generation. A similar process occurs for subsequent generations after day 35 and after day 63 pe. The result of all this is a useful redial burden ranging from 28 to 43 live rediae at day 80 pe in snails originating from different populations of *G. truncatula* living on acidic soils (the shell height of adults is often less than 8 mm). However, for the highest shell heights (up to 12 mm) of *G. truncatula*, the burden may rise up to an average of 56 live rediae, essentially by a numerical increase of third- and fourth-generation rediae (Rondelaud and Barthe, 1987a; Belfaiza *et al.*, 2004b). When the first redia of the first generation dies, daughter redia production is ensured by another free redia, either the second mother redia of the first generation (first variant of abnormal development), or the first daughter redia of the first cohort of the second generation (second variant). However, the number of daughter rediae produced by this substitute redia is lower (only 2–6) and occurs later over time (the first free rediae emerge after day 30 pe at 20°C). As a result, the average burden of free and living rediae in *G. truncatula* at day 80 pe ranges from 12 to 25 larvae in the first variant and from 12 to 23 in the second variant (Rondelaud *et al.*, 2009a). In single-miracidium infections of *G. truncatula*, the first developmental variant was seen in about 20% of infected snails, whereas the second variant was very scarce: less than 0.1% (Rondelaud and Barthe, 1982b).

In snail species such as *Austropeplea tomentosa* and *Pseudosuccinea columella*, which showed a rapid increase in the height of their shells during the experiment (up to 12.6 mm for *A. tomentosa* and 15.6 mm for the other lymnaeid), the formation of rediae belonging to the first cohort of the second generation (23–26 rediae) was ensured by two mother rediae, i.e. the first two rediae that emerged from the sporocyst (Rondelaud and Barthe, 1987b). In *P. columella* (4 mm high) when exposed to miracidia, the number of mother rediae producing the second generation

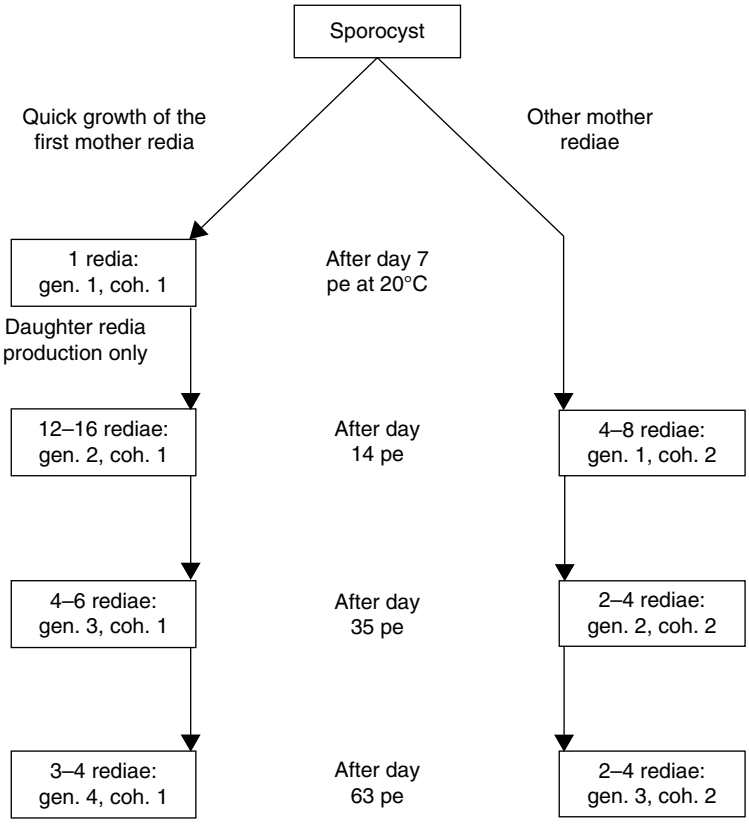

Fig. 2.3. Normal development of *Fasciola hepatica* redial generations in single-miracidium infections of *Galba truncatula*, with an indication of redial numbers for each redial cohort. In this case, the first redia of the first generation remained alive at least up to day 49 post-exposure (pe) at 20°C. coh., cohort; gen., generation. From: Rondelaud, Belfaiza, Vignoles, Moncef and Dreyfuss (2009a): Redial generations of *Fasciola hepatica*: a review. *Journal of Helminthology*, 83, 245–254 (Fig. 2, page 248). RightsLink Printable License no. 4666941365625. With permission from Cambridge University Press.

was higher: two per *P. columella* (versus one redia in *G. truncatula*) in single-miracidium groups and 3.1 (versus 1.9) in the two-miracidia groups (Dar *et al.*, 2014). In juvenile *P. columella* measuring 1 mm in height at exposure, a single mother redia producing only daughter rediae of the second generation was noted in each snail, while the group measuring 2 mm at exposure showed the presence of a single mother redia (43 snails/71) or two (28 snails/71) within their bodies (Dar *et al.*, 2015).

A more surprising development of redial generations (Belfaiza *et al.*, 2005) was noted in Moroccan *G. truncatula* infected by a sympatric isolate of *F. hepatica* miracidia. In parent snails, all free mother rediae produced second-generation rediae. However, in the F1 and F2 snails, daughter redia production was progressively ensured by the first mother redia. This abnormal development does not seem to be another variant, but rather the result of adaptation between two sympatric partners.

2.3.2.3 Factors influencing the developmental pattern of redial generations

These factors are listed in Table 2.1. Their effects on the development of the first three generations of *F. hepatica* were only studied up to day 49 pe at 20°C. Whatever the factor studied, the three generations were found in all snail groups. However, for some factors, variations in the numbers

Table 2.1. Impact of different environmental or biotic factors on the redial generations of *Fasciola hepatica*. From Rondelaud *et al*. (2009a,b) and Dreyfuss *et al*. (2015b).

Effect of factor	Reference
Increased patent period; the three generations present	
Low temperatures	Rondelaud and Barthe, 1978a
Limitation in redial numbers in the three generations	
Scarce natural encounters between snails and parasite	Rondelaud, 1993
Species of the definitive host	Rondelaud and Dreyfuss, 1995; Vignoles *et al.*, 2004
Infection of juvenile snails	Vignoles *et al*., 2002b
Snail infection by two digenean species	Hourdin *et al.*, 1992; Hourdin *et al.*, 1993
Infection of surviving snails after poisoning	
Drought of snail habitat for 15 days	Rondelaud, 1995
Presence of snail predators	Rondelaud, 1994
Susceptibility or resistance of adult flukes to triclabendazole	Rondelaud *et al.*, 2002b; Walker *et al.*, 2006
Limitation in redial numbers in generations 2 and 3	
Geographical origin of *G. truncatula*	Rondelaud and Barthe, 1982a
Multiple-miracidium infections	Rondelaud and Barthe, 1987b Rondelaud and Barthe, 1982a.
Limitation in redial numbers in generations 2 (cohort 2) and 3	
Snail co-infection with an iridovirus	Ruellan and Rondelaud, 1992.

of free and live rediae were noted. Drought of the snail habitat for 35 days (Rondelaud, 1994), as well as seven biotic factors, had the effect of limiting redial numbers in the three generations. Among the biotic factors, the most important are the species of the definitive host from which eggs of *F. hepatica* are collected for snail infections (miracidia originating from eggs collected from cattle, sheep or nutria had a greater infectivity than those coming from lagomorphs: Rondelaud and Dreyfuss, 1995; Vignoles *et al*., 2004), the susceptibility or the resistance of adult flukes to triclabendazole (Walker *et al.*, 2006) and the frequency of encounters between snails and the parasite in the field (Rondelaud, 1993). Limitation in redial numbers is different for the other three biotic factors. Indeed, the second and third generations are affected in the case of the geographical origin of snails (Rondelaud and Barthe, 1982a, 1987a) and the number of miracidia used per *G. truncatula* (Rondelaud and Barthe, 1982a), whereas only the second cohort of the second generation and the third generation are affected if snails are already highly infected with an iridovirus at the time of miracidial exposure (Ruellan and Rondelaud, 1992).

This limitation in redial numbers is often due to the degeneration of free rediae. Two types of redia degeneration have been observed on histological sections of infected snails (Rondelaud and Barthe, 1980a). The 'mummified rediae' are cadavers of small larvae that have degenerated after their exit from the sporocyst or a parental redia. Most of them are found in the inter-visceral spaces of the snail foot, sometimes in the mantle, rarely in the visceral cavity. Their nuclei are often triangular and pycnotic, with strongly condensed chromatin. Another scarcer case of degeneration concerns intra-redial germinal masses whose nuclei persist while the cytoplasm of their cells has disappeared. This last mode has not yet been satisfactorily explained. However, as this affects all rediae in a snail, it is questionable whether this type of degeneration might be due to a hyperparasite such as the microsporidian *Nosema eurytremae* (Canning *et al.*, 1974, 1979).

2.3.2.4 Redial generations in natural infections of snails

The development of redial generations was studied in 731 *G. truncatula* naturally infected with *F. hepatica* and collected over a period of 10 years in 168 farms located in two nearby French departments (Rondelaud *et al.*, 2004b). Snails were dissected under a stereomicroscope and counts of rediae were made under a microscope

according to their physiological state, their generation and their contents (Table 2.2). Single-sporocyst infections were found in numerous snails, either with a normal development of redial generations (50.4%), or with an abnormal development (38.4%). Two-sporocyst infections were scarcer: 8.9% and 2.2%, respectively. The first three generations of rediae were present in all snails. In each type of *F. hepatica* infection (one- or two-sporocyst), the numbers of first-generation rediae were close. The rediae belonging to the second cohort of the second generation and the third generation were few in numbers and this fact may be due to the characteristics of snail samples used for this study, i.e. snails harbouring cercariae-containing rediae. In spite of these fluctuations in the numbers of free rediae, the sequence of three redial generations developing one after the other was noted in all naturally infected snails, thus demonstrating the constancy of this process in all populations of *G. truncatula* when infected with *F. hepatica*.

2.3.2.5 *Consequences of redial development patterns on cercarial productivity*

The existence of several developmental patterns for the rediae of *F. hepatica* and the lower numbers of rediae found during an abnormal development have consequences for cercarial productivity. Table 2.3 gives the results obtained at day 63 pe (at 20°C) in experimentally infected snails originating from acid soils. Snails showing a normal development of redial generations produced more cercariae than those with an abnormal development (a total of 343.2 cercariae up to day 63 pe instead of 233.9 in single-sporocyst infections, for example). Most cercariae in the case of a normal development were formed by the first cohort of the second generation,

Table 2.2. Distribution of live rediae of *Fasciola hepatica* in 731 naturally infected *Galba truncatula*. From Rondelaud *et al.* (2004, 2009a).

Type of *Fasciola* infection (number of snails)	Mean values (SD) of rediae in snails			
	All	Gen. 1 (cohort 2)	Gen. 2 (cohort 1)	Gen. 2 (cohort 2) + gen. 3 (cohort 1)
Single-sporocyst, normal development of redial generations (369)	15.8 (1.7)	6.3 (1.3)	8.5 (2.6)	1.1 (0.8)
Single-sporocyst, abnormal development (281)	10.3 (1.2)	5.2 (1.1)	1.2 (0.7)	4.0 (1.9)
Two-sporocyst, normal development (65)	25.7 (5.2)	11.1 (5.4)	13.8 (7.3)	0.8 (1.6)
Two-sporocyst, abnormal development (16)	19.2 (4.3)	9.4 (2.7)	9.3 (5.7)	0.5 (0.5)

Gen., generation.

Table 2.3. Assessment of *Fasciola hepatica* cercarial productivity in the case of single and two sporocyst infections in *Galba truncatula* at day 63 post-exposure (pe) at 20°C. From Rondelaud *et al.* (2009a).

Redial generation (cohort)	Number of *Fasciola hepatica* cercariae (%)			
	Single-sporocyst infections		Two-sporocyst infections	
	Normal development	Abnormal development[a]	Normal development	Abnormal development[a]
Other rediae of generation 1 (the first redia not included)	97.2 (28.3)	161.8 (69.4)	240.8 (3.2)	203.9 (49.4)
Generation 2 (first cohort)	216.0 (62.8)	47.5 (20.4)	399.4 (55.1)	146.9 (35.6)
Generation 2 (second cohort) + generation 3 (first cohort)	30.7 (8.9)	23.9 (10.2)	84.7 (11.7)	61.6 (15.0)
Total	343.9	233.2	724.9	412.4

[a]First variant of abnormal development.

whereas they were produced by the second cohort of mother rediae when redial development was abnormal. In both cases, the role of the second cohort of the second generation and that of the first cohort of the third generation in cercarial production were limited up to day 63 pe: less than 12% (single-sporocyst infections) or less than 16% (two-sporocyst infections). In long-surviving snails (at least up to day 102 at 20°C), the role of these two cohorts was also limited, as they produced 17% of the whole cercarial production in single-sporocyst infections and only 13.1% in two-sporocyst infections (Belfaiza *et al.*, 2004a).

2.3.2.6 Concordance between these redial generations and literature data

Four generations of *F. hepatica* rediae may develop one after the other within the intermediate host. This succession was verified in naturally infected snails as well as in those that were subjected to miracidia under laboratory conditions, thus suggesting that it is species dependent. As Thomas (1883a,b), Leuckart (1879–1886), Kendall (1964, 1965), Saint-Guillain (1968) and Wilson and Draskau (1976) only described the presence of two redial generations, i.e. mother rediae and daughter rediae, in the life cycle of F. *hepatica*, an explanation must be given. In their reports, Thomas (1883a,b) and Leuckart (1879–1886) did not describe the rapid growth of the first mother redia and the production of only daughter redia, so that the mother rediae described by these authors might correspond to the group constituted by the rediae of the first generation and those belonging to the first cohort of the second generation. In the same way, the daughter rediae reported by Thomas (1883a,b) might correspond to rediae that became free after day 35 pe, i.e. the second cohort of the second generation and the first cohort of the third generation. This interpretation might also partly explain the variations that several authors (Thomas, 1883a,b; Leuckart, 1879–1886; Kendall, 1965; Styczynska-Jurewicz, 1965; Wilson and Draskau, 1976) have noted in daughter redia production when infected snails were under adverse conditions. In contrast, the studies performed in our laboratory did not confirm the succession of three or more cohorts into each redial generation of *F. hepatica*, as reported in the review by Ginetsinkaya (1988).

The role of the first redia of the first generation in daughter redia production has already been reported in the life cycle of *Fasciola gigantica* (Rakotondravao *et al.*, 1992) and *Echinostoma paraensei* (Sapp *et al.*, 1998). This redia has a great importance in the larval development of *F. hepatica*, as the daughter rediae produced (first cohort of the second generation) led to the development of most cercariae in the case of a normal development. However, its role in daughter redia production might change. According to Belfaiza *et al.* (2005), each mother redia, after its exit from the sporocyst, formed several rediae of the second generation (the two cohorts cannot be identified) when Moroccan *G. truncatula* were exposed to miracidia of a sympatric isolate of *F. hepatica*. Under these conditions, one may wonder about the reasons for such changes for these mother rediae and two perhaps complementary hypotheses may be proposed. The first is to admit that regulation of the redial population would be ensured by the host snail, and particularly by its size, as there is a relationship between the shell height of snails and the quantity of free rediae found within them (Zischke, 1967). However, evolution in the role of these mother rediae over time by adapting to a new snail host or to an unusual snail cannot be excluded.

2.4 Cercarial Shedding of *Fasciola hepatica*

The cercariae are tadpole-like with a discoidal body and a long tail. The body measures 250–350 µm and the tail is twice as long. There is an oral sucker and a ventral sucker in the centre of the body. Each cercaria contains a pharynx, lined with numerous cystogenous cells, an oesophagus and a forked intestine (Andrews, 1999). Cercariae accumulate glycogen and fatty acids during their intra-redial stay. Most of the cercarial glycogen is accumulated in the tail, while fat is accumulated in the body (Ginetsinkaya, 1988). When exiting the snail and swimming outside in the water, the cercariae do not feed but use first glycogen and then fat for energy. Cercaria survival outside is determined by the amount of glycogen and fat, as well as by environmental factors (Graczyk and Fried, 1999).

2.4.1 Emergence and outcome of cercariae

Free cercariae present in the body of the snail exit from it between the 5th and 12th week of infection, according to the environmental temperature. The shedding of these larvae occurs through the perianal region of the snail via a passive mode (Kendall and McCullough, 1951; Kendall, 1965). According to these authors, the cercariae gather, at the time of expulsion, in the space surrounding the posterior intestine of the snail. The integument of the perianal region becomes soft and forms a protuberance. The closure of the pneumostome and the contraction of the mantle cause a tear in the integument of the protuberance, thus allowing the exit of cercariae from their host. Dreyfuss (1994) noted that 94.1% of these cercariae encysted on a support (settled metacercariae) and 5.8% formed cysts floating on the surface of water. A few cercariae (0.14%) died after their exit from the snail or during the formation of their cyst (Dreyfuss, 1994). Rarely, metacercariae, not fixed on the bottom of Petri dishes, have also been observed in several experimental infections (Dreyfuss, 1994).

Several factors are necessary for cercarial shedding: (i) the need for the snail to be in water; (ii) an ambient temperature of at least 10°C; (iii) the stimulating action of fresh water on shedding; and (iv) the favourable action of the transition from drought to humidity after an inundation or the first post-summer rains, for example (Kendall and McCullough, 1951). The cercarial emergence of *F. hepatica* from the snail host may be triggered by a decrease in temperature (Kendall and McCullough, 1951; Kendall, 1965). This stimulating effect of decreasing temperature was used by subsequent authors to induce this process by subjecting cercariae-containing snails to sudden changes in temperature (Hodasi, 1972b; Malone and Yilma, 1999; Rondelaud *et al.*, 2013, for example, for *F. hepatica*).

However, other factors may also trigger cercarial exit. The stimulating effect of light on cercarial emergence was reported in several snail–parasite models and particularly in the case of schistosomes (case of *Schistosoma mansoni*: Théron, 1984, 1989). According to Combes (1995), the different schistosome species showed remarkably different circadian patterns of emergence corresponding with light–dark cycles. Contrary to schistosomes, the effect of light on cercarial emergence of *F. hepatica* was never demonstrated before the 2010s. By subjecting *F. hepatica*-infected *G. truncatula* to a regular thermal shock during the patent period, Vignoles *et al.* (2014b) demonstrated a significant effect of natural light level on cercarial emergence, whereas the intensity of artificial light did not influence this process. According to these authors, the number of cercariae shed by snails was significantly higher for low natural light levels (601–1200 lux) and the highest nebulosity values (7–8 octas).

2.4.2 Dynamics of cercarial shedding

Cercarial shedding can occur continuously or can be discontinuous. In general, most cercariae are released during shedding waves of several days each and these waves are separated from each other by time intervals with no exit of cercariae (interwave). When snails were reared in a semi-natural habitat with daily fluctuations from 8°C to 22°C, seven waves of cercarial shedding were noted and a single wave was observed in 38.1% of *G. truncatula*. Under constant conditions (20°C), up to 14 waves were observed and the highest frequencies concerned snails that shed their cercariae during three, four, or five waves. The number of cercariae released during each wave showed no significant variation, whatever the numbering of this wave. Snails that shed their cercariae during several waves are considered well adapted to the parasite. In contrast, those that released their larvae in a single wave, sometimes for 2 or 3 days, would still be incompletely adapted with the digenean (Dreyfuss, 1994). As both types of *G. truncatula* (one wave or several waves of shedding) are present in the same snail population, one may wonder whether other factors such as the physiological state of *G. truncatula* during the patent period might play a decisive role in the dynamics of cercarial shedding.

According to Kendall and McCullough (1951), the only parameter determining the emergence of these larvae is the period of the day where the maintenance of infected snails (change of water) is performed. However, as for *F. gigantica* (Albaret *et al.*, 1980; Rakotondravao and Rondelaud, 1991), a circadian rhythm in

the cercarial shedding of *F. hepatica* has been demonstrated in pre-adult snails infected with cattle-derived miracidia and reared in semi-natural breeding (Audousset *et al.*, 1989). The highest cercaria output was noted during the night (8 pm–4 am) with a distinct peak between 12 midnight and 1 am. It was more reduced during the other hours of the day, with a period of low production between 12 noon and 2 pm. The existence of this circadian rhythm was verified by Rondelaud and Dreyfuss (1995) in pre-adult *G. truncatula* kept under constant conditions of temperature (20°C). When snails were exposed to cattle-derived miracidia, there was a peak in cercarial shedding between 12 midnight and 1 am. However, if miracidia came from eggs collected from sheep or rabbits, the peak was observed between 12 midnight and 2 am (sheep group) and between 4 am and 8 am (rabbit group). According to Rondelaud and Dreyfuss (1995), the disturbances observed in the circadian rhythm of cercariae in the rabbit group could be due to the definitive host species from which *F. hepatica* eggs are collected. In contrast to the above, no circadian rhythm was noted by Bouix-Busson *et al.* (1985a) during cercarial shedding when juvenile snails such as *Omphiscola glabra* were infected by *F. hepatica*.

An infradian-type rhythm was also reported by Audousset *et al.* (1989) in *G. truncatula* maintained in a semi-natural habitat. According to these authors, there was a clear rhythm with a periodicity of about 7 days. In contrast, this rhythm was not found in experiments carried out by Dreyfuss (1994) and Dreyfuss and Rondelaud (1994a) when infected snails were reared under constant conditions (20°C). A more detailed study on this last point was performed by Vignoles *et al.* (2006). According to these authors, the periodicity of 6.8–7.8 days did not involve all the snails of an experimental group kept at a constant temperature of 20°C, but only a few *G. truncatula* with a long survival. In reality, this periodicity is due to the length of the interwaves which separate two successive shedding waves and might correspond to the infradian-type rhythm reported by Audousset *et al.* (1989). However, as these snails with regular waves of shedding are not very numerous, one may wonder about the reasons for this process: is it a very high degree of adaptation between the snails and the parasite, as Boray (1978) suggested, or is it a larval development of *F. hepatica* that is slower than usual?

Little information on the distance that cercariae travel once they exit the snail before encysting is available in the literature. In watercress beds on acidic soil, most of the cercariae were encysted near the host snail (usually within a length of 50 cm) (Rondelaud, 2004). But several cercariae managed to swim up to an average of 5 m in slow-flow waters before their encystment, whereas this distance was only 4 m in faster waters (Rondelaud *et al.*, unpublished data).

2.4.3 Metacercariae of *Fasciola hepatica*

In infected dead snails that do not shed, encystment of several cercariae can occur in the body of the snail (Vareille-Morel *et al.*, 1993b; Dreyfuss *et al.*, 2009), According to Vareille-Morel *et al.* (1993b), such internal cysts are formed immediately prior to, or following, the death of infected snails. This process could result from mature cercariae within the host tissues reacting to pathological changes associated with this event (Morley, 2015).

2.4.3.1 Metacercariae settled on a support

The cercaria swims for several minutes and searches for a place to encyst and form its cyst. Due to its negative geotropism and negative phototropism (Pécheur, 1967), this larva usually settles near the surface of the water. Observations on the cercariae choice of colour of the support on which they settled are contradictory (Morley, 2015). Pécheur (1967, 1974) reported that cercariae are not attracted by yellow light or any coloured surface, while Jimenez-Albarran and Guevara-Pozo (1980) found a slight preference for a glass surface covered with yellow cellophane compared with other surfaces covered with green, red, blue or colourless. Cercariae seem to prefer certain species of plants for their settlement (Morley, 2015). According to Pécheur (1967), three species of plants, out of the nine that this author had tested, were chosen by the cercariae for their encystment, and the author related this fact to the smooth surfaces of these plants, which would allow a good adhesion of *F. hepatica* cysts. Pécheur (1967) also found that *F. hepatica* encystment occurred on the upper or

lower surface of the leaves belonging to several plant species. On the contrary, Hodasi (1972b) found that *F. hepatica* encysted mainly on the lower surface of submerged leaves of the grass, *Dactylis glomerata*. Green parts of plants, rather than brown decaying parts, were also preferred by *F. hepatica* and the majority of *F. hepatica* metacercariae occured on the leaves rather than the stems of grass (Hodasi, 1972b).

A vertical distribution of metacercariae on plants growing in water also occurs (Morley, 2015). The majority of *F. hepatica* cercariae encysted just below the water surface, typically less than 2 cm depth (Sinitsin, 1914; Dreyfuss *et al.*, 2004), and the frequency of cysts in the other submerged sections decreased gradually with depth. It has also been reported that a small number of *F. hepatica* cercariae also encysted up to 2 cm above the water surface (Sinitsin, 1914; Hodasi, 1972b; Dreyfuss *et al.,* 2004) but one can wonder if it is a real ability of the cercariae to be so encysted, a simple growth of the plant or a decrease in the thickness of the water layer during the experiments that these authors performed. The metacercariae of *F. hepatica* are also able to form aggregates (Morley, 2015). These aggregates consist of three or more cysts separated from each other by a distance of 0.2 mm or less (Abrous *et al.*, 2001b). In a laboratory experiment, these authors investigated the number of these metacercarial aggregates using Petri dishes (14 cm in diameter) and infected snails, each placed in a container with holes 2 mm in diameter. On the walls of the dishes, the number of metacercariae per aggregate went up to 21 and two peaks were noted, the first with 10 cysts and the other with 15 cysts per aggregate. In aggregates constituted of floating metacercariae, the number of cysts per aggregate could be up to 14 and the number of these groups decreased as the amount of larvae per aggregate was increasing (Abrous *et al.*, 2001b).

After its settlement, the cercaria loses its tail and quickly undertakes the formation of its cyst (Thomas, 1883a). The average diameter of each cyst is about 250 µm. The colour of each cyst is initially whitish and changes in the hours that follow to become flaxen and then tawny. The envelope is formed by two walls, each with two layers. Using histochemical stains, Dixon (1965, 1966), Dixon and Mercer (1964, 1967) and Mercer and Dixon (1967) identified the chemical nature of these different layers. In the outer wall, the external layer consisted of a tanned protein that became yellow by a quinonic tanning phenomenon, while the internal layer was fibrous with mucoproteins and acid mucopolysaccharides. The inner wall itself consisted of an external mucopolysaccharidic layer (with three sublayers) and an internal keratinized layer. This last layer thickened in the ventral region of the cercaria to form a plug. If the metacercaria was separated from the outer wall with a thin needle, the inner wall had a characteristic pearly appearance.

To our knowledge, no epidemiological study has been carried out on the distribution of metacercariae settled on grassland vegetation on acid soils. In contrast, this type of investigation was carried out for 15 years in 59 wild watercress beds in order to find *F. hepatica* metacercariae on samples of *Nasturtium officinale* and *Apium nodiflorum*. The total number of metacercariae found in the 59 wild beds varied each year, with a minimum value in 1991 (70 cysts) and a maximum in 1999 (241 cysts). The amount of encysted larvae on both plant species was low: an average of 2.6–6.3 per bed. The distributions of larvae on *N. officinale* and *A. nodiflorum* were close to each other: 1165 cysts on watercress (51.5%) compared with 1097 on false watercress (Dreyfuss *et al.*, 2005).

2.4.3.2 Floating metacercariae

The existence of floating metacercariae in the life cycle of *F. hepatica* was first reported by Sinitsin (1914), while the characteristics of this larval stage were only studied by our team from 1989. Each cyst is surrounded by a collar formed by the external layer of the outer wall. This collar consists of dissociated fibrils separated from each other by air-filled lacunae, which allows the cyst to float on the surface of the water. In the ventral part of the cyst, the fibrils are bristled towards the depth, giving the appearance of a 'hairy' collar when the cyst is examined under a light microscope (Esclaire *et al.*, 1989). Consequently, the diameter of a floating metacercaria was significantly higher than that of a cyst settled on a support (an average of 346 µm instead of 226.5 µm), while their heights showed no significant difference (128 and 134 µm, respectively). The average radius of the collar was 60.3 µm

and its thickness was 16.3 µm when measured at the base of the cyst (Dreyfuss, 1994).

Floating cysts are produced at the same time as the metacercariae that settle on a support and represent 6.8% of the total metacercarial production. Their numbers are maximal between 12 midnight and 1 am and they could float for more than 3 months in stagnant waters (Esclaire *et al.*, 1989). Photoperiod, light intensity, experimental Petri dish diameter, depth of water, presence/absence of plant and the number of water changes do not influence production of floating metacercariae. On the other hand, a diurnal variation of the temperature between 12°C and 25°C significantly increased the number of cysts compared with a constant temperature (Vareille-Morel and Rondelaud, 1991). However, thermal shock due to cold (3 h at 12°C once a week) did not significantly affect the average number of floating metacercariae produced by *F. hepatica* (Rondelaud *et al.*, 2013; Morley, 2015).

Floating cyst production varied considerably with the host snail species and showed a significant increase in the highest-shelled snails when subjected to experimental infection (Vareille-Morel *et al.*, 1994a). The proportion of floating metacercariae was particularly high at the onset of emergence and accounted for 25–36% of all metacercariae counted during the first 2 days of the patent period. Afterwards, the production decreased considerably during successive waves (Vareille-Morel *et al.*, 1994b). In spite of their collar, these cysts floated only on stagnant water, because the presence of running water induced their fall to the bottom. Laboratory and field experiments revealed that more than half of these metacercariae had fallen to the bottom of the running water, and only a small proportion was deposited on emerged vegetation (*Nasturtium officinale*), most of them dying at the same time as the plant (Vareille-Morel *et al.*, 1993a; Rondelaud *et al.*, 2004b).

Floating cysts are also infectious for the definitive host, as experimental infections of rabbits have shown (Dreyfuss, 1994). According to Vareille-Morel and Rondelaud (1991), the cercaria at the origin of a floating cyst would suffer lesions during its shedding. Dreyfuss (1994) suggested that lesions might occur during the passage of larvae through the perianal region of infected snails prior to release in the environment. This crossing would be perilous for cercariae that migrate first in this area and would result in the death of some cercariae after the loss of their tail or during the formation of their floating cyst for other larvae. If these two hypotheses are valid, it is necessary to reconsider the idea that the exit of the larvae during shedding would be passive (Kendall and McCullough, 1951). In our opinion, the first cercariae that emerge from the snail during each shedding wave play an active role in the formation of the tear that occurs in the integument of the perianal region of the snail.

2.5 Snail Infection by the Parasite

2.5.1 Characteristics of infection

2.5.1.1 Parameters studied

Three parameters are commonly used in epidemiological and epizootiological studies on the transmission of *F. hepatica* by intermediate hosts (Roberts and Suhardono, 1996; Graczyk and Fried, 1999). They are therefore calculated in naturally infected snails as well as in those that are subjected to experimental infections in the laboratory. According to Margolis *et al.* (1982), the prevalence (= infection rate) corresponds to the number of infected snails (with live larval forms or cercariae, according to the authors) and is calculated in relation to the number of snails collected in the field or in a breeding box at a given date. The intensity of infection corresponds to the larval burden (free and live rediae, free cercariae) which develops in the body of the snail. The enumeration of cercarial shedding can be used to assess the ability of the snail as an intermediate host, i.e. by studying the number of shed cercariae and the dynamics of shedding over time.

The other parameters only concern experimental infections of the snails by *F. hepatica* (Rondelaud *et al.*, 2009b; Dreyfuss *et al.*, 2015b). The survival rate is usually determined at a given date (on day 30 or day 45 pe, according to the authors). The growth of infected snails during the experiment, the length of the prepatent period (phase of larval differentiation until the first cercarial shedding) and that of the patent

period (phase during which the cercariae exit from the snail) are also frequently taken into account. Both types of metacercariae (settled on a support or floating cysts) are sometimes considered, to determine the proportion of one type or the other compared with total metacercarial production.

The term prevalence is sometimes used by some authors to represent the number of molluscs in which *F. hepatica* DNA is detected using molecular markers. As this genetic material can come from an infection with live larvae or an abortive infection, this approach cannot reliably determine if the snail is a potential intermediate host of the parasite capable of shedding cercariae. In order to avoid this difficulty, the term prevalence should be used only in infected molluscs in which live larval forms have been observed, while another expression, such as the frequency of snails containing *F. hepatica* DNA, might be used in molecular biology studies.

2.5.1.2 Methods used

Microscopic examination is the most frequently used technique to detect *F. hepatica* in the intermediate host. Three classical methods have been used to diagnose trematode infections in snails: (i) observation of cercarial shedding; (ii) dissection; and (iii) crushing, followed by microscopic examination (Kaplan *et al.*, 1997). Although these techniques are simple, fast and very cheap, they have low sensitivity and/or specificity. Indeed, prior to the release of rediae from the sporocyst and their subsequent migration through the tissue of the snail (around day 21 pe), the detection of the parasite is difficult. Nevertheless, young rediae might be seen from 9 to 10 days after infection (at 20°C). However, the morphology of larval forms often does not allow the differentiation of trematode species between them before cercariae develop in rediae or second-generation sporocysts (Kaplan *et al.*, 1995). Examination of serial sections of infected snails allows an accurate count of the different larval forms of *F. hepatica*, the evaluation of their physiological state and their location within the snail, but the use of the histological technique needs a prior identification of the parasite species on the larval morphology and is also time-consuming (Caron *et al.*, 2008). For these reasons, other techniques have been proposed. A thin-layer starch gel electrophoresis was used by Van Aken and Brandt (1987) to detect larval forms of *F. hepatica* in the snail through the study of the glucose-6-phosphate isomerase of the parasite. Several DNA- or RNA-based techniques were also developed (Shubkin *et al.*, 1992; Rognlie *et al.*, 1994; Kaplan *et al.*, 1995; Krämer and Schnieder, 1999; Mostafa *et al.*, 2003; Magalhaes *et al.*, 2004; Cucher *et al.*, 2006). These techniques have generally been found to be more specific and more sensitive than microscopic methods (Kaplan *et al.*, 1997; Caron *et al.*, 2008). However, there are some limitations in their use; for example, the presence of inhibiting factors in snails can reduce PCR sensitivity (Cucher *et al.*, 2006). It is also important to ensure that putative PCR primers specific to trematodes do not amplify the DNA of snails (Beesley *et al.*, 2018). A number of other trematode species, including those that infect birds and amphibians, have been isolated from *G. truncatula* (Rondelaud *et al.*, 2016). Some of these trematodes have little or no published DNA sequence available, making it difficult to ensure that the PCRs are specific to *F. hepatica*. In addition, few PCRs published on *F. hepatica* have been validated for use with snails (Beesley *et al.*, 2018). According to several authors, these DNA- or RNA-based techniques would be suitable, for example, for monitoring seasonal transmission during epidemiological studies (Rognlie *et al.*, 1994; Kaplan *et al.*, 1997; Caron *et al.*, 2008).

The question often arises as to which method should be used to determine the parameters of infection in molluscs. The choice of this method depends on the purpose of parasitological investigations in snails. The crushing and dissection of snails are simple methods and allow the direct detection of larval forms in naturally or experimentally infected snails. However, identification of the digenean on the morphology of its larvae is necessary. Prevalence and intensity of infection are easy to calculate, but these methods are slow, laborious, tedious and only feasible in highly infected snails. In addition, they need an experienced microscopist. Monitoring cercarial shedding is a very useful method that provides information regarding the ability of the snail as an intermediate host and to contaminate the pasture. Histological methods allow an accurate count of the larval forms in the snail. Among these four classical

techniques, the most commonly used methods to detect patent infection in studies on infected snails are snail crushing and snail dissection (Caron *et al.*, 2008). The enzymatic electrophoresis used by Van Aken and Brandt (1987) is interesting because of its low cost but its low sensitivity (the parasite cannot be detected until the fifth day of infection) limits its use in researching possible intermediate hosts of digeneans whose life cycle is partly known. Blotting is not acceptable, because this technique is complicated and involves the use of radiolabelled molecules or chemiluminescence. However, this allows evaluation of new sequences that can be used to establish a PCR. Despite its high sensitivity and specificity, classical PCR provides no information on the intensity of infection and cercarial production. Therefore, a very low level of detection brings little information in the epidemiology of fasciolosis, because at least one sporocyst will be present in the snail. In addition, abortive infections can skew the results if new intermediate hosts are sought. Real-time PCR can provide very accurate data on the intensity of infection but its cost limits its use (Caron *et al.*, 2008).

At the present time, there is no simple, robust, reproducible and cheap technique for field use to study snails. As PCR accurately detects parasite invasion and microscopy reveals successful infections in the intermediate host, both techniques could be used together to better understand the epidemiological situation in a given area and to assess the ability of intermediate hosts to sustain larval development of the parasite.

A research pathway under development is to study the DNA that host snails and free larval stages of *F. hepatica* (miracidia, cercariae) leave in the outdoor environment. The study of this environmental DNA may be a valuable tool for identifying the presence of snails and parasites, and subsequently for estimating the risk of infection to livestock grazing pasture. Source water samples are filtered to collect excreta/secretions of snails and parasite larval forms, and the DNA resulting from these organisms is extracted and amplified with PCR. Using these techniques, Rathinasamy *et al.* (2018) were able to detect the DNA of the host snail (*Austropeplea tomentosa*) and that of *F. hepatica* in water samples from Australian pastures where these snails lived. Similarly, Jones *et al.* (2018) could detect the presence of *G. truncatula*, *F. hepatica* and another parasite, *Calicophoron daubneyi*, in field water samples. According to these last authors, this technique could detect DNA from snails or parasites in other environmental samples such as soil samples.

2.5.2 Natural infections of snails

2.5.2.1 Technique for collecting snails in the field

When snails are collected from the field for detecting naturally infected individuals, the prevalence of *F. hepatica* infection may vary depending on the technique used. In the case of *G. truncatula*, for example, there is no standard technique and so the protocols used are subject to question. In general, the number of snails taken from the field is performed per unit time or per unit area (Leimbacher, 1973). The reproduction periods of the species are often not taken into account and several authors take monthly samples of *G. truncatula* during the year when conditions are favourable. In addition, all age groups in the population are affected. The first consequence is a great variability in the results, because adult snails are often more affected than pre-adults (2–4 mm high for *G. truncatula*) and juveniles. Another consequence is the frequent disappearance of the snail population after several years of collection, as the remaining individuals are no longer able to reconstitute the population to its original size. Some populations of *G. truncatula* disappeared in the French department of Haute Vienne between 1970 and 1980 due to excessive collection.

Given these facts, it was necessary to redefine the conditions for collecting snails in the field. As the maximum shell height of adult *G. truncatula* is usually 12 mm, the selection of individuals 4–12 mm high for collecting this species is quite obvious as they are in the adult stage. Two sampling periods can be selected for lowland populations of *G. truncatula* in temperate zones: March or April for overwintering snails (sampling date depends on local climatic conditions), and September or October for snails of the spring generation that have survived the summer drought. In addition, the selection of

snails for the sample should be carried out in all the habitats present on the pastures of a farm by taking the same number of adult individuals in each habitat. This technique has been used by our team since 1976 for the populations of *G. truncatula* living in central France on acid soils. Comparing the results noted in this region with data obtained with another method is rather difficult, because the natural prevalence among adults will always be higher than that recorded in the whole population of snails. However, as juveniles and pre-adults are not involved in snail sampling, this technique allows monitoring of populations over a long period of time to collect snail samples regularly, as in the survey conducted by Rondelaud (2004) and Dreyfuss *et al.* (2005) for 15 successive years in 59 wild watercress beds of Haute Vienne.

2.5.2.2 *Natural infections in* Galba truncatula

Because of the wide geographical range of *G. truncatula* (Seddon *et al.*, 2014) and its role as the main intermediate host of *F. hepatica*, many authors have collected specimens of this species to determine the prevalence of its natural infection. To our knowledge, the most complete set of results was reported by Ollerenshaw (1969, 1971) in Great Britain, with the collection and dissection of more than 52,000 *G. truncatula* between 1960 and 1969. According to this author, the prevalence of active infection was generally less than 2% but could increase up to 20% and sometimes even more when conditions for hatching of fluke eggs were favourable. Also in Great Britain, Smith (1984) found a prevalence of 4.6% in 2652 snails he dissected between 1973 and 1975. In other countries, variable values of prevalence were reported in the Old World: less than 3%, for example, in a total of 5676 snails collected by Khallaayoune and El Hari (1991) and Khallaayoune *et al.* (1991) in Morocco, 11.4% in 5486 snails coming from the Porma River basin in Spain (Manga-González *et al.*, 1991), 54.7% in 530 snails collected in the Tunisian oasis of Tozeur (Diawara *et al.*, 2003) and 19.2% in 1346 snails coming from the Gafsa oases in the same country (Hammani *et al.*, 2007).

Several parasitological surveys were conducted by our team on the acid soils of central France to determine the characteristics of *F. hepatica* infection in snails and their relationship with infection rates in the definitive host. A first survey was carried out by Mage *et al.* (2002) over a 12-year period and a total of 18,792 snails collected in 141 farms. An average prevalence of 5.0% was noted, with annual percentages varying from 4.7% in 1989 to 7.2% in 1993 and then decreasing to 3.3% in 2000. In view of the first results of this survey, further investigations were performed to determine the prevalence of natural infection in the definitive host and that found in *G. truncatula*. Snail samples were thus collected from other farms known for their ruminants having high (> 70%) or low (< 30%) infections with *F. hepatica*. In farms with highly infected ruminants, the prevalence noted in 1778 snails ranged from 4.6% to 33%, whatever the sampling period (Abrous *et al.*, 1999a). In contrast, values found in *G. truncatula* did not exceed 7.6% (out of 1424 snails) when the rate in ruminants was low or moderate (Abrous *et al.*, 2000). Contrary to what is currently admitted (the infection rate of livestock with *F. hepatica* is independent of that observed in snails), there was a relationship between the two types of prevalence in cattle and snails studied in central France. A second survey was conducted in wild watercress beds, because cases of human fasciolosis had been detected since 1955 in 860 people after consumption of contaminated cress (Rondelaud *et al.*, 2000). Snail samples were collected between 1970 and 1989 from 59 sites to search for possible *F. hepatica* larvae in their bodies. According to Rondelaud (1991), the prevalence ranged from 0.5% to 5.4% in beds occupied only by *G. truncatula* and from 14% to 66% when *G. truncatula* and *Omphiscola glabra* lived together in the same sites (the number of *G. truncatula* in these mixed communities was always low). These beds were systematically investigated from 1990 for over 15 years. Larval forms of *F. hepatica* were found in 346 snails (out of 19,249 *G. truncatula*) and the overall prevalence was only 1.7% (Rondelaud, 2004; Dreyfuss *et al.*, 2005). These authors did not find any relationship between the annual prevalence (which ranged from 1.2% to 2.4%) and the different years of the study. Thirdly, a retrospective study on naturally infected snails found between 1970 and 2006 in Haute Vienne was conducted to determine if there were areas at risk for fasciolosis

(Vignoles *et al.*, 2017b). Of the 108,481 snails collected in 151 municipalities, the overall prevalence of infection was 3.8% but varied according to the municipalities from which samples were taken. The prevalence of *F. hepatica* infection in snails significantly decreased when the average altitude and annual rainfall of municipalities increased. In contrast, it significantly increased with the increase in the average annual temperature of municipalities. The risk of infection for local livestock would be greater in the Haute Vienne below 400 m altitude and would gradually decrease when the altitude of these lands increased (Vignoles *et al.*, 2017b). Similarly, the distribution of *G. truncatula* populations is closely related to the altitude and climatic conditions as these populations decrease in frequency when the average altitude and annual rainfall of municipalities are increasing (Dreyfuss *et al.*, 2018). These results raise the question of whether this decrease in the prevalence of *F. hepatica* infection and in the frequency of snail populations, when altitude and precipitation increase, is a local process related to acidic soils or a general phenomenon that can be observed in any altitude zone in temperate countries, whatever the geology of the subsoil.

Compared with the behaviour of European *G. truncatula* living in the lowlands, the populations of the Bolivian Altiplano are more aquatic and are rarely encountered on the mud, out of water. A significant number of emerged snails was only observed in 12% of habitats studied by Mas-Coma *et al.* (2009). Snails infected with *F. hepatica* showed a longer period of cercarial shedding and higher cercarial production, related to greater survivability of these Altiplanic snails (Mas-Coma *et al.*, 2001). Higher survival of infected snails and longer lifespan were also noted by Vignoles *et al.* (2002c) in five *G. truncatula* populations from the French Alps and the Massif Central, whereas higher cercarial production was noted in only three of them. According to these last authors, the long survival times of highland snails in the laboratory could be an adaptation of these *G. truncatula* to the more extreme climate in altitude. The better ability of highland snails to sustain larval development of the parasite suggests that they would be better intermediate hosts in the life cycle of *F. hepatica* than lowland populations.

2.5.2.3 *Natural infections in other snail species*

At least 20 species of Lymnaeidae have been reported as potential intermediate hosts in the life cycle of *F. hepatica*. Among these, two species are important as host snails at the regional level.

Pseudosuccinea columella is of North American origin and is known as an invasive species that has been introduced into many countries (Pointier *et al.*, 2007). Boray *et al.* (1985) reported the presence of some naturally infected individuals in Australia. Prepelitchi *et al.* (2003) provided a prevalence of 8.8% (out of 500 snails collected) in Argentina, while percentages up to 5.2% were reported by Coelho and Lima (2007) in Brazil. In Cuba, the situation is much more complex because two species of lymnaeids, *Galba cubensis* and *P. columella*, have been identified in the island (Gutiérrez *et al.*, 2000, 2001). In addition, populations of *P. columella* naturally susceptible to the parasite and others resistant to infection (with encapsulation of miracidia after their penetration into the snail) have been reported (Gutiérrez *et al.*, 2003b, 2005). These two types of populations identified in Cuba have genetic and phenotypic differences such as mantle pigmentation (Gutiérrez *et al.*, 2003a,b). The prevalence of natural infection in *G. cubensis* averaged 2.9% in 2015 among the 816 snails that Vázquez *et al.* (2015) dissected, whereas it was 4.1% and 7.4% among the 767 snails that Alba *et al.* (2016) studied first by dissection, then by multiplex PCR. In *P. columella*, natural infection was reported for the first time in 2011 in a parasite-susceptible population (Gutiérrez *et al.*, 2011) and was confirmed in 2015 with a prevalence of 1.6% among the 300 snails dissected by Vázquez *et al.* (2015).

In central France, rediae and cercariae of *F. hepatica* were observed from 1996 in another lymnaeid, *Omphiscola glabra* (Vignoles *et al.*, 2017a). The juveniles of this species (< 2 mm high) were already known for their ability to support larval development of the parasite, while pre-adults and adults were refractory to this infection (Boray, 1978; Busson *et al.*, 1982). However, the co-infection of juveniles and pre-adults by *F. hepatica* and another digenean, *Calicophoron daubneyi*, or vice versa, allows the complete larval development with cercarial shedding for one of the parasites, the other, or both (Abrous *et al.*, 1998). As a result, several parasitological surveys were

conducted in the field for collecting adult *O. glabra* in June–July. When *O. glabra* was the only species of lymnaeid in swampy meadows (four farms) the infection rate ranged from 6.1% to 14.1%. In contrast, if *O. glabra* lived with *G. truncatula* on the same surface drainage system (three farms), prevalence ranged from 13.3% to 21.8% in *G. truncatula* and from 0% to 2.6% in *O. glabra* (Abrous *et al.*, 1999a). When the two species of lymnaeids occur in the same grasslands, the prevalence of natural *F. hepatica* infection is therefore lower in *O. glabra* than in the pastures where this species lives alone. In order to verify these results, a second survey was carried out on 11 farms where the two species of snails live on the same meadows. In *O. glabra*, prevalence in June–July ranged from 0% to 2.6%, while in *G. truncatula* prevalence ranged from 2.0% to 7.8% (Abrous *et al.*, 2000).

The extent of fasciolosis in the various habitat types that *O. glabra* colonizes and the prevalence of infection in snails were determined in three other surveys. The natural infection of *O. glabra* with *F. hepatica* in the wild watercress beds was regular from 1996 until the end of the third survey (2004), with an annual prevalence ranging from 0.4% to 1.8% (Dreyfuss *et al.*, 2005). In addition, this infection has progressively affected the various habitat types colonized by the snail on acidic soils and the prevalence increased slightly until 2002. At this date, the prevalence in *O. glabra* was 2.1% in marshy meadows, 1.4% in road ditches, 1.2% in fenced ponds and 1.0% in walled water points (Dreyfuss *et al.*, 2003). A similar increase in the prevalence of natural infection was observed when *O. glabra* adults were transplanted from their natural habitats to new sites on acidic or sedimentary soils (Dreyfuss *et al.*, 2010). The problem with this snail is whether the *Fasciola* infection observed in this lymnaeid is a local process limited to acidic soils in central France or whether it is a general phenomenon that affects the various populations of *O. glabra* living in Western Europe. Novobilsky *et al.* (2014) did not detect *F. hepatica* DNA in the 138 specimens of *O. glabra* they studied in Sweden.

This variability in the prevalence of natural infection according to snail species was also found in intermediate hosts other than Lymnaeidae. In northern Tunisia, the prevalence in *Bulinus truncatus* was 39% in a total of 163 adult snails collected for one year (Hamed *et al.*, 2009). An infected planorbid (*Anisus leucostoma*) was also found among the 457 adult specimens that Dreyfuss *et al.* (2002) collected in a wild watercress bed in central France (prevalence: 0.1%). The presence of fasciolosis in these two sites might depend on frequent contacts between the snail and *F. hepatica* (*B. truncatus*) or co-infection of the snail with *C. daubneyi* (*A. leucostoma*).

2.5.3 Experimental infections of snails over several successive generations

In central France, the co-infection of *O. glabra* by *C. daubneyi* and *F. hepatica* did not explain all cases of bovine fasciolosis in farms where *G. truncatula* was lacking. Several *F. hepatica* infections of *O. glabra* were observed by our team on seven of these farms prior to the 2000s, while paramphistomosis had not yet been detected in cattle. Similarly, Dreyfuss *et al.* (2003) reported natural infections of *F. hepatica* in *O. glabra* living in gardens surrounded by walls, while only hares and rabbits, often infected with the parasite, were present in these sites (Rondelaud *et al.*, 2001). In view of these facts, one might wonder whether another mode of snail infection with *F. hepatica* existed in the case of *O. glabra*. This approach was supported by two reports. Sanabria *et al.* (2012) used two successive generations of *Lymnaea viatrix* (= *Galba viatrix*) hatched from eggs laid by their parents already infected with *F. hepatica* and in turn infected them with the same digenean. These authors noted a gradual increase in cercarial production from parents to the F2 generations. Similarly, Vignoles *et al.* (2014a) reported a progressive increase in the prevalence and cercarial production in five successive generations of *Lymnaea cubensis* (= *Galba cubensis*) infected with *Fascioloides magna*, a parasite species relatively close to *F. hepatica*.

To test this hypothesis, seven successive generations of *O. glabra* 4 mm high, originating from two populations, were subjected to bi-miracidial infections to count rediae and cercariae in the first six generations and to follow the dynamics of cercarial shedding in the F7 generation (Table 2.4). The overall prevalence of *F. hepatica* infection significantly increased with increasing snail generation. In each population considered separately,

Table 2.4. Characteristics of *Fasciola hepatica* infection in six generations of *Omphiscola glabra* subjected to individual bimiracidial exposures. The snails were reared at 20°C and dissected on day 42 post-exposure (pe). These descendants are derived from eggs laid by their infected parents between weeks 2 and 5 of the infection. From Rondelaud *et al*. (2015a).

Snail population and generation	Number of surviving snails on day 30 p.e. (%)[a]	Number of infected snails with				Overall prevalence of infection (%)
		Immature rediae	Cercariae-containing rediae	Free cercariae	Cercarial shedding	
Population A						
F1	16 (32.0)					0
F2	22 (44.0)	1				4.5
F3	19 (38.0)	4	1			26.3
F4	23 (46.0)	2	3	1	1	30.4
F5	18 (36.0)	2	3	4	1	55.5
F6	22 (44.0)	2	2	6	3	59.0
Population B						
F1	19 (38.0)					0
F2	15 (30.0)					0
F3	18 (36.0)	1				5.5
F4	21 (42.0)	3	2			23.8
F5	24 (48.0)	3	3	1		33.3
F6	23 (46.0)	2	5	4	1	52.1

[a]Fifty snails per population and generation at miracidial exposure.

three (population A) or four (population B) successive generations of snails were necessary to obtain the first cercarial shedding from an infected snail. In the seven generations of controls issuing from uninfected parents and subjected to miracidia, no larval forms of *F. hepatica* were found (Rondelaud *et al*., 2015a). The characteristics of this infection in *O. glabra* were compared with those found in *G. truncatula* infected according the same protocol. Compared with *G. truncatula*, the prevalence of infection and the total number of cercariae were significantly lower in *O. glabra,* while the prepatent period in both populations of *O. glabra* was significantly longer (Rondelaud *et al*., 2015a).

This mode of infection with *F. hepatica* through several successive generations of snails did not seem specific to *O. glabra*, as similar results have been reported for *Radix balthica*, *Stagnicola fuscus* and *S. palustris* (Rondelaud *et al*., 2014b; Dreyfuss *et al*., 2015a).

2.5.4 Factors affecting larval development of *Fasciola hepatica* in the snail

According to Boray (1966, 1969, 1978), all populations of a lymnaeid species susceptible to *F. hepatica* are able to sustain the larval development of the parasite. However, the susceptibility of these snails to parasite infection varies according to their geographical origin, which induces fluctuations in the prevalence between populations of this lymnaeid and also between different individuals of the same population. In addition, there are several factors that can have an effect on the success of parasitic infection in the snail. According to Smyth and Halton (1983), three factors may be involved: environmental factors, snail-related factors and parasite-related factors.

2.5.4.1 Interpopulation and intrapopulation variability in snail susceptibility

Several experiments were carried out by our team with the snail *G. truncatula*. First, Rondelaud (1993) reported variability in infection rates among 17 snail populations living in the same district and subjected to single-miracidium exposures. This author considered four groups of populations according to their natural contacts with the parasite, from frequent contacts (snails living in swampy grasslands) to exceptional contacts (snails on the banks of rivers). In

populations with rare or exceptional contact with the parasite, there was a marked decrease in the number of infected snails, a reduced redial burden, a delay in cercarial differentiation and a longer patent period. The number of infected snails that died without shedding increased strongly, while that of settled metacercariae was significantly lower (Table 2.5). Previous contacts between the snail population and the parasite in the field may therefore have an influence on the success of an experimental infection. Secondly, this variability has even been observed within the same population of *G. truncatula* according to the different individuals that constitute it. When infected with *F. hepatica*, not all snails succeed in ensuring larval development of the digenean up to the cercaria stage. Vignoles *et al.* (2002a) noted a relationship between the prevalence of parasite infection and the ability of snails to sustain complete larval development of *F. hepatica* (Table 2.6). When the prevalence was greater than 20%, the highest frequency concerned snails that died after cercarial shedding. When prevalence ranged from 5% to 19%, the highest percentages were observed for snails harbouring free cercariae, rediae containing cercariae, or immature rediae. Below 5%, only immature rediae and/or sporocysts were observed.

Important variations in prevalences among populations were also reported in *Galba cubensis* infected by *F. hepatica* (Vázquez *et al.*, 2015). According to these authors, these differences might be due to environmental conditions and/or to differences in compatibility between snail population and parasite isolates. Intrapopulation variations could be due to genetic differences in compatibility or resistance to the parasite, as experimentally shown in *G. truncatula* by Meunier (2002), and/or to differences in snail age or physiological condition at the time of infection.

2.5.4.2 *Environmental factors*

The development of lymnaeid populations and, consequently, of *F. hepatica* larval forms is dependent on the climate in the region or country

Table 2.5. Characteristics of experimental infections in 17 populations of *Galba truncatula* subjected to single-miracidium exposures with *Fasciola hepatica* (origin, cattle) in relation to the frequency of natural contacts between snails and the parasite. Only mean values are given. From Rondelaud (1993).

Natural contact of snail population with the parasite	Frequent	Rather frequent	Rare	Exceptional or no contact
Number of populations	6	3	4	3
Snail mortality (%) on day 30 (20°C)	41.0	47.0	63.0	75.0
Infected snails (%)	57.0	47.0	35.0	32.0
Total number of rediae	32.0	29.8	22.5	14.1
Patent period				
Beginning	Day 53	Day 51	Day 57	Day 61
Length (days)	18.6	21.2	23.3	28
Number of settled metacercariae	132.0	124.5	83.1	67.1

Table 2.6. Frequency of *Galba truncatula* experimentally infected with *Fasciola hepatica* in relation to prevalence of infection and larval development stage (five groups). From Vignoles *et al.* (2002a).

	Mean frequency (%) for each group of infected snails				
Prevalence of infection (number of populations)	Cercarial shedding	No shedding. Presence of free cercariae	Cercariae-containing rediae	Immature rediae and sporocysts	Sporocysts only
> 60% (17)	56.8	18.6	10.7	8.4	5.5
40–59% (15)	53.5	21.1	10.2	6.0	9.2
20–39% (23)	50.1	18.1	14.2	9.3	10.3
5–19% (11)	14.0	35.3	22.7	19.5	8.5
< 5% (4)	0	0	0	61.3	38.7

where this lymnaeid lives. The most favourable conditions are represented by a temperature ranging between 10°C and 25°C and a high relative humidity depending on atmospheric precipitation. As a result, the disease is common in temperate regions, like most European countries. This relationship between climate and the parasitosis was demonstrated by the observations of Weybridge researchers in England (Ollerenshaw and Rowlands, 1959; Ollerenshaw and Smith, 1969; Ollerenshaw, 1974). Other authors have broadened this relationship between parasitosis and climate by incorporating other factors such as vegetation growth, local climatic variations (microclimate) and pasture topography (Malone *et al.*, 1998, 2001; Malone and Yilma, 1999). The existence of this relationship has since been confirmed by various authors that have studied the relationship between climate and fasciolosis (Mas-Coma *et al.*, 2009; Fox *et al.*, 2011; Olsen *et al.*, 2015).

A favourable temperature, the need for light so that there are abundant nutrients in the habitat, sufficient moisture and a type of soil with enough calcium for the snail to build its shell are needed for population development and optimal growth of individuals (Taylor, 1965). The range of temperatures compatible with maximum snail growth depends on the species of the lymnaeid and also on the location and type of habitat in which each population lives. In the case of lowland populations of *G. truncatula* in temperate countries, this scale of favourable temperatures ranges from 10°C to 25°C with an optimum between 18°C and 21°C because the algae, which constitute their main food source, then have a strong development. Above 25°C, *G. truncatula* usually emerges on the mud, thus creating unfavourable conditions (Kendall, 1953). The adult stage of this snail is attained from 4 mm in height, i.e. at the end of the fourth week of life (Kendall, 1953). A temperature range of 18–21°C and a high nutrient content result in cercarial shedding from the eighth week of infection in *G. truncatula* (Kendall, 1949b, 1964; Kendall and Ollerenshaw, 1963). Below 10°C, the animal remains active to a temperature of 1.5°C but it is no longer feeding in general (Kendall, 1953). During summer drought and high temperatures, the snail attaches to a support and quiesces with retraction of its body into the shell (Kendall, 1949a). During these two periods, larval development is clearly slowed down (Rondelaud and Barthe, 1978a; Rondelaud, 1994). In *Radix viridis*, a temperature between 20°C and 23°C and a diet with unicellular algae allowed it to obtain a size of 12 mm in 37 days (Lee *et al.*, 1994) and cercarial shedding from the 27th day post-exposure (Lee *et al.*, 1995).

The presence of water and favourable temperatures are necessary for the penetration of miracidia into the body of the host snail. These two parameters are also important for cercarial shedding of the parasite. Cercarial release from *G. truncatula* usually occurs above 10°C (Kendall and McCullough, 1951). Thermal shock from 20°C to 6–8°C for 30 min or 12°C for 3 h, followed by a gradual return to 20°C, can trigger cercarial shedding in infected *G. truncatula* but often leads to the death of the snails because of a massive release of cercariae (Abrous *et al.*, 1999b; Vignoles *et al.*, 2014b). The presence of water and/or relative humidity in the environment, when the water disappears, is important for the longevity of *F. hepatica* metacercariae (Torgerson and Claxton, 1999).

2.5.4.3 Factors depending on the snail

The factors that depend on the intermediate host are not yet all accurately known. This is the case, in particular, of the effect that the fitness of the snail at miracidial exposure may have on the success of an infection. The paragraphs below only relate to those that are currently well identified.

Not all age groups of a susceptible lymnaeid are able to ensure the complete larval development of *F. hepatica*. In the case of *G. truncatula*, snails 4 mm high (4 weeks of life at 20°C) are the most suitable, due to their high prevalence (Gold, 1980). Both older and younger age groups showed a gradual decrease in prevalence in relation to the increase or decrease in the height of their shells, respectively (Gold, 1980). Parasitological investigations focused mainly on juveniles (< 2 mm in height) to determine their potential role in the transmission of fasciolosis (Kendall, 1950; Berghen, 1964; Boray, 1966, 1969, 1978). According to Busson *et al.* (1982), the prevalence of infection varied according to lymnaeid species and infected individuals had reduced growth compared with that of unexposed

controls of the same age. In the field, the introduction of miracidia into the habitats of *Omphiscola glabra* during the spring egg-laying period resulted in a prevalence of 12.3% and a shell height of infected snails ranging from 2.1 mm to 5 mm (Bouix-Busson and Rondelaud, 1986). In contrast, if this introduction of miracidia was carried out for four successive years in the habitats of *Stagnicola palustris*, the prevalence and height of infected individuals gradually increased with the number of years (Dreyfuss *et al.*, 1994b). Cercarial production by these juveniles remains low (less than 20 per snail, whatever lymnaeid species) and no rhythm in cercarial production has been noted (Busson *et al.*, 1982; Bouix-Busson *et al.*, 1985b).

Kendall (1949b) and Kendall and Ollerenshaw (1963) demonstrated the effect of a high-quality diet on the redial and cercarial production of *F. hepatica*. They used unicellular algal cultures to feed snails and found a significant increase in the number of rediae and cercariae produced by well-fed *G. truncatula*. Other high-quality foods have been proposed to obtain optimal cercarial production (Kendall, 1949b; Malek, 1962; Boray, 1969; Pécheur, 1974; Bruce and Liang, 1992). The lowest cost prices for 100 metacercariae of *F. hepatica* were noted when algae or Boray's diet were used as food for *G. truncatula* (Rondelaud *et al.*, 2002b). The use of a commercial diet (Tetraphyll™ fish food) improved the production of cercariae by *G. truncatula* (Belfaiza *et al.*, 2004c) and also increased the number of cercariae released during each wave of shedding (Belfaiza *et al.*, 2004a). However, the contact of *G. truncatula* with this food must be limited in time (several hours twice a week) because of the snail's palatability for this type of diet, which may result in the death of some infected *G. truncatula* (Belfaiza *et al.*, 2004a).

Stressing *G. truncatula* just before its exposure to miracidia was used by our team to study consequences on the characteristics of *F. hepatica* infection. Discordant results, probably related to the intensity of the method used to generate stress, have been reported. Rondelaud (1994, 1995) analysed the effects of a 10-day experimental fast or exposure to a sublethal dose of cupric chloride on infected snails. In both cases, the prevalence of infection was lower than that of controls that were infected but not exposed to stress. The redial burden was decreased in stressed snails. Given these first results, Abrous *et al.* (2001a) used less aggressive methods to generate stress, such as fasting the snail for 3 days, immersing it in water at 6–8°C for 15 min, or exposing it to a very low dose of detergent. The survival of snails and the prevalence of infection were better in the stressed groups than in the infected controls not exposed to stress, while the redial burden and the cercarial production did not show any significant difference (Abrous *et al.*, 2001a). The action of predator-induced stress on the snail during parasite infection also has effects by decreasing the length of patent period and the number of cercariae shed by surviving *G. truncatula* (Rondelaud *et al.*, 2002b). Infection of *Lymnaea ovata* (= *Radix balthica*) from a population subject to regular herbicide pollution resulted in individuals up to 8 mm in height with active infection, while infected individuals were observed only in groups 1 mm and 2 mm of five control populations (Dreyfuss *et al.*, 2000). According to these authors, the intensity of the method or agent used to create stress could disrupt the snail's defence system and facilitate the larval development of *F. hepatica*.

The characteristics of *F. hepatica* infection are better in highland populations of *G. truncatula* than in the lowland populations (Mas-Coma *et al.*, 2001; Vignoles *et al.*, 2002c). According to these authors, the results could be an adaptation of these snails to the more extreme climate that prevails in altitude. In addition, populations of *G. truncatula*, known for their natural infections with *Haplometra cylindracea*, are better intermediate hosts for *F. hepatica* because infected individuals have high cercarial production, due to the length of the patent period (Vignoles *et al.*, 2007). This latter result has not been satisfactorily explained.

2.5.4.4 Factors depending on the parasite

The infectivity of miracidia is conditioned by the species of the definitive host from which *F. hepatica* eggs originate. According to Euzeby (1971), the hatching of eggs collected from rabbits is significantly lower than that of eggs from cattle (30% instead of 60–85%) and this author wondered about the role of these lagomorphs in the epidemiology of fasciolosis. Rondelaud and Dreyfuss (1995) infected three groups of *G. truncatula* with *F. hepatica* miracidia from three different

definitive hosts (cattle, rabbits and sheep) to test whether the mammalian species from which the miracidia originated did or did not modify the larval development in the snail. The redial burden and cercarial production were significantly lower in snails infected by rabbit-derived miracidia than in those with miracidia coming from cattle: an average of 3.7 live rediae and 54.5 cercariae instead of 29.7 and 216.6, respectively. The degenerate rediae were also more numerous in the rabbit group than in the two others (Rondelaud and Dreyfuss, 1995). As the development of a trematode within its definitive host varies according to the mammalian species (Haroun and Hillyer, 1986), it is necessary to admit, as pointed out by Euzeby (1971), that the development of *F. hepatica* up to the adult form would be inhibited in lagomorphs, thus leading to the laying of poorly fertile eggs by the parasite. The same experiment was repeated by Vignoles *et al.* (2001) using miracidia from four definitive hosts (cattle, nutria, rabbits and sheep). In this case, the redial burden in the nutria group was similar to that found in the bovine group, while the production of cercariae was much higher (Vignoles *et al.*, 2001). In view of these results, one may wonder whether this high cercarial production in the nutria group might be the result of a high infectivity of miracidia when the population of *G. truncatula* is allopatric. An argument in support of this hypothesis comes from reports by Gasnier *et al.* (2000), Goumghar *et al.* (2001), Sanabria *et al.* (2012, 2013) and Dar *et al.* (2013). According to these authors, the characteristics of *F. hepatica* infection in *G. truncatula* were better when the flukes, from which the miracidia originated, came from a foreign country (allopatric infections) than the country in which the snails were collected.

The influence of the definitive host on the subsequent larval development of *F. hepatica* in snails was also demonstrated when conditions of parasitism in mammals changed. The use of specific drugs such as triclabendazole to eliminate flukes from local livestock since 1990 (Mage *et al.*, 2002) is probably responsible for two phenomena: (i) unfertilized eggs were regularly found among other *F. hepatica* eggs at the slaughterhouse (up to 5% of eggs collected from cattle in 2006; Dreyfuss *et al.*, 2007); and (ii) some miracidia of *F. hepatica* did not hatch after egg incubation, even if eggs were exposed to sunlight for 2 h or more (Dreyfuss *et al.*, 2007). These disturbances might be the cause of a change in infectivity of cattle-derived miracidia. To test this hypothesis, Dreyfuss *et al.* (2007) used four populations of *G. truncatula* for their experiments between 1971 and 2006 and these authors noted two results (Table 2.7). First, the prevalence in single-miracidium infections was significantly higher in 2006 than in experiments prior to 1980, whereas snail survival on day 30 pe did not show a significant difference. Secondly, in two-miracidium infections, snail survival on day 30 in two snail populations was significantly lower in 2006 than in experiments carried out between 1981 and 1990, whereas prevalence values in the four populations of *G. truncatula* did not significantly differ from each other. As the average cercarial production for each infected snail was approximately the same, whatever the year of study and the number of miracidia used for each *G. truncatula*, a change in infectivity of the miracidium must be admitted. According to Dreyfuss *et al.* (2007), miracidia that succeeded in hatching from eggs in 2006 would be more aggressive towards local snails, so that a single miracidium for each

Table 2.7. Survival of snails on day 30 post-exposure, prevalence of *Fasciola hepatica* infection and number of cercariae shed by four populations of *Galba truncatula* depending on the year of the experiment and the number of miracidia used for each snail. From Dreyfuss *et al.* (2007).

Number of miracidia per snail	Experiments	Snail survival (%)	Prevalence (%)	Number of cercariae per snail
1	Before 1980	66.8–78.0	37.4–52	96.4–132.5
	2006	60.0–81.2	86.6–90.1	98.3–124.6
2	1981–1990	46.5–61.3	74.2–89.3	92.9–121.3
	2006	23.7–51.2	88.5–94.7	86.4–134.7

G. truncatula would be sufficient at the present time to have a good snail survival on day 30 pe and a high prevalence of infection.

The question also arises whether the treatment of the definitive host to control fasciolosis may affect the subsequent development of *F. hepatica* larval forms when the host snail is infected with miracidia from eggs laid by the surviving flukes. The results are currently contradictory. Walker *et al.* (2006) reported that snails infected with miracidia from triclabendazole-resistant flukes shed four fold more cercariae than those infected with miracidia from susceptible flukes. In contrast, cercarial production appeared to be higher in *G. truncatula* exposed to miracidia coming from triclabendazole-susceptible flukes than in snails infected with the other isolate (Hodgkinson *et al.*, 2018). As the breeding technique and diet can have an effect on cercarial production (Belfaiza *et al.*, 2004a; Rondelaud *et al.*, 2014a), further experimental infections are still needed to answer this question.

Another point concerns snail infections with several miracidia. Rondelaud and Barthe (1982a) carried out multiple-miracidium exposures and serial exposures of *G. truncatula* with *F. hepatica*. The same type of experiment was performed by Alba *et al.* (2018) in *Pseudosuccinea columella* susceptible to the parasite. These latter authors noted an increase in prevalence and redial burden when the number of miracidia per exposure or the number of successive exposures increased. Is it necessary to subject the host snail to more miracidia for significant cercarial production? This point is always debatable. Indeed, when the number of miracidia per snail increased from one to 20, the survival of *G. truncatula* on day 30 pe showed a corresponding decrease (Rondelaud and Barthe, 1982a), so that in the 20-miracidium group there were only a few infected snails that shed their cercariae. The most successful experimental infections carried out by our team were performed with one or two miracidia per snail, which is consistent with the results observed in natural infections of *G. truncatula* with *F. hepatica*. Another problem is the growth of the snail host shell during multiple-miracidium infection, as many immature rediae cannot differentiate in the snail and so cercarial production is generally lower.

2.5.5 Snail resistance to the parasite

The resistance of a mollusc to the parasite is difficult to demonstrate in the absence of histological sections. Resistance can only be established by the presence of fibro-amoebocytic granulomas formed around sporocysts or immature rediae. Sazanov (1972) found that infections of *Lymnaea stagnalis*, *Radix ovata* and *Stagnicola palustris* by Russian isolates of *F. hepatica* were rapidly stopped by a tissue reaction against the sporocyst. Granulomas were also observed in *S. palustris* (McReath *et al.*, 1982) and in non-susceptible populations of *Pseudosuccinea columella* (Gutiérrez *et al.*, 2003b) when these lymnaeids were subjected to experimental infections. The presence of these granulomas in *G. truncatula* was rare (Rondelaud and Barthe, 1980b, 1981; Diawara *et al.*, 2003) and most of them were observed around xiphidiocercariae after penetration into the foot of the snail (Rondelaud *et al.*, 2009b).

Experimental infections of a snail species are necessary to detect resistance: (i) by varying the age of the snail, the geographical origin of the miracidial isolate and the definitive host species at the origin of these larvae; and (ii) through monomiracidial or plurimiracidial infections and series of exposures with one or more miracidia. Of the 317 populations of *G. truncatula* that our team has subjected to an experimental infection during the past 45 years, only three showed abortive infections. The eight sets of experimental infections conducted from 1974 to 1981 with the first population using single-miracidium exposures were negative with high snail mortality (> 65% on day 30 to 20°C). The dissection of the surviving snails in two sets and the histological examination of about 20 individuals showed the presence of dead sporocysts and rediae, forming 'mummified cadavers' (Rondelaud *et al.*, 2009a). A similar finding was noted for two populations living along the banks of a river, because their exposure to the parasite only resulted in abortive infections (Rondelaud *et al.*, 1997). Since these snail populations had high mortality following miracidial exposure, it is difficult under these conditions to conclude resistance to the parasite.

The case of *Galba schirazensis* shows how difficult it is to demonstrate resistance to infection

with *F. hepatica*. Bargues *et al.* (2011) found no natural infection among the 8572 snails that these authors collected in different countries. In the same way, they obtained only abortive infections (with the exception of two individuals harbouring a few rediae) among the 171 snails infected experimentally with miracidial isolates from Egypt, Peru and Poland. This set of results led the authors to conclude that *G. schirazensis* was refractory to infection with *F. hepatica* (Bargues *et al.*, 2011). However, this lymnaeid is able to be infected and develop independent rediae and cercariae within its body after several generations of laboratory-reared snails and experimental exposures (Dreyfuss *et al.*, 2015c), indicating a gradual increase in snail susceptibility to the parasite. This result was confirmed in 2017 by the discovery of independent rediae in 26 field-collected snails (prevalence 1.7%) among the 1482 that Caron *et al.* (2017) studied by crushing, while the prevalence was 6% among the same individuals studied by a multiplex PCR. The role of *G. schirazensis* as a potential host snail of *F. hepatica* should not be totally ignored, especially if local adaptation occurs, as suggested in the report by Dreyfuss *et al.* (2015c).

2.6 Snail Co-infections

The term co-infection refers to the parasitism of a mollusc by two species of digeneans or a digenean and a protostrongylid. When both parasites enter the snail simultaneously, they can develop to a specific stage, but this process often results in the dominance of one parasite over the other. However, the infection of a snail with a parasite may be advantageous for the larval development of the second species. This facilitation effect has been observed for several species of digeneans in snail populations normally resistant to these parasites (Lie *et al.*, 1977a,b; Lie and Heyneman, 1979).

2.6.1 Co-infections by *Fasciola hepatica* and *Calicophoron daubneyi*

Vassilev and Samnaliev (1976) performed several cross-infections of *G. truncatula* with *F. hepatica* first and *C. daubneyi* second, or vice versa. According to these authors, the simultaneous development of both digeneans in the snail is possible because both types of cercariae were shed by two individuals. The best results obtained by Chipev *et al.* (1985) with *G. truncatula* came from infections with the *C. daubneyi*/*F. hepatica* sequence and a 24 h interval between the two monomiracidial exposures. Cross-infections of *G. truncatula* and *O. glabra* with the two parasites were also performed by our team by exposing pre-adult snails to *C. daubneyi* and then to *F. hepatica* 4 h later. These infections resulted in the larval development of *C. daubneyi*, *F. hepatica*, or both until the release of cercariae (Abrous *et al.*, 1996; Augot *et al.*, 1996; Abrous, 1998). Such co-infections of *G. truncatula* and *O. glabra* with live larval forms of the two digeneans were also found in the field during several malacological investigations in the French departments of Corrèze, Creuse and Haute Vienne (Abrous *et al.*, 1999a, 2000; Rondelaud, 2004). Other co-infections with the two parasites were noted by Jones *et al.* (2017) in Welsh farms. Among the 892 *G. truncatula* these authors collected, two snails were co-infected, the first with *F. hepatica* cercariae and *C. daubneyi* DNA and the other with the DNA of both parasites.

Rondelaud *et al.* (2004b) collected a total of 24,764 *G. truncatula* for 10 years (1993–2002) and found only 111 (0.4%) snails co-infected with live larval forms of *C. daubneyi* and *F. hepatica*. According to these authors, the number of alive and free rediae was low: an average of 7.1 for *C. daubneyi* and 6.4 for *F. hepatica*. Experimental infections of *G. truncatula* with both digeneans also revealed that each co-infected snail provided an average of 52.7 metacercariae for *C. daubneyi* and 83.7 for *F. hepatica* during the same shedding waves (Rondelaud *et al.*, 2009b). A retrospective study was conducted by Vignoles *et al.* (2017c) on the different samples of *G. truncatula* and *O. glabra* that these authors collected between 1993 and 2010. Experimental infections of both snail species during the same period were also analysed to study the metacercarial production of each digenean in co-infected snails. In natural co-infections, the prevalence was 0.7% in *O. glabra* (186/25,128 dissected snails) and 0.4% in *G. truncatula* (137/31,345). Low redial burdens were observed in these snails and *F. hepatica* rediae were significantly more numerous in *O. glabra* than in

G. truncatula (7.5 per snail instead of 5.2). The numbers of *C. daubneyi* rediae in the two lymnaeids were close to each other (4.3 in *O. glabra* and 3.0 in *G. truncatula*). In experimental co-infections, the prevalence was 3.0% in *O. glabra* (24/800 snails exposed to miracidia) and 6.3% in *G. truncatula* (38/600). The prepatent period was shorter and the number of metacercariae was lower in *O. glabra* (an average of 17.4 for *C. daubneyi* and 27.2 for *F. hepatica*) than in *G. truncatula* (37.3 and 78.5, respectively). In the two lymnaeids, both types of cercariae were released during the same waves and several peaks during the patent period were synchronous. These last results can be explained by two perhaps complementary hypotheses. First, cercariae of the first digenean (*C. daubneyi* or *F. hepatica*) would dig one or several tunnel-shaped lacunae through the snail's perianal region to exit from their intermediate host and these lacunae would be used by cercariae of the other digenean. The other hypothesis is derived from the report by Kendall and McCullough (1951), who noted an accumulation of well-differentiated *F. hepatica* cercariae around the snail's posterior intestine before cercarial exit. In these co-infected snails, the accumulation of both digenean cercariae within this space would progressively increase parasite pressure on the snail's close viscera and finally trigger simultaneous release of cercariae (Vignoles *et al.*, 2017c).

2.6.2 Co-infections with *Fasciola hepatica* and other parasites

Other examples of natural co-infections have been reported in the literature. Samnaliev *et al.* (1978) noted co-infections of *G. truncatula* with *Calicophoron microbothrium* and *F. hepatica*, or with *C. microbothrium* and *Echinostoma lindoense*. Manga-González *et al.* (1994) found three *G. truncatula* co-infected with *F. hepatica* and *Plagiorchis elegans*, and another snail with *Notocotylus reynai* and *Opisthoglyphe ranae.* Rondelaud *et al.* (2004b) reported two *G. truncatula* co-infected with *F. hepatica* and *Haplometra cylindracea,* and two others with *F. hepatica* and an unidentified echinostomatid. Six co-infected *G. truncatula* (five with *C. daubneyi* and *F. hepatica*, and the other with *C. daubneyi* and *O. ranae*) were found by Rondelaud *et al.* (2016) in the Brenne Natural Regional Park (central France) between 2012 and 2014. On the other hand, Vázquez *et al.* (2015) did not find any co-infection in *Galba cubensis* during their parasitological investigation in Cuba. Similarly, no co-infection in *O. glabra* was noted in the Brenne Natural Regional Park in 2014 and 2015 (Rondelaud *et al.*, 2015b).

Experimental co-infections of *G. truncatula* were also performed by our team. First, snails shedding cercariae of *Haplometra cylindracea* were exposed to *F. hepatica* miracidia. On the 42nd day of the experiment, 31–37% of *H. cylindracea* sporocysts were degenerating without formation of granulomas, while the number of *F. hepatica* rediae ranged from 9 to 15 and their differentiation was slower (Moukrim *et al.*, 1992b, 1993). Secondly, co-infections of *G. truncatula* with *F. hepatica* and *Muellerius capillaris* (Protostrongylidae), or vice versa, were performed with an interval of 4 h or 7, 14 or 30 days between the two exposures (Hourdin *et al.*, 1992, 1993). Snails with *F. hepatica* were the most numerous (a total of 86), while snails with *M. capillaris* or both parasites were 14 and 9, respectively). Several third-stage larvae of *M. capillaris* were found in the snail foot from day 35 or day 42. The number of both types of larvae was reduced, whatever the parasite species. The lack of domination of one parasite over the other can be explained by the mode of development of these larval forms (polyembryonic for *F. hepatica* and monoembryonic for *M. capillaris*).

Chaetogaster limnaei limnaei is known to be a predator of *F. hepatica* miracidia and cercariae (Rajasekariah, 1978; Ibrahim, 2007). Conflicting results have been reported in the literature. Peruvian *G. truncatula* carrying *C. l. limnaei* and subjected to *F. hepatica* miracidia showed a prevalence of 13.3% on day 40 pe, while the infection rate was 70% in non-carrier snails (Muñiz-Pareja and Iturbe-Espinoza, 2018). In contrast, French *G. truncatula* carrying this worm and exposed to *F. hepatica* died between the 14th and 21st day after exposure, while the non-carrier controls sustained complete larval development of the digenean (Rondelaud *et al.*, 2009a). In these conditions, one may wonder whether this ectoparasite, by its presence, would not weaken the snail and cause its death when it is subjected to *F. hepatica*.

2.7 Consequences of Parasitism on the Snail

2.7.1 Visceral pathology in the snail

Penetration of a miracidium or first-stage protostrongylid larva into *G. truncatula* induces the development of epithelial necrosis, followed by reconstitution. Four viscera, namely the digestive gland, gonad, albumen gland and kidney, are particularly affected by these processes during an experimental infection. The other organs are clearly less affected. These lesions are not specific to parasitism, as they have also been observed when the snail is exposed to metal salt (Rondelaud *et al.*, 1976) or the organochlorine pesticide Thiodan™ (Cergiz *et al.*, 2005). Similarly, this visceral pathology has been noted in other Lymnaeidae such as *Lymnaea stagnalis*, *Omphiscola glabra*, *Radix balthica* or *Stagnicola palustris* when infected with a digenean (Sindou *et al.*, 1990a,b, 1991).

2.7.1.1 Description of tissue lesions

The digestive gland was the most studied viscus in *F. hepatica*-infected snails (Rees, 1931; Barber, 1962; Southgate, 1969; Hodasi, 1972a; Humiczewska, 2001a,b). In *G. truncatula*, epithelial necrosis appeared at week 2 of snail infection at 20°C and developed until week 7. Peripheral digestive tubules ceased their growth. Necrosis first affected the calcium cells of each tubule. Numerous pockets of vacuolated cytoplasm, coming from the secretory cells, were then released in the tubular lumen from week 3, with a peak at week 6. When reconstitution began from week 6, the number of tubules increased again, but their diameter was always smaller than that found in the glands of unexposed controls. All types of epithelial cells showed hyperplasia. Due to their number and volume, hyperplastic calcium cells formed convex protuberances on the outer side of the tubules and anisocaryosis was observed in most of them (Rondelaud and Barthe, 1978c).

In the gonad of *G. truncatula*, all follicular and germ cells disappeared from week 4 after penetration of a redia (one-third of the cases). The atrophy of this gland was often followed by the formation of a fibrous tract. In the other snails, a multifocal necrosis appeared from week 2 and first affected the germinal epithelium close to the rediae. Most oocytes and spermatozoa were then removed from the gland, whose diameter and number of acini gradually decreased. Necrosis became generalized from week 4 and was followed by reconstitution from week 7. The diameter of the acini and the number of oocytes gradually increased, but their values were always lower than those observed in the gonads of unexposed controls. The thickness of the epithelium was often reduced (Rondelaud and Barthe, 1980c). In juvenile snails, the atrophied gonad could sometimes disappear from the body. Epithelial necrosis might also result in the disappearance of the male or female germinal line (Bouix-Busson *et al.*, 1985a). Epithelial necrosis and reconstitution also affected the albumen gland. The lesions were more difficult to identify in the annex glands of the snail's genital system, i.e. the three segments of the oviduct and the prostate (Rondelaud and Barthe, 1980c).

In the kidney of *G. truncatula*, several changes were observed in weeks 4 and 5 of infection. Numerous free concretions, coming from necrotic nephrocytes, and nodules consisting of disorderly fibroblasts and nephrocytes, have been noted in the renal lumen. The lamellae were always dilated and their length reduced. From day 28 of infection, nephrocytes showed hyperplasia with a decrease in their height and the presence of small-sized concretions. Another necrosis-reconstitution cycle was observed between day 35 and day 56 of infection, but these changes were heterogeneous (Rondelaud and Barthe, 1983). Intra-lamellar deposits and/or oedema were also observed in these kidneys (Sindou *et al.*, 1990a,b).

During the first weeks of infection, circulating amoebocyte proliferation has sometimes been observed in the visceral cavity of snails (Rondelaud and Barthe, 1980b, 1981). In *G. truncatula* infected with *F. hepatica*, the number of neurons in the dorsal lobes of cerebroid ganglia and in the pedal ganglia decreased during the time of infection. Numerous necrotic neurons and others showing anisocaryosis have been noted in both types of ganglia (Szmidt-Adjidé *et al.*, 1996).

Beesley *et al.* (2018) considered the pathogenic effect of *F. hepatica* on the snail to be relatively low. Although this pathology does not lead to the death of the snail, it is highly destructive in the infected snail: there is a decrease in the number of digestive tubules, atrophy or destruction of the gonad, atrophy of the albumen gland and decrease in the number of renal lamellae (Rondelaud *et al.*, 2009b).

2.7.1.2 Development of tissue lesions and parasite infection over time

In the infected snail, lesions affecting the four viscera developed over the same time interval. It was therefore useful to compare the development of this visceral pathology with that of the larval forms of *F. hepatica* in the snail body. Figure 2.4 was prepared with the data on *G. truncatula*, as it is the species for which our team made the most numerous observations on serial sections of infected snails. Necrosis appeared rapidly in the kidney and albumen gland, while it began later in the digestive gland and gonad. Apart from the kidney, for which epithelial reconstitution occurred from day 21, necrosis developed in the other three viscera during the period of cercarial differentiation in the body of parental rediae. The epithelial reconstitution only began from day 42, while the first cercariae became free in the body of the snail.

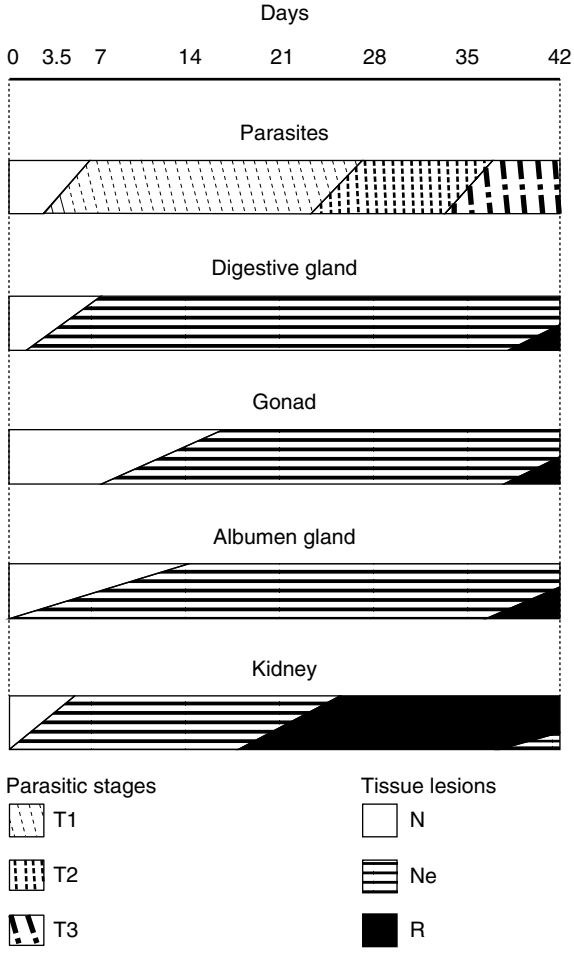

Fig. 2.4. The response of four viscera in *Galba truncatula* infected with *Fasciola hepatica*. The chronology of tissue lesions and the larval developmental stages of the parasite are given up to day 49 post-exposure. Parasitic stages: T1, immature rediae; T2, cercaria-containing rediae; T3, free cercariae within the snail's body. Tissue aspects: N, normal aspect of the organ; Ne, epithelial necrosis; R, epithelial reconstitution. From Rondelaud, Dreyfuss and Cabaret (2003) Les mollusques d'intérêt vétérinaire, in: Provost, Uilenberg, Blancou and Lefèvre, eds., Maladies Infectieuses et Parasitaires du Bétail des Régions Chaudes. Editions Lavoisier (Fig. 6, page 190). With permission from the Editions Lavoisier, Cachan.

In the case of the kidney, this date corresponded to the appearance of a new wave of necrosis.

The above results suggest that epithelial necrosis is caused by rediae when they differentiate near the digestive gland, gonad and/or albumen gland. Direct consumption of the digestive tubules by rediae has sometimes been observed on serial sections of infected snails. But this is not the only reason and it is necessary, according to Euzeby (1971), to relate the presence of these lesions to the development of these larvae and also to local toxic action. In the case of the kidney, this viscus is involved in the purification of the haemolymph of most wastes by means of its nephrocytes, which collect these products in their apical concretions. This action causes a rapid development of necrosis, followed by a reconstitution that appears earlier than in the other three organs, whereas the kidney continues its functional activity. According to Graczyk and Fried (1999), digestion of the snail tissues into glycogen, amino acids and fatty acids by differentiating rediae was also the cause of necrosis.

Epithelial necrosis and reconstitution were also found in *G. truncatula* infected with *C. daubneyi* (Moukrim and Rondelaud, 1992a). They were also observed in the case of protostrongylidae, while the larva only developed in the foot of the snail (Hourdin *et al.*, 1990).

2.7.2 The other consequences of parasitism

Many authors have used several snail–parasite models to study the biochemical changes that occur in the intermediate host during the development of larval forms (see for example Ginetsinkaya, 1988 and Thompson, 1997). According to Graczyk and Fried (1999), four processes can be observed in *F. hepatica*-infected snails: (i) infected snails release up to three times more heat than unexposed snails; (ii) increased oxygen consumption; (iii) increased metabolism; and (iv) the size of snails could increase, leading to the notion of 'gigantism' in several papers (Hodasi, 1972a; Wilson and Denison, 1980; Chappuis, 2009). The larval development of a digenean causes a decrease in the glycogen present in the digestive gland and foot of the snail, as larvae transform it into glucose (Cheng, 1963; Cheng *et al.*, 1963). Similarly, in the case of *F. hepatica*, lipids and in particular fatty acids are used as an energy source, with a gradual decrease of these elements in the snail and a corresponding accumulation in rediae and differentiating cercariae (Southgate, 1970; Moore and Halton, 1973; Humiczewska and Rajski, 2005).

According to Thompson (1997), the increase in the size of the infected snail is the result of an increase of soft masses and shell. Wilson and Denison (1980) considered that 'gigantism' would result from the switch of nutrients from reproduction to the growth of somatic tissues and parasites. It would be of nutritional rather than endocrine origin. An argument supporting this interpretation is parasitic castration, as the gonad has been destroyed in many infected snails, either by penetration of one or several rediae inside, or by compression exerted by rediae present around the gland.

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3 Development of *Fasciola hepatica* in the Mammalian Host

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3.1 Introduction

In the 20 years since the first edition of this book was published there have been considerable advances in our understanding of *Fasciola* biology, largely at the molecular level. Nevertheless, a detailed understanding of the fine structural organization of the major organ systems in the fluke, and the changes that these undergo during development within the mammalian host, remain as relevant as ever by providing a structural context for understanding temporal changes in gene expression, protein production and even post-translational modification (e.g. glycosylation). The aim of this chapter is to present the current understanding of *Fasciola* anatomy at the ultrastructural level and will cover the tegument, the parenchyma, the musculature, the nervous system, the cytoskeleton, the gut and the excretory system. The reproductive system, a complex of male and female organs, is examined separately in Chapter 4 of this volume. Each tissue will be examined in turn and supplemented with representative micrographs to create an 'atlas' of *Fasciola* ultrastructure. Finally, advances in the use of *in vitro* models of *Fasciola* infection will be reviewed with emphasis on how these are driving our current understanding of fluke development at cellular and molecular levels.

3.2 Tegument

The tegument is the layer in most intimate contact with the host's tissues and body fluids. As such, it represents a site where considerable biochemical, physiological and immune interplay takes place between the fluke and its host. It is a syncytial, protoplasmic layer connected by cytoplasmic strands to nucleated cell bodies located in the general body parenchyma below the main somatic muscle layers. Moreover, it is a metabolically active layer specialized to carry out a number of functions: synthesis and secretion of various substances; absorption of nutrients; osmoregulation; protection against the host's (and parasite's) digestive enzymes and the surfactant properties of bile; and protection against the host's immune response. Furthermore, it possesses a sensory role essential in migration, growth and development. In this section, the basic organization of the tegument will be described first, then the developmental changes, before discussing the different functions.

3.2.1 Surface features

The adult fluke has a leaf-like shape, is dorsoventrally flattened and has fluted margins. At the

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anterior end is the oral cone, with the oral sucker at its tip and the ventral sucker at the oral cone–main body junction. The two suckers are the main organs of attachment, used to grip the lining of the bile duct and to assist the migratory movements of the fluke. The genital pore, which is the common opening for the male and female reproductive systems, lies just anterior to the ventral sucker.

The general body surface is covered by spines, which are closely packed, dorsoventrally flattened and point posteriorly. The spines help to maintain the position of the fluke in the bile duct; they also serve to erode the epithelium and puncture blood vessels, in this way playing a role in feeding. On the oral cone, the spines occur in a regular pattern of 60 rings, each with 60–70 spines, whereas those on the posterior body are scattered without any pattern of rings (Bennett, 1975a,b). The number of spines in the posterior region of the body doubles (from 3000 to 6000) during the first week post-infection (pi) and increases up to eightfold by 3 weeks pi. The spines also elongate during development of the fluke to maturity – the anterior spines approximately 8 times, the posterior spines approximately 24 times. Between 2 and 3 weeks pi, all the spines on the body surface metamorphose from being single-pointed to multi-pointed, by division at the spine tips. The anterior spines have between 10 and 15 points, whereas the posterior spines have up to 30 points, but the overall shape comes to resemble a child's mitten (Bennett, 1975b). In the adult fluke, the spines on the ventral surface of the oral cone are tightly packed and broad, with a number of 'spinelets', or serrations, along their tips. The spines on the dorsal surface of the oral cone are also tightly packed with posterior serrations, but they are generally narrower and slightly longer than those on the ventral surface. Both surfaces of the midbody region are covered in spines of a similar morphology that are typically broader than those on the oral cone and are not as closely arranged, and the serrations along their tips are not as well-defined. The spines on the tail surface, again, are very similar on both surfaces and are smaller, particularly in terms of length, than the spines covering the rest of the fluke's surface (McConville *et al*., 2009) (Fig. 3.1).

Also scattered over the body surface are groups of sensory papillae, which are most numerous in the anterior region, around and inside the suckers (where spines are missing) and on the ventral surface. The papillae are conical and have a shallow pit into which the tip of a cilium projects. In the suckers there are low, conical papillae which do not have an exposed ciliary tip; they may be mechanoreceptors, whereas the ciliate type may be either mechano- or chemoreceptors (Bennett, 1975a).

3.2.2 Fine structure

The following account is based on the definitive studies by Threadgold (1963, 1967) (Fig. 3.2). The tegument comprises a surface syncytial layer of cytoplasm, joined by cytoplasmic connections to nucleated tegumental cell bodies situated beneath the fibrous basal lamina and the circular and longitudinal muscle layers. The syncytium is 15–20 μm thick and the surface is folded into a series of broad plateaux separated by deep valleys. The surface area is further increased by small, flask-shaped intuckings of the apical plasma membrane, known as apical invaginations. The apical membrane is covered by a thick glycocalyx (see below). The basal plasma membrane is invaginated to form long, parallel-sided structures known as basal infolds which reach almost to the apical plasma membrane. Associated with the basal infolds are ill-defined membrane-less vacuoles of relatively low electron density which contain mucopolysaccharide (Threadgold and Brennan, 1978). The basal lamina (or basement membrane) comprises three layers: the lamina lucida, lamina densa and lamina reticularis (Stoitsova and Gorchilova, 1997). The lamina densa consists of amorphous electron-dense material which protrudes into the basal infolds. The lamina reticularis appears as a dense network of fibrils. Anchoring fibrils cross this layer and form loops. Along their length they contact hemidesmosomes of muscles, thus connecting muscle to muscle and muscle to tegument. Where tegumental spines reach the basal lamina, the contact is reinforced by numerous hemidesmosomes that connect to the lamina densa and to anchoring fibrils that reach towards the underlying muscles. It has been suggested that spine orientation and movement are under the control of tegumental–muscle contractions by means of the anchoring fibrils (Stoitsova and Gorchilova, 1997).

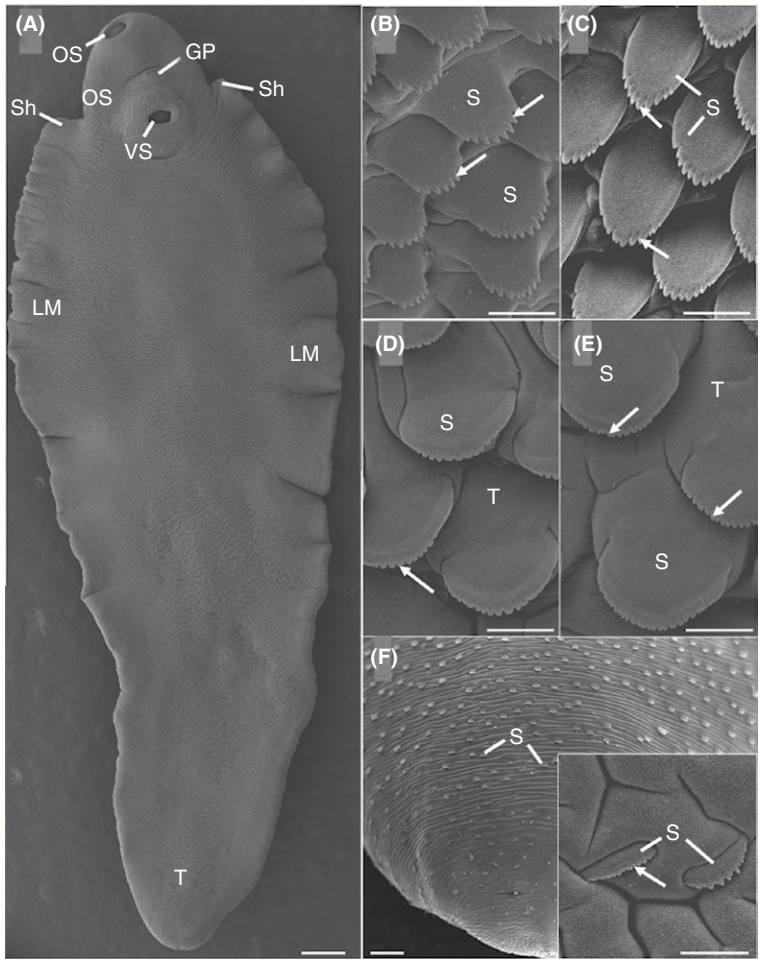

Fig. 3.1. Scanning electron micrographs (SEMs) of adult *Fasciola hepatica*. **(A)** Low-power SEM of the ventral surface of an adult liver fluke showing the oral sucker (OS), genital pore (GP) and ventral sucker (VS). So-called 'shoulders' (Sh) are at the very anterior of the midbody region. The entire tegumental surface of the fluke is covered in posteriorly directed spines. LM, lateral margin; T, tail. Bar 1 mm. **(B)** Tightly packed, posteriorly pointing spines (S) from the ventral surface of the apical cone region. The spines are broad with distinctive serrations along their tips (arrows). Bar 25 µm. **(C)** Spines (S) from the dorsal surface of the apical cone region. Spines are tightly packed, longer than they are broad, and have distinctive serrations along their tips (arrows). Bar 25 µm. **(D)** Large, wide spines (S) with shallow serrations (arrow) along their tips cover the ventral surface of the midbody region. Between spines, the tegument (T) is visible. Bar 25 µm. **(E)** Dorsal surface of the central midbody region showing large, broad spines (S) with shallow serrations (arrows) along their tips. T, tegument. Bar 25 µm. **(F)** SEM of the dorsal surface of the tail region, showing a low density of spines (S) adorning the tegumental surface. Bar 100 µm. Inset shows a high-magnification SEM of spines (S) from the tail region. The spines are small and blunt in appearance, but have a few, irregular serrations along their tips (arrow). Bar 12.5 µm (McConville *et al*., 2009).

The cytoplasm of the syncytium contains numerous mitochondria, concentrated basally and which are often arranged in vertical chains parallel to the basal infolds (Fig. 3.3). In the adult fluke, the syncytium also contains two types of secretory body, designated the T1 and T2 secretory bodies. The T1 bodies are round and uniformly electron-dense and occur in a

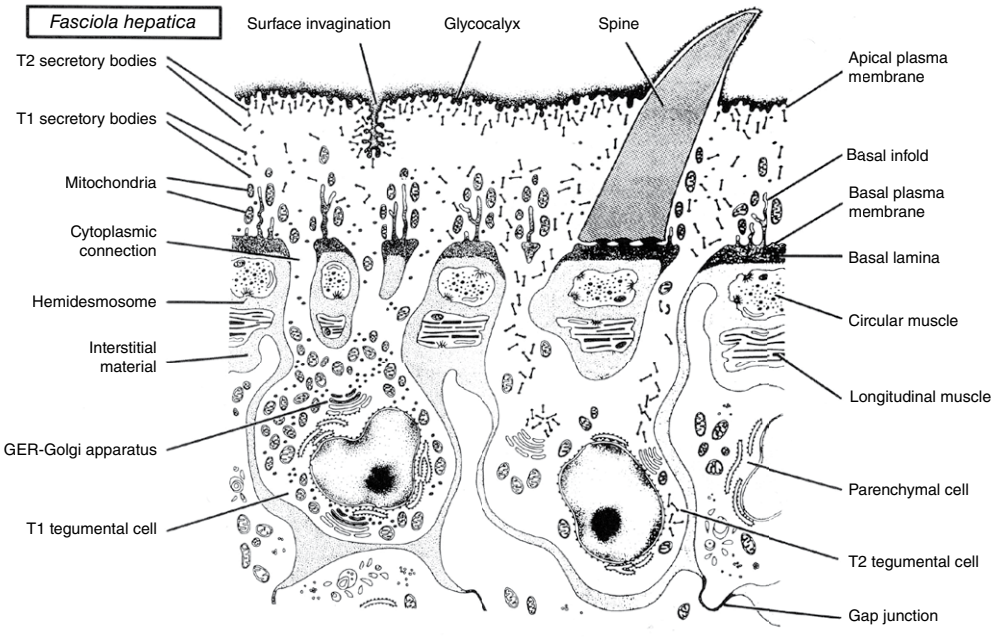

Fig. 3.2. Diagram showing the fine structural organization of the tegument and related structures in *Fasciola hepatica* (based on Threadgold, 1963, 1967; Smyth, 1994).

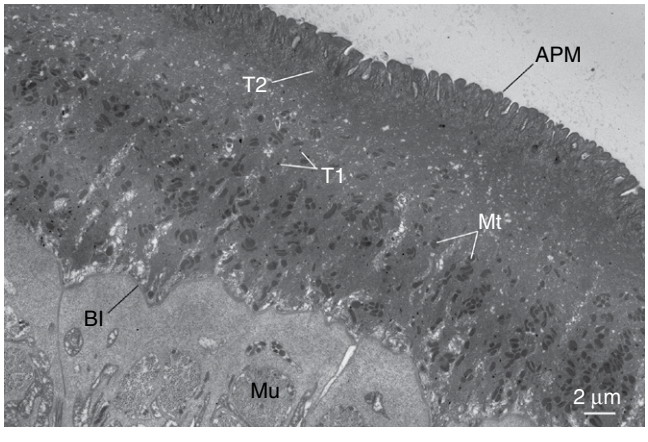

Fig. 3.3. Transmission electron micrograph of the tegument of adult *Fasciola hepatica* showing the characteristic invaginations of the apical plasma membrane (APM) and numerous T1 and T2 secretory bodies. Note also the chains of mitochondria (Mt), the basal lamina (Bl) and the underlying subtegumental muscle blocks (Mu).

gradient within the syncytium, with the greatest number situated basally. The T2 bodies have the shape of a biconcave disc with a less dense centre and have a gradient opposite to that of the T1 bodies, being concentrated apically, where they often lie with their long axis at right angles to the apical plasma membrane. A third type of secretory body, the T0 body, is present in the newly excysted juvenile (NEJ), but once the juvenile enters the liver the T0 cells metamorphose into T1

cells. The T0 bodies are very dense biconcave discs (Bennett and Threadgold, 1973, 1975). The glycoprotein nature of the secretory bodies, developmental changes in their production and their antigenic roles are discussed below. The spines project well above the general surface of the tegument but are completely enclosed within the apical and basal plasma membranes. The spines are firmly attached to the basal plasma membrane by dense bodies resembling hemidesmosomes. They have a crystalline structure and have been shown to be composed of a form of actin, with a molecular mass of 15 kDa (Stitt *et al.*, 1992a) (see also section 3.4).

In his original account of tegument ultrastructure, Threadgold (1963) described the presence of small, membrane-bound vesicles. It is only in the past few years that the role these vesicles play in fluke biology has become understood. They have been termed extracellular vesicles (EVs) and are formed in the syncytium itself and released directly from the apical plasma membrane (Marcilla *et al.*, 2012; Cwiklinski *et al.*, 2015a; de la Torre-Escudero *et al.*, 2016) via a mechanism akin to the formation of surface blebs produced in response to the stress induced by drug treatment (de la Torre-Escudero *et al.*, 2016; see also Chapter 7, this volume) (Fig. 3.4). Structures resembling multivesicular bodies (MVBs), the main source of endosomal-derived EVs in mammalian cells, have also been observed within the tegumental syncytium (Wilson *et al.*, 2011; de la Torre-Escudero *et al.*, 2016). However, despite the large number of ultrastructural studies performed on *F. hepatica*, these putative MVBs have not often been observed, suggesting

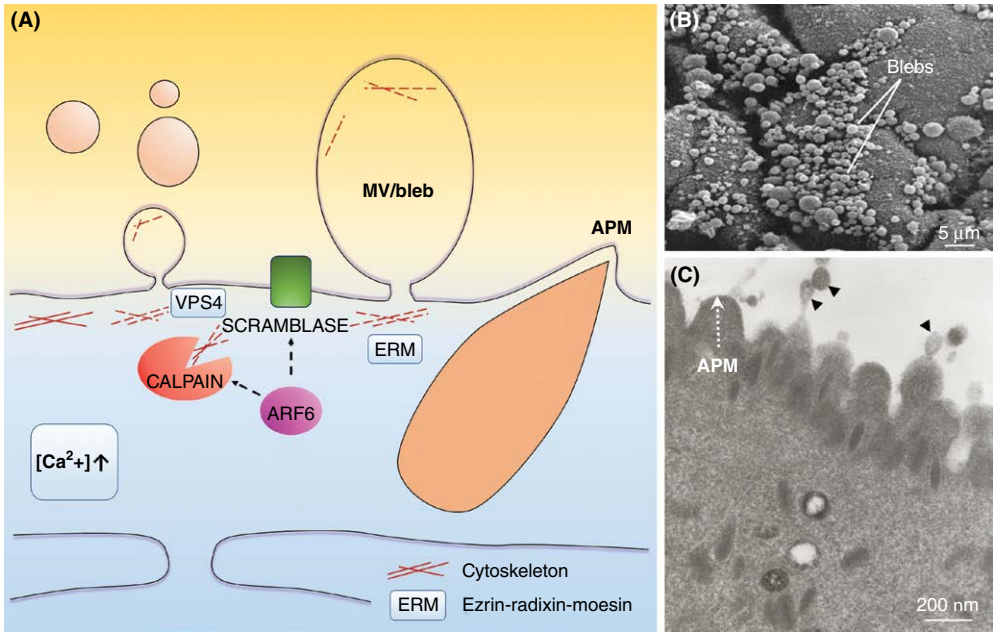

Fig. 3.4. Microvesicle/bleb release from the apical plasma membrane of the *Fasciola hepatica* tegument. **(A)** Schematic representation of the proposed mechanism of bleb/microvesicle (MV) formation and release from the apical plasma membrane (APM) of the *F. hepatica* tegument. An increase in intracellular Ca^{2+} concentration triggers a signalling cascade that promotes the excision of MVs/blebs directly from the tegmental surface. This 'budding' process requires a state of membrane asymmetry, conducted by phospholipid translocases (e.g. scramblase) which is driven further by calpain-dependent cleavage, and rearrangement, of the actin cytoskeleton. The actin-binding proteins ezrin, radixin and moesin (ERM) are also involved in the reassembly of the cytoskeleton following MV/bleb formation. Although the final abscission mechanism of the MV is not fully understood, the AAA-ATPase VPS4 may be involved. **(B)** SEM of the surface of the *F. hepatica* tegument showing profuse blebbing. **(C)** TEM of the *F. hepatica* tegumental syncytium showing the pinching of blebs/MVs from the apical plasma membrane and their release from the tegumental surface. Arrowheads; MV/blebs (de la Torre-Escudero *et al.*, 2016).

that they are transient (and thus difficult to capture by transmission electron microscope) or that this EV biogenesis pathway is of minor importance in liver fluke. The tegument is one of a number of tissues in which EVs are produced: they have been localized in the gut (see section 3.7), excretory system and parenchyma as well (Bennett *et al*., 2020a). Orthologues of sphingomyelinase (SMase) and other components of EV formation in mammalian cells have been demonstrated in *F. hepatica* EVs, suggesting that similar biogenesis mechanisms operate in the fluke (Bennett *et al*., 2020a; Bennett *et al*., 2020b). While the vesicles are present in all mammalian life cycle stages (Sanchez-Lopez *et al*., 2020), the cargo molecules they contain are developmentally regulated and therefore variable (Cwiklinski *et al*., 2015a). Proteomics analysis has revealed a large number of protein molecules, including a range of immunomodulators, which have been localized within the vesicles by means of immunogold labelling (Marcilla *et al*., 2012; Cwiklinski *et al*., 2015a; de la Torre-Escudero *et al*., 2016; Cameron *et al*., 2017). The protein and carbohydrate composition of the vesicle membrane has also been characterized by means of proteomics techniques and fluorescently labelled lectin microarrays (de la Torre-Escudero *et al*., 2016, 2019). The surface proteins are antigenic and they direct internalization of EVs by host cells (de la Torre-Escudero *et al*., 2019). Mannose-containing oligosaccharides predominate on the vesicle surface (de la Torre-Escudero *et al*., 2019), as is true of the tegumental glycocalyx itself (see section 3.2.4, below). Following their release, the vesicles are internalized by host cells (e.g. macrophages) and this allows for two-way communication between parasite and host (Marcilla *et al*., 2012; Cwiklinski *et al*., 2015a; de la Torre-Escudero *et al*., 2019). This may facilitate fluke migration through host tissues and help to counteract host immune attack; EVs are recognized as having a major immunomodulatory role (Roig *et al*., 2018). Treatment of flukes with GW4869, an inhibitor of SMase activity, has been shown to inhibit the release of tegumental EVs; it also caused severe vacuolation of the tegumental syncytium and a decrease in the numbers of T2 secretory bodies (Bennett *et al*., 2020a). Vacuolation and disruption of the gut, parenchyma and protonephridial epithelium were also observed (Bennett *et al*., 2020a). This result has provided the first experimental evidence that targeting EV biogenesis and release is a valid strategy for future therapeutic development. Blocking the uptake of EVs by host cells represents an alternative approach (Cwiklinski *et al*., 2015a).

The nucleated tegumental cells below the subtegumental muscle layers are of two types, the T1 and T2 cells, corresponding to the type of secretory body they produce. The cells occur in groups, with the T1 cells being more numerous: in adults, the T1:T2 ratio is approximately 4:1. The T1 cells contain a large nucleus with a prominent nucleolus, numerous mitochondria, a network of cisternae of granular endoplasmic reticulum (GER) and a number of distinct Golgi complexes which produce the secretory bodies. All these features are typical of an active secretory cell (Fig. 3.5). The T2 cells are similar, but have a less extensive GER system, fewer and less

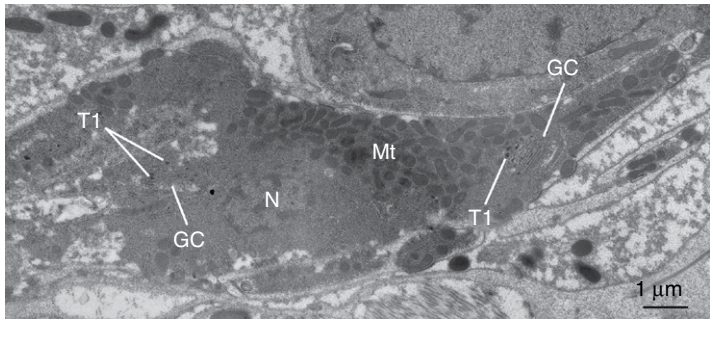

Fig. 3.5. Transmission electron micrograph of a *Fasciola hepatica* T1 tegumental cell showing the nucleus (N) and in the cytoplasm a number of Golgi complexes (GC) giving rise to T1 secretory bodies (T1). Note also the numerous mitochondria (Mt) in the cell.

well-defined Golgi complexes and fewer mitochondria but greater numbers of secretory bodies in the cytoplasm.

A number of enzymes have been demonstrated in the tegument, including acid phosphatase, Na^+/K^+-ATPase, esterase, peroxidase, cytochrome oxidase and succinic dehydrogenase. Acid phosphatase activity, for example, occurs in the syncytium and is mainly associated with the apical plasma membrane, either close to, or in contact with, its inner component; greater activity is evident in the dorsal than in the ventral tegument (Threadgold, 1968; Fujino *et al.*, 1983). Na^+/K^+-ATPase activity is particularly associated with the invaginations of the apical plasma membrane and the infoldings of the basal plasma membrane; this is indicative of the presence of ion pumps on the tegumental membranes. The enzyme is inhibited by ouabain, a known inhibitor of Na^+/K^+-ATPase activity, and there is greater activity in the ventral than in the dorsal tegument (Threadgold and Brennan, 1978; Skuce *et al.*, 1987). The role of ion pumps in osmoregulation is discussed in section 3.2.6.2.

Tegumental preparations of adult fluke have been subjected to proteomic analysis, revealing a rich variety of proteins, including membrane enzymes, membrane-spanning transporters and associated ATPases, cytoskeletal proteins, surface defence proteins, enzymes of energy metabolism and elements of secretory pathways (Wilson *et al.*, 2011; Haçariz *et al.*, 2012, 2014; Cameron *et al.*, 2017). Identification of proteins in the tegument of *F. hepatica* will further our understanding of the roles of the tegument at the host/parasite interface and identify potential candidates for vaccine development.

Tegumental protein changes in the NEJ have been monitored following incubation with host intestinal epithelial cells. The host intestine is the first barrier that must be overcome by the fluke in order to establish infection and, after incubation, proteomic analysis showed that many proteins in both parasite preparations and host cells were up- or downregulated (González-Miguel *et al.*, 2020). The changes reflect the dynamic interaction that occurs between parasite and host and, by gaining a greater understanding of the processes involved, this might lead to the identification of novel drug and vaccine targets.

While the tegument of *F. hepatica* is a syncytial layer, there is evidence to suggest that there are regional differences within the tegument and its associated structures. Anterior/posterior and dorsal/ventral differences in the morphology and distribution of spines and the distribution of acid phosphatase and Na^+/K^+-ATPase activities have already been mentioned. The sensory receptors associated with the tegument also exhibit regional differences in their distribution; these differences and the fine structure of the three types of receptor will be described later. There are regional differences, too, in the susceptibility of the tegument to anthelmintic-induced changes; this point is discussed in Chapter 7 of this volume. While the available evidence is limited, it suggests that the tegument does not function uniformly throughout the body of the fluke.

3.2.3 Developmental changes

The tegument of NEJs contains only one type of secretory body, the T0 body, and the cells are packed with these bodies (Bennett and Threadgold, 1973). The tegument of flukes recovered from the abdominal cavity of mice 12 h pi contains greater numbers of T0 bodies in the syncytium, but fewer bodies in the T0 cells; moreover, the glycocalyx on the apical plasma membrane is more prominent than at 0 h (Bennett and Threadgold, 1975). At 1 day pi, the numbers of T0 bodies in the cells recover, and at 2–3 days pi – with the flukes still within the abdominal cavity – differentiation of T2 cells from neoblast (undifferentiated) cells in the parenchyma begins, although connections with the syncytium are not yet established (Bennett and Threadgold, 1975). The connections are complete by 4–5 days pi, soon after penetration of the liver by the juvenile flukes and T2 bodies are observed in the syncytium. Between 5 days and 3 weeks, the synthesis of T0 bodies gradually declines, being replaced by the formation of T1 bodies, and the metamorphosis of T0 cells into T1 cells is complete by 3 weeks pi. By this time, the numbers of T1 bodies predominate over T0 bodies, the basal infolds and their associated vacuoles in the syncytium are increased in number and size, and there are greater numbers of mitochondria (Bennett and Threadgold, 1975). These changes continue after entry into the bile duct (4 weeks onwards), being accompanied by

invagination and folding of the apical plasma membrane to adopt the morphology typical of the adult. Only T1 and T2 bodies are present at this stage, being produced by the T1 and T2 cells, respectively (Bennett and Threadgold, 1975). A similar change from T0 to T1 body occurs during the development of *Fasciola gigantica* (Hanna *et al*., 2019a). In this study, newly excysted metacercariae were compared with gut-penetrated juveniles; the latter were obtained using a 'gut loop' technique originally pioneered by Hanna *et al*. (1975) (see section 3.9). The T0 bodies in *F. gigantica* were of a different shape (ovoid rather than biconcave disc-like) and were smaller and less electron-dense than those in *F. hepatica* (Hanna *et al*., 2019a).

Developmental changes in the tegument of *F. hepatica* have been linked to immune responses by the host to fluke infection and thus have highlighted a potential role for the secretory bodies. The contents of the latter have been shown to be released at the apical plasma membrane and added to the glycocalyx, in this way presenting an antigenic stimulus to the host's immune system. Immunofluorescent studies using sera from infected sheep have shown that the level of T1 antigen in the tegument peaks at 3–5 weeks pi (in rat fluke infections), while that for T2 reaches a plateau after 10–12 weeks, T2 antigen being first detected only after 6 weeks pi (Hanna, 1980b). The results suggest that, although T2 bodies begin to appear in the syncytium 1–2 weeks pi (in mice), they do not express their contents at the surface until flukes are in the bile ducts (Hanna, 1980b) (Fig. 3.6). In separate fluorescent antibody-labelling experiments, it has been shown that NEJs become coated with host immunoglobin (IgG) when incubated in immune serum. The coat is sloughed when the flukes are transferred to medium lacking antibody, although when re-exposed to immune serum they acquire a new layer of IgG (Hanna, 1980a). This suggests that the glycocalyx had been replaced by an antigenically similar coat, by the release of (T0) secretory bodies at the apical plasma membrane. The ability of the fluke to continually replace the surface glycocalyx represents an important mechanism whereby it can evade the host's immune response; that is, it can slough off attached host antibody and replace damaged surface membrane via addition of the membrane of secretory bodies following exocytosis (Hanna, 1980a). The evasion mechanism is particularly important in the early, migratory phase of infection and the large stores of T0 bodies in the tegumental cells of the metacercaria may be a pre-adaptation, enabling the NEJ to counteract immune attack by the host (Hanna, 1980a). The stores are mobilized rapidly after excystation, but then replaced (Bennett and Threadgold, 1975). The immunoprotective role of T0 bodies is taken over by T1 bodies in the liver parenchyma, because they contain similar antigenic determinants although they are morphologically distinct (Hanna, 1980b) (Fig. 3.6). Once in the immunologically 'safe' environment of the bile duct, protection is no longer required and the rate of glycocalyx turnover declines, although it continues throughout the life of the fluke. The role of the T2 bodies is uncertain but may be concerned with maintaining the structural integrity and nutritive function of the apical plasma membrane. Their expression at the tegumental surface coincides with the entry of the fluke into the bile duct and may be related to structural reorganization of the apical membrane required following the switch from a tissue to a fluid (bile) environment. The accumulation of T2 bodies in the tegument during migration through the liver parenchyma may be a pre-adaptation to the new environment (Hanna, 1980b) (Fig. 3.6). Therefore, the combined morphological and immunological data have illustrated an intriguing and important functional link between tegumental secretory body production, migration of the fluke and evasion of the host's immune response.

3.2.4 Glycocalyx

The glycocalyx is a labile structure that is not fully preserved by conventional fixation techniques. It comprises two layers: a continuous inner layer, approximately 25 nm thick, lying next to, and tightly bound to, the apical plasma membrane; and an outer fibrillar layer, 25–35 nm thick (Threadgold, 1976). The glycocalyx is largely composed of glycoprotein, with projecting side-chains of oligosaccharides containing terminal sialic acid residues. It is polyanionic throughout its thickness and has a net negative charge (Threadgold, 1976). Lectins have been

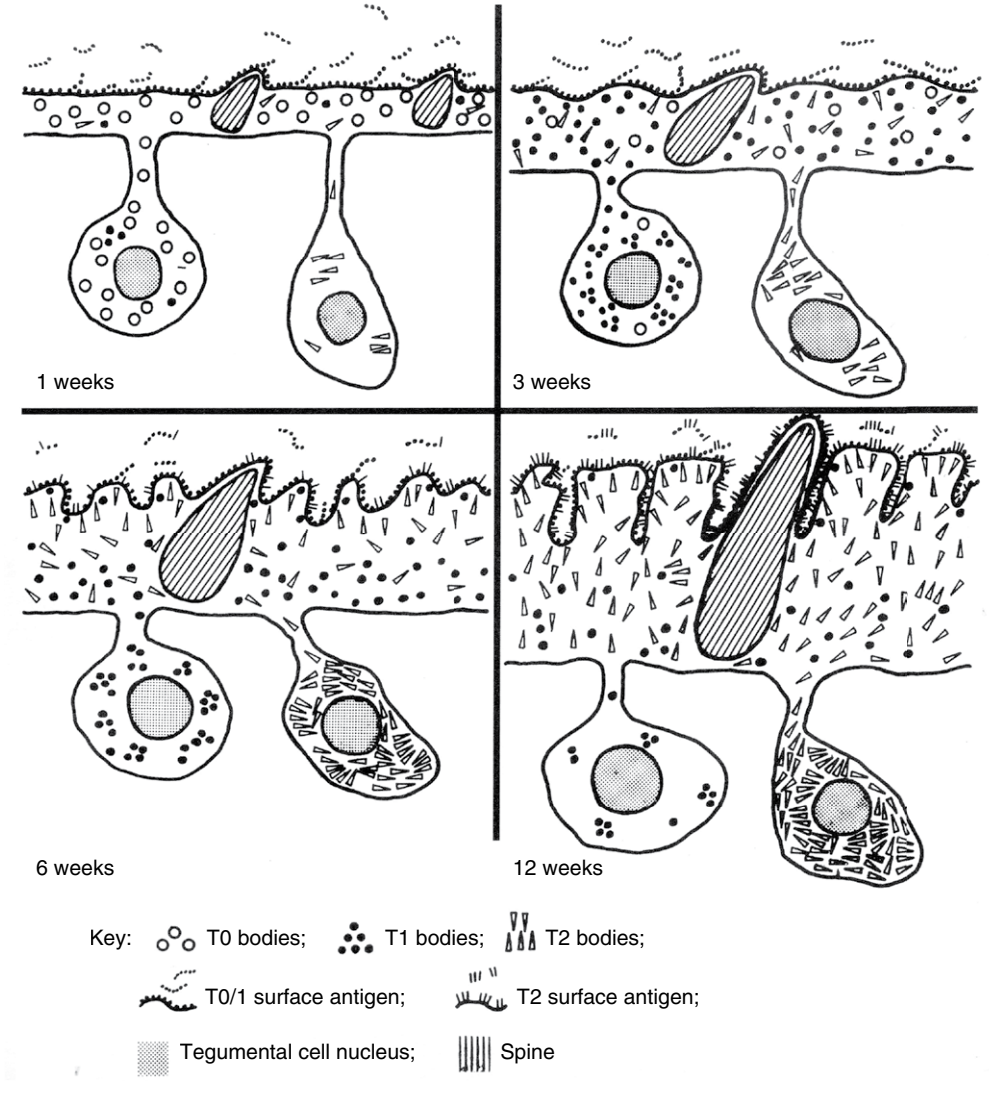

Fig. 3.6. Diagram summarizing the proposed changes in the antigenicity of the tegument of *Fasciola hepatica* during development in the mouse as related to ultrastructure (grossly simplified, not to scale) (Hanna, 1980b).

used to characterize the carbohydrate components of the glycocalyx, since individual lectins bind to specific sugars. For example, incubation of adult flukes in concanavalin A led to a number of tegumental changes, including surface blebbing, formation of microvillus-like projections, deepening of the apical invaginations, increased numbers of T2 secretory bodies in the apical cytoplasm, evidence of accelerated release of secretory bodies and swelling of the basal infolds. The effects of concanavalin A could be prevented by addition of α-methyl-D-mannoside to the incubation medium. Similar, but less pronounced, changes were induced by wheat germ agglutinin binding. The combined data indicate the presence of mannose, glucosamine or glucose moieties and of *N*-acetylglucosamine in the adult fluke glycocalyx (Rogan and Threadgold, 1984). Stereological analysis of the morphological changes observed by

scanning electron microscope (SEM) and transmission electron microscope (TEM) confirmed the increase in apical plasma membrane surface area, the apical concentration of T2 bodies and greater numbers of 'open' bodies (which are indicative of the release of the contents of the secretory bodies) (Rogan and Threadgold, 1984).

In a recent study, lectin microarrays were utilized to characterize more fully the carbohydrate motifs present in the glycocalyx of the adult fluke. The tegumental glycoproteins were shown to be predominantly oligomannose type *N*-linked oligosaccharides. Following application of lectin histochemistry, the glycoproteins were localized to the spines, suckers and the 'tegumental coat' of *F. hepatica*. The tegumental glycoproteins were isolated and subjected to further proteomic analysis. The analysis revealed a rich variety of proteins (Ravidà *et al*., 2016).

The importance of an intact glycocalyx to the normal functioning of the tegument has been demonstrated in experiments involving the use of poly-L-lysine, to neutralize its net negative charge (Threadgold, 1985). Pre-incubation with poly-L-lysine decreased the normal ability of the tegument to resist the action of enzymes such as pepsin and α-amylase. Treatment with poly-L-lysine followed by amylase had the most drastic effect, resulting in the total loss of the tegument, perhaps due to the action of the enzyme on the glucoside linkages of the oligosaccharide components of the glycocalyx (Threadgold, 1985). The changes induced by poly-L-lysine plus pepsin were less severe: surface blebbing, formation of microvilli and deep surface invaginations, swelling of the basal infolds and limited breakdown of the surface. The swelling of the basal infolds may be due to an osmotic effect, pepsin attacking the protein component of the apical plasma membrane and glycocalyx and thus altering the semipermeable properties of this combined structure (Threadgold, 1985). Surface blebbing, formation of microvilli and accelerated release of the secretory bodies appear to be features of the response of the tegument to a stress situation. They have been observed following lectin binding (as described above), following enzyme attack (as just described), following immunological attack (see below) and in response to anthelmintics (see Chapter 7, this volume). Poly-L-lysine pre-treatment did little to potentiate the action of bile, which had minimal effect on the tegument on its own (Threadgold, 1985).

Characterization of the glycocalyx of the NEJ of *F. hepatica* has also been carried out by mass spectroscopy. The NEJ is the first stage that comes in contact with host tissues in the mammalian host and therefore is important in relation to the establishment of the infection and avoiding the host's immune response. High mannose and oligomannose *N*-glycans were shown to be the most dominant glycan types in the NEJ, and were localized on the tegumental surface, and on the oral and ventral suckers (Garcia-Campos *et al*., 2016a). The blocking of mannose-type *N*-glycans with lectins significantly inhibited the migration of NEJs across the distal jejunum, suggesting that these carbohydrate moieties may be involved in the initial stage of the infection process and they may provide a potential target for therapeutic intervention. The blockage could have an effect on feeding or secretion or on adhesion to host intestinal epithelial cells (Garcia-Campos *et al*., 2017). The glycocalyx of newly excysted metacercariae and gut-penetrated juveniles of *F. gigantica* has been studied by ultrastructural cytochemical techniques (Hanna *et al*., 2019b). No comparable studies have been carried out for these stages in *F. hepatica*. An outer hyaline layer of mucopolysaccharide is present as a component of the glycocalyx in the early stages of *F. gigantica* development, but it is not present in that of adult *F. hepatica*. This may represent a specific adaptation to facilitate tissue invasion, whilst also serving to evade the host's immune responses and protecting the surface of the fluke against enzyme attack (for example, proteases derived from both host and parasite) (Hanna *et al*., 2019b).

3.2.5 Synthetic activity

The secretory activity of the tegumental cells has been studied by a number of techniques: autoradiography, stereology, immunocytochemistry, cytochemistry and inhibitor studies. Incubation of adult fluke slices in [^{3}H]leucine for up to 3 h showed that the label in the tegumental cells was initially incorporated into protein being synthesized by the GER, then moved into the Golgi complexes, becoming associated with the T1 secretory bodies being packaged by the

complex. Labelled secretory bodies moved into the cytoplasmic connections leading to the syncytium and reached the base of the syncytium after 1 h. Here they remained for some time, for label did not appear in the apical cytoplasm until after 3 h: at this time, the label was associated with the apical plasma membrane and its glycocalyx and also occurred throughout the tegumental syncytium (Hanna, 1980c). The results demonstrate that the process of protein synthesis in the tegumental cells of *F. hepatica* follows the typical GER–Golgi complex–exocytosis pathway observed in other secretory cells and that the entire process of secretory body synthesis, transport and release requires approximately 3 h for completion. Moreover, transport appears to be a three-step process, with initial movement through the cytoplasmic connections to the basal region of the syncytium, where the secretory bodies are stored for some time before undergoing a rapid transit to, and discharge from, the apical surface, where the glycoprotein content of the vesicles is incorporated into the glycocalyx. Radiolabelled galactose has also been shown to be incorporated into the glycoprotein being packaged into secretory bodies by the Golgi complexes in the T1 cells (Hanna, 1976). Labelled secretory bodies were also observed in the cytoplasmic connections but, over the 60 min incubation period, no label was observed in the syncytium, a result which emphasizes the relatively slow nature of the transport process. Evaginations of the parenchymal cells which penetrate the muscle layers and end close to the base of the tegument were often heavily labelled with [^{3}H]glucose; this observation supports the concept that the tegument is the main route for glucose transport into the fluke (Hanna, 1976).

The effects of certain metabolic inhibitors on the synthesis of secretory bodies by the T1 cells have been analysed by stereological methods. Cycloheximide, an inhibitor of protein synthesis, inhibited the synthesis of secretory bodies but had no effect on the transport and discharge of those already present prior to drug treatment (Hanna and Threadgold, 1976). However, with longer incubation periods, the number of secretory bodies in the syncytium declined, due to the fact that they could not be replaced by synthesis in the cell bodies (Hanna and Threadgold, 1976). Treatment with iodoacetate (an inhibitor of glycolysis) and 2,4-dinitrophenol (an inhibitor of oxidative phosphorylation) did not lead to a drop in number of secretory bodies in the tegumental cells; instead, the numbers stayed relatively constant at a level above normal. The numbers of secretory bodies in the syncytium dropped less in treated material than in the controls (Hanna and Threadgold, 1976). The results were interpreted as demonstrating an inhibition of the synthesis, transport and release of the secretory bodies (Hanna and Threadgold, 1976).

Monoclonal antibodies have been raised against an antigen present in the tegumental syncytium and glycocalyx of juvenile flukes and used in immunolocalization studies (Hanna and Trudgett, 1983). Gold labelling occurred over the T1 secretory bodies in the syncytium and over the glycocalyx, the latter being the result of exocytosis of the contents of the secretory bodies, replacing the glycocalyx as it sloughs from the surface. In the T1 cells, the secretory bodies were heavily labelled, whether lying free or forming in the Golgi complex. Antibody binding also occurred over the GER and ribosomes to some extent. Labelling was confined to T1 bodies in adult and juvenile flukes (Hanna and Trudgett, 1983). The monoclonal antibodies also bound to T0 secretory bodies in the tegumental syncytium and cell bodies of metacercariae, suggesting that the T0 and T1 bodies share a common antigen. Labelling also occurred in the gut and excretory system, being associated with the glycocalyx lining the lamellae in both sites. In the gut cells, labelling was associated with the secretory vesicles, Golgi complex and GER. The results indicated that the gut and excretory system have antigenicity in common with the tegument (Hanna and Trudgett, 1983). The epitope bound by the monoclonal antibody has been shown to be present in the polypeptide component of the glycoprotein and is not altered by glycosylation in the Golgi complex. The protein has a molecular mass of 50 kDa and may be linked to smaller entities with molecular masses of 25–40 kDa (Hanna and Trudgett, 1983).

Movement of secretory bodies from the tegumental cells to the apex of the syncytium is dependent on microtubules. The evidence is twofold. First, immunocytochemical studies using tubulin antibodies have shown that immunoreactivity is present in the tegumental cell bodies, in the cytoplasmic connections linking

the cells to the syncytium, and in the syncytium itself. In the latter, immunostaining was evident as a distinct band beneath the apical plasma membrane, the band following the contours of the tegumental folds. Immunofluorescence occurred throughout the syncytium, with a marked increase in intensity of fluorescence just above the basal plasma membrane. Strong fluorescence was evident around the spines, specifically in the syncytium around the base of the spines (Stitt *et al*., 1992b; Buchanan *et al*., 2003; McConville *et al*., 2006). Secondly, movement of secretory bodies is blocked by the microtubule inhibitors colchicine and tubulozole-C, and this result adds further support to the idea of a role for microtubules in the transport process (Stitt and Fairweather, 1993). The effects of tubulozole-C are more rapid and severe than the changes induced by colchicine. Thus, tubulozole-C inhibits the synthesis of secretory bodies, an action stemming from a marked vacuolation of the GER cisternae, which retract towards the cell nucleus, and the migration of Golgi complexes to the cell periphery, followed by their gradual disappearance. These changes are classic cell responses to microtubule inhibition, because microtubules are responsible for the organization, distribution and movement of the GER, Golgi complexes and other cell organelles (for references, see Stitt and Fairweather, 1993). Inhibition of secretory body formation and transport, not unexpectedly, have serious effects on the tegument (Stitt and Fairweather, 1993).

Microfilaments may also play a role in the movement of secretory bodies, because the microfilament inhibitor cytochalasin B causes a block of the two transport phases involved in this process. There is a gradual decline in their production, too, and this may exacerbate the situation (Stitt and Fairweather, 1991). Microfilaments have been demonstrated in the tegumental cell bodies of *F. hepatica* and their connections with the surface syncytium, and this supports their postulated role in secretory body transport (Stitt *et al*., 1992a).

3.2.6 Functions of the tegument

The synthetic activity of the tegument has been discussed above. It is also responsible for the uptake of small molecules – amino acids, solely by diffusion (Isseroff and Read, 1969), whereas monosaccharide uptake is via a carrier-mediated process that has been interpreted as facilitated diffusion rather than active transport (Isseroff and Read, 1974). The importance of an intact glycocalyx in protecting the fluke against digestive enzymes has already been discussed. The tegument is also the first line of defence against anthelmintics, for both adult and juvenile flukes. This is because the tegument of adults will be exposed to anthelmintics as they are being excreted in the bile, while that of juvenile flukes is bathed in blood as they are migrating through the liver parenchyma. The interaction of the tegument with anthelmintics is discussed in Chapter 7 of this volume.

3.2.6.1 Immune protection

The role of the tegument in shielding the fluke from the host's immune response has already been discussed: in relation to the sloughing of the glycocalyx and repair of the surface membrane, a process that involves the T0 and T1 secretory bodies (section 3.2.3); the composition of the glycocalyx and the consequences of its disruption following treatment with lectins (section 3.2.4); the production of immunomodulatory molecules by the EVs (section 3.2.2); and the immunolabelling of a tegumental antigen (section 3.2.5). The importance of the tegument to the fluke in resisting immune attack from the host has also been illustrated by the extent of immune-related 'damage' observed in a number of experimental situations. For example, incubation of immature juvenile (16-day-old) flukes in immune serum *in vitro* has been shown to cause surface changes leading to complete destruction of the tegument; the phenomenon was dependent on IgG_1 and IgG_2 antibodies (Eckblad *et al*., 1981). Attachment of eosinophils to the surface of NEJs *in vitro* occurs in the presence of immune serum. The binding is independent of complement and does not affect the viability of the fluke, as measured by its ability to infect naive rats (Doy *et al*., 1980; Doy and Hughes, 1982). Attachment of eosinophils and neutrophils to NEJs is mediated by IgG_1 and IgG_2 antibodies and is dependent on F_c receptors. In the presence of excess antibody, aggregations of antigen/antibody complexes build up over the surface of the parasites and are shed into the

medium. Again, no disruption was observed and the viability of the flukes was unaffected (Duffus and Franks, 1980). In contrast to these light microscope studies, in which no disruption was detected, more detailed electron microscope studies have shown that, in the presence of immune serum *in vitro*, eosinophils become attached to NEJs in those regions not covered by antigen/antibody precipitates. Subsequent degranulation of the eosinophils leads to vacuolation of the syncytium in these regions, thus showing that disruption does take place (Glauert *et al.*, 1985).

Coating of NEJs with antibody occurs *in vivo* as well as *in vitro*. It can occur within the lumen of the intestine of sensitized (that is, resistant) rats. All classes of antibody are involved, especially IgG and IgM (Burden *et al.*, 1982). The coating did not appear to cause any disruption (Burden *et al.*, 1982, 1983) and was shed when the flukes were incubated in culture medium *in vitro* for 3 h (Burden *et al.*, 1982). The antibody coating is 'wiped off' as the fluke penetrates through the gut wall but, on entering the peritoneal cavity, the fluke is coated with antibody and host cells – initially eosinophils, but later neutrophils and macrophages. Degranulation of the eosinophils created pits in the tegument, leading to its erosion (Burden *et al.*, 1983). The results were confirmed by a separate study involving injection of NEJs into the peritoneal cavity of sensitized rats. The flukes were dead within 6 h of entry. Once the tegument had been breached by eosinophils, neutrophils were seen to attach to the internal tissues (Davies and Goose, 1981).

Introduction of adult flukes into the peritoneal cavity of sensitized rats led to their death after 9–12 h (Bennett *et al.*, 1980). In contrast to the mechanism of tegumental destruction described above for the NEJs, the host cells appear to penetrate the syncytium of the adult fluke and prise it off. The fluke attempts to replace the tegument with the formation of a new one underneath. In the early stages after transfer, there is an accelerated movement of T2 secretory bodies to the surface, followed by their release. In addition, there is evidence of blebbing of the surface membrane and formation of microvilli. These features are all indicators of a stress response by the fluke, to shed and replace damaged membrane, as described previously. The number of secretory bodies eventually declines, and host cells start to penetrate the syncytium, but this is not seen until the supplies of T2 bodies become depleted (Bennett *et al.*, 1980).

Therefore, it should be apparent that an intact glycocalyx and syncytium are vital to the survival of the fluke. Once they are breached, this leads to more widespread disruption to the internal tissues, and eventually the death of the fluke. The mechanism by which the fluke protects itself against immune attack is via the continual turnover and replacement of the apical plasma membrane and glycocalyx as a result of the synthesis and release of secretory bodies, particularly the T0/T1 bodies in the early migratory phases of development. The mechanism allows the fluke to shed antigen/antibody complexes and resist attack by immune effector cells; details have already been given.

3.2.6.2 Osmoregulation

F. hepatica appears to carry out little or no water regulation in media of various concentrations, suggesting that it behaves like an osmoconformer. It is able to tolerate a fairly wide range of osmotic pressures *in vitro* (Δ 0.40–0.81°C). The fluke's natural environment is bile, which has a relatively high osmolarity: 290–320 mosmol/l in sheep. Bile contains ions such as Na^+, K^+, Ca^{2+}, Mg^{2+} and Cl^- at concentrations that are believed to be 2–6 times higher than those within the fluke itself (Threadgold and Brennan, 1978). Consequently, the fluke will be subject to either an influx of ions or efflux of water. In addition, the flame cells of the excretory system are continuously operating, resulting in the extrusion of large volumes of fluid.

These facts suggest that the fluke has a high requirement for water, as well as a need to reduce levels of certain ions. Evidence suggests that the tegument plays a role in ion and water control. As described previously, the tegument is characterized by amplification of both the apical and basal plasma membranes and the Na^+/K^+-ATPase activity associated with them is indicative of the presence of ion pumps (Threadgold and Brennan, 1978; Skuce *et al.*, 1987). Mitochondria are associated with the basal infolds, as are polymorphic masses of mucopolysaccharides. The basal infolds are particularly long and respond to changes in external osmolarity by swelling in hypotonic media and collapsing in hypertonic media. The responses are not simply

passive ones, because the infolds return to near-normal configuration within 1 h in either medium, even though the whole fluke may be shrunken or turgid, depending on the osmolarity of the medium (Threadgold and Brennan, 1978). Thus, the tegument has many of the features of a transporting epithelium and a model has been put forward to account for its role in osmoregulation (Fig. 3.7). According to this idea, ions from the bile would enter the tegument by diffusion and could be pumped out again by the ion pumps associated with the apical plasma membranes and its ATPase. Ions that escaped the pumps would enter the cytoplasm of the syncytium and could be pumped out into the lumen of the basal infolds by their associated ion pumps. This could result in a lumen that was hypertonic to the surrounding cytoplasm, thus forming a standing gradient of the forward type. The energy required for the pumping would be supplied by the many mitochondria closely associated with the basal infolds and oriented along their long axes. Because of the standing gradient in the lumen of the infolds, water would be drawn from the tegumental cytoplasm and presumably be replaced by water movement into the tegument from the bile. Some of this bile-derived water could be trapped and held by the

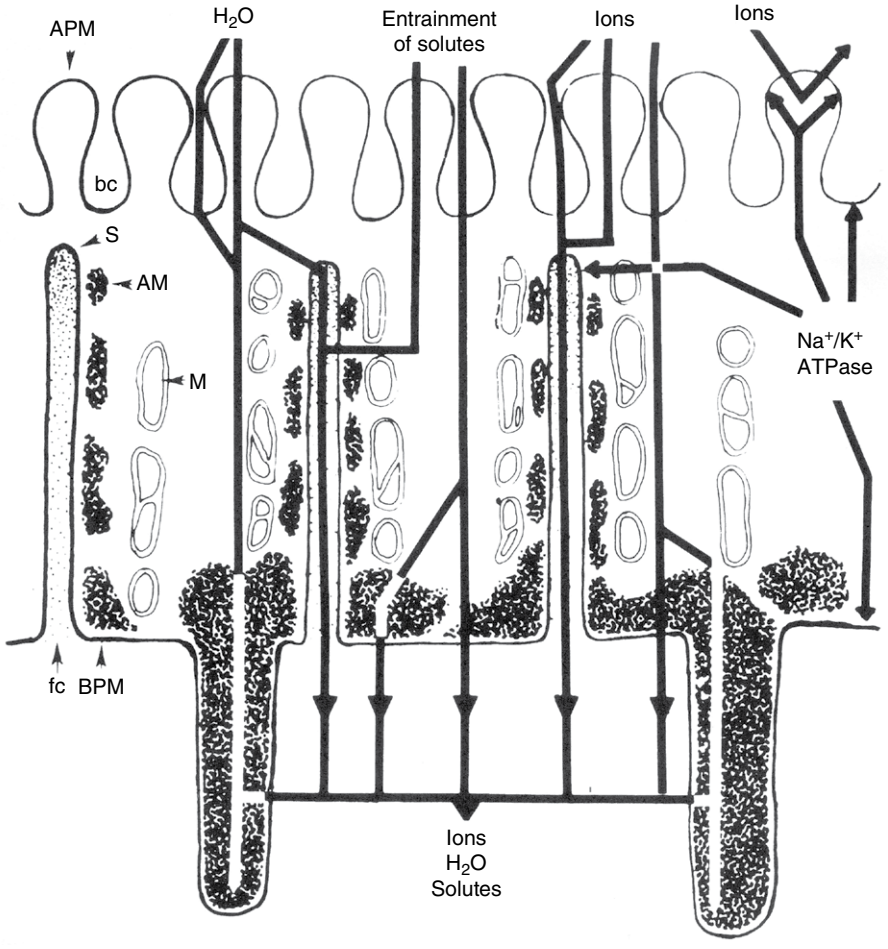

Fig. 3.7. Diagrammatic representation of the ion, water and solute flow through the tegument of *Fasciola hepatica*. AM, associated acidic mucopolysaccharide (vacuoles); APM, apical plasma membrane; bc, backward channel; BPM, basal plasma membrane; fc, forward channel; M, mitochondria; S, basal invagination (Threadgold, 1979).

mucopolysaccharide masses associated with the basal infolds. These masses would then function as a water sink and so act as a buffer against sudden changes in fluid content of the bile, which might either dehydrate or flood the tegument. The establishment of such a standing gradient, down which water could flow, would result in the drag or entrainment of solutes such as monosaccharides and amino acids through the tegument and into the fluke. If small organic molecules and ions were swept into the flask-like invaginations of the apical plasma membrane, this would increase their chance of being 'trapped' by the glycocalyx or of being transported. A further gradient for water, ions and small molecules could be established between the base of the tegument and the excretory system. This gradient could cause water solutes and metabolites to diffuse through the parenchyma and other organ systems and also transport wastes or fluids into the excretory system (Threadgold and Brennan, 1978). As indicated above, this hypothesis was originally based on the assumption that the levels of ions in the bile are higher than those in the fluke. However, subsequent studies have shown that, of Na^+, K^+, Ca^{2+} and Mg^{2+} ions, only Na^+ ions are present at a higher level in the bile than in the fluke; this was true of both bovine and ovine bile and their respective flukes (Caseby *et al.*, 1995). Consequently, some revisions of the tegumental osmoregulatory model may be required.

3.2.6.3 Sensory perception

In addition to the sensory papillae described previously (section 3.2.1), the tegument contains three types of sensory receptor, suggesting that it plays a role in sensory perception. There is a ciliated type that occurs between the spines on the anterior ventrolateral surfaces; a pair of these receptors also occurs in the mid-anterior dorsal surface. Each consists of a bulbous body containing mitochondria and vesicles and is joined to the surrounding cytoplasm by means of a septate desmosome. Within the bulb, adjacent to the desmosome, are two electron-dense collars. The bulb also contains a basal body with striated rootlet, from which arises the single cilium, which projects to the same height as the surrounding spines. This type of receptor presumably serves as a tangoreceptor (Bennett and Threadgold, 1973; Bennett, 1975a). A second, non-ciliated and 'domed' type occurs within the spine-free tegument overlying the oral and ventral suckers. A pair of these receptors occurs on the dorsal lip of the oral sucker and six more are regularly spaced around the ventral sucker. Each comprises a bulb containing two electron-dense collars and a basal body with striated rootlet, although no cilium is present. Again, it is connected to the surrounding cytoplasm by means of a septate desmosome. It may serve as a pressure or contact receptor (Bennett, 1975a). The third type of presumed receptor consists of a spiral of tegument apparently shielding a pit. A group of three of these receptors occurs on each side of the oral sucker. This type has not been observed by transmission electron microscopy, so its internal structure is not known. It may serve as a chemoreceptor, allowing the fluke to 'taste' the substrate over which it is moving (Bennett, 1975a). The complement of receptors enables the fluke to detect changes in the external environment and pass on this information to more central regions of the nervous system to formulate an appropriate response. The data on receptors is limited, mostly confined to the NEJ as later stages have not been examined.

3.3 Parenchyma

The parenchymal cells fill the spaces between the organ systems in the fluke. The cells are separated from each other, and from other organ systems, by interstitial material (believed to be synthesized and secreted by the parenchymal cells themselves) composed of fibres in a homogeneous matrix. The interstitial material probably functions as a flexible cytoskeleton, the elastic fibres allowing considerable distortions of body shape. It also provides anchorage for muscle fibres (Threadgold and Gallagher, 1966; Gallagher and Threadgold, 1967).

Originally viewed as a simple packing tissue, the parenchyma is now considered to carry out important synthesis and transport functions and to be involved in carbohydrate metabolism. The observation that parenchymal cells do not contain uniform amounts of fatty acid-binding proteins (FABPs) (Sirisriro *et al.*, 2002) suggested that distinct cell types exist and led Pankao *et al.* (2006)

to revisit the parenchymal tissue of *F. gigantica.* They were able to describe three distinct parenchymal cell types (Pc_1, Pc_2 and Pc_3 cells) that were classified according to their ultrastructural features and histochemical staining reactions (Fig. 3.8).

Pc_1 cells are large ovoid-shaped cells that display a weakly basophilic cytoplasm and strong PAS staining indicative of significant amounts of carbohydrate. The major discerning feature of Pc_1 cells at the ultrastructural level is the electron-lucent cytoplasm, which contains very few organelles, with some mitochondria and cisternae of GER found near the nucleus. Numerous glycogen deposits are dispersed within the cytoplasm and the cytoplasmic processes. The Pc_2 cells are smaller than Pc_1 cells and have a more basophilic cytoplasm. When viewed by TEM, the Pc_2 cell cytoplasm appears to be more electron-dense than in Pc_1 cells and contains numerous mitochondria, but only a small amount of glycogen particles, in line with the weak PAS staining seen with this cell type. Large congregations of lipid droplets are evident and autophagic vacuoles can also be seen in the Pc_2 cell cytoplasm. The Pc_2 cells in particular have pronounced cytoplasmic processes, which pass between the muscle layers to make contact with other tissues, including the gut and excretory system. Due to their high FaBP content and accumulation of lipid droplets, Pc_2 cells are thought to participate in the storage and metabolism of fatty acids and other lipids. Pc_3 cells present with an elongated ovoid shape and display the strongest basophilia of all three parenchymal cell types. At the ultrastructural level, the cytoplasm appears dense and is packed with numerous ribosomes, cisternae of GER and mitochondria. Golgi complexes with small secretory bodies are also prominent in the Pc_3 cell type. The relative levels of immunoperoxidase staining for FaBPs were Pc_2 cells > Pc_1 cells > Pc_3 cells (which appeared negative). Whilst *F. hepatica* parenchymal cells have not been studied at the ultrastructural level in any detail since they were first described in the 1960s (Threadgold and Gallagher, 1966; Gallagher and Threadgold, 1967), recent observations suggest that they follow the same classification proposed by Pankao *et al.* (2006) for *F. gigantica* (Bennett, 2019).

Based on these structural observations, it is proposed that Pc_1 cells specialize in glycogen storage and thus serve to regulate glucose metabolism. This is in agreement with earlier studies in which *F. hepatica* parenchymal cells were found to contain considerable quantities of α- and β-glycogen (Threadgold and Gallagher, 1966). Incorporation of [^{3}H]glucose and [^{3}H] galactose into glycogen storage areas of the parenchymal cells was demonstrated by Hanna (1976). Particularly dense labelling occurred in the terminals of parenchymal cell extensions that pass between the muscle layers to lie close against the base of the tegument, reinforcing the view that glucose enters the fluke mainly across the tegument (Hanna, 1976). Starvation and refeeding experiments carried out by Threadgold and Arme (1974) confirmed a role for parenchymal cells in carbohydrate metabolism. During starvation, mobilization of glycogen reserves (glycogenolysis) occurs (at least in part) by a process of autophagy, although the internal membranes involved are derived from the mitochondria, rather than from the endoplasmic reticulum which is more typical. Protrusions from the mitochondria become pinched off to form M bodies; the M bodies fuse to form smooth membranous cisternae (SMC) which engulf portions of cytoplasm containing glycogen, giving rise to β bodies. The β bodies fuse with primary lysosomes derived from the Golgi complexes to form secondary lysosomes and the glycogen is broken down by lysosomal enzymes to release glucose. Refeeding of starved flukes leads to replenishment of glycogen stores, although the mechanism involved is uncertain since no morphological correlates of glycogenesis have been observed. It has been suggested that the enzymes required for glycogen synthesis and the intermediates involved exist in soluble form in the cytoplasm (Threadgold and Arme, 1974). Whilst the enzymes required for this and other carbohydrate metabolic pathways (including glycolysis, gluconeogenesis and glycogenesis) are conserved in *F. hepatica* (Cwiklinski *et al.*, 2015b; Cwiklinski *et al.*, 2018), they require biochemical characterization and confirmation of their tissue and cellular locations.

Metacercariae are non-feeding stages and must rely upon endogenous glycogen stores to support them during excystment and initial establishment of infection. In the NEJ, the parenchymal cells are filled with large stores of α- and β-glycogen and contain numerous mitochondria

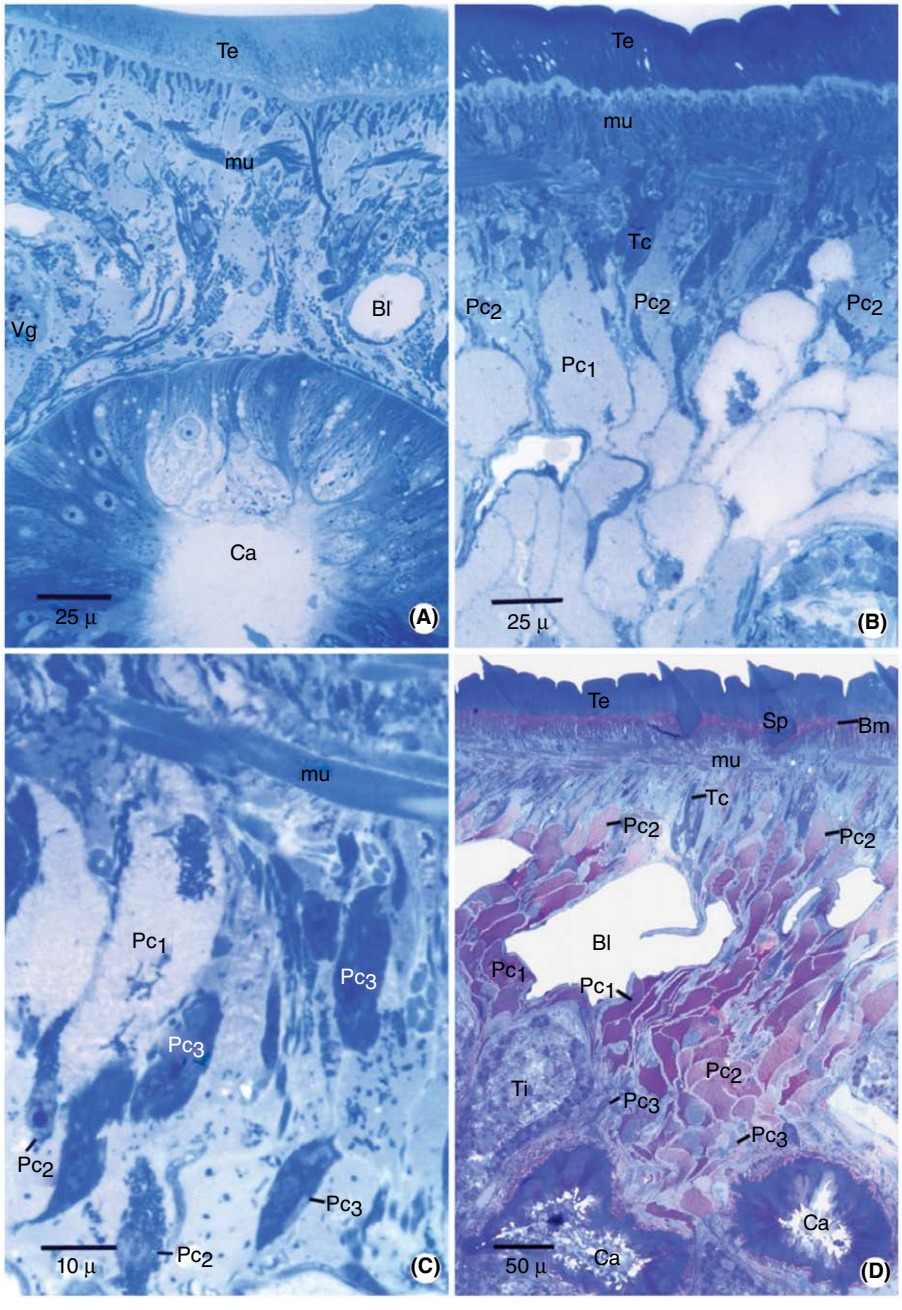

Fig. 3.8. Light micrographs showing the histochemical classification of parenchymal cells in adult *Fasciola gigantica*. **(A)** The general location of the parenchymal tissue between various organs that include tegument (Te), muscle (mu), caecum (Ca), bladder (Bl) and vitelline gland (Vg). **(B, C)** The three types of parenchymal cells are shown: type 1 parenchymal cells (Pc_1), type 2 parenchymal cells (Pc_2) and type 3 parenchymal cells (Pc_3), which show increasing basophilia with methylene blue staining. **(D)** Section stained with periodic acid-Schiff (PAS) showing intense carbohydrate deposits in Pc_1 but moderate levels in Pc_2. Pc_3 are unstained with PAS. Tc, tegumental cell; Te, tegument; mu, muscle; Sp, spine; Ti, testis; Bm, basement membrane (Pankao *et al*., 2006).

and some lipid droplets (Bennett and Threadgold, 1973; Bennett, 1977). The limiting plasma membranes of the cells are much invaginated. By 12 h pi, the cells are greatly increased in volume and the glycogen stores have become depleted. The latter show signs of an increase by 24 h pi and are replenished by 2–3 days pi (Bennett, 1977). The increase in volume of the parenchymal cells is most likely to be due to rehydration after excystment during the metacercarial stage, and the initial mobilization of glycogen reserves may be linked to the migration of the NEJ across the gut wall (Bennett, 1977). These observations are supported by transcriptome analysis of *F. hepatica* metacercariae and NEJs at 1 h, 3 h and 24 post-excystment, in which the expression of key enzymes involved in glycogen metabolism and biosynthesis were found to be tightly regulated (Cwiklinski *et al*., 2018). Specifically, genes associated with metabolism of glycogen were expressed more highly in metacercariae and NEJ 1 h post-excystment compared with genes related to glycogen catabolism and synthesis which, in contrast, were upregulated in the NEJs 24 h post-excystment. Again, these findings are consistent with the free-living metacercariae being reliant on endogenous glycogen reserves as a source of energy. Initially, in the NEJs, the parenchymal cells only form junctional complexes with the excretory system and tegument; contacts with the caecal cells of the gut do not develop until 24 h pi (Bennett, 1977). Morphological evidence of glycogenolysis, as described above, is present at all stages of juvenile development, although in this study (Bennett, 1977) the parenchymal cells were never seen to synthesize or secrete any substances; this does not support the previous suggestion that these cells are responsible for production of the interstitial material (Threadgold and Gallagher, 1966).

In places, the interstitial material between the parenchymal cells is absent, allowing very close contact between the membranes of adjacent cells; the cells are joined by desmosome-like structures (Threadgold and Gallagher, 1966). Similar contacts are made between the parenchymal cells and the gut, excretory system, tegument and reproductive system. For example, pseudopodia-like processes from the parenchymal cells pass between the muscle blocks surrounding the gut, penetrate the interstitial material and basement membrane and extend for a short distance into the base of the gut cells. Similar projections from parenchymal cells make contact with the flame cells and larger ducts of the excretory system and with the tegumental cell bodies and their connections with the surface syncytium (Gallagher and Threadgold, 1967). In the reproductive system, parenchymal cells form junctional complexes with the nurse cells within the vitelline follicles and with the seminal vesicle and ejaculatory duct (Irwin and Threadgold, 1970; Threadgold, 1975). The junctional complexes permit the exchange of ions, metabolites and excretory products and, in the absence of a circulatory system, the parenchyma could serve as a means of transporting substances around the body of the fluke (Threadgold and Gallagher, 1966; Gallagher and Threadgold, 1967). In support of this is the observation that numerous proteins that are found in *Fasciola* excretory/secretory (ES) products, such as FaBPs, glutathione *S*-transferase (GST), helminth defence molecule (HDM), peroxiredoxin and 14-3-3 proteins, localize to the parenchymal cells (Creaney *et al*., 1995; Chaithirayanon *et al*., 2006; Pankao *et al*., 2006; Martínez-Sernández *et al*., 2014; Sangpairoj *et al*., 2014). Due to the various, and intimate, connections between the parenchymal cells and other fluke tissues, it is conceivable that proteins synthesized in parenchymal cells (most likely the Pc_3 cell type, which appears to be secretory) are trafficked to other destinations for eventual release from the parasite. For instance, it has been proposed that the immunomodulatory molecule HDM is initially produced in the parenchymal cells of *F. hepatica*, but is released trans-tegumentally (Martínez-Sernández *et al*., 2014. Similarly, a variety of EV marker proteins, including TSG101, Ral-A, SMPD2, ALIX, TIP, DM9 and SMPDL3, were localized to cells resembling the Pc_2 cell type in adult *F. hepatica* (de la Torre-Escudero *et al*., 2019; Bennett *et al*., 2020a). The labelled vesicles were often seen outside the parenchymal cells (around their periphery or in the interstitial material) and in close proximity to the gut, tegumental cell bodies and excretory duct epithelial cells, suggesting that EVs may also be produced in this tissue before being trafficked to other sites for extra-corporeal release (Bennett *et al*., 2020a). Vesicles bearing the same markers were also detected within the rear extensions of the

gastrodermal cells that protrude into the parenchyma, suggesting that there may be a movement of vesicles between the parenchyma and the basolateral membrane of the gastrodermis. This mechanism, termed adsorptive-mediated transcytosis, allows EVs shed by red blood cells to cross the blood–brain barrier (Matsumoto *et al.*, 2017), although this route requires experimental investigation in *F. hepatica*. Further support for a parenchymal origin of *Fasciola* EVs comes from ultrastructural observations following treatment of fluke with GW4869, a chemical inhibitor of SMase, which is a key driver of EV biogenesis in mammalian cells. The SMase activity associated with EVs isolated from *F. hepatica* culture supernatants was susceptible to GW4869 and incubation of adult flukes with the inhibitor led to a significant reduction in the release of EVs *in vitro*, suggesting that an SMase-dependent pathway drives EV biogenesis in liver fluke (Bennett *et al.*, 2020a). Whilst non-lethal, GW4869 treatment did cause considerable and widespread ultrastructural disruption to adult flukes *in vitro*. The most severe changes occurred in the parenchymal cells, which appeared to be highly disorganized, with numerous large electron-lucent vacuoles dominating the tissue. Although some vacuolation of the excretory duct epithelial cells was evident, the lipid droplets within the duct lumen appeared to be normal (Bennett *et al.*, 2020a). Whilst it is not known if the ultrastructural changes induced by GW4869 were directly due to inhibition of sphingomyelin hydrolysis, they are similar to those seen in sphingomyelin storage diseases, such as Niemann-Pick disease, where sphingomyelin accumulates in the cell (Elleder, 1989).

3.4 Muscle

The main somatic musculature is situated beneath the tegument and has been visualized in whole-mount preparations (Mair *et al.*, 1998) and isolated muscle fibres (Kumar *et al.*, 2003) of adult *F. hepatica* using fluorescein isothiocyanate (FITC)-labelled phalloidin (as a probe for filamentous actin) and examined by confocal microscopy. The morphology of isolated muscle fibres that form individual muscle bundles has also been examined by SEM (Kumar *et al.*, 2003). The somatic musculature comprises two main layers: an outer circular muscle layer and an inner longitudinal layer. The antagonistic actions of these two layers are responsible for the rhythmic waves of contraction and relaxation that take place along the lateral margins of the fluke. The oral and ventral suckers are highly muscular organs, too, of vital importance to the adult fluke in maintaining attachment to the lining of the bile duct and (for the oral sucker) in the suctorial feeding movements of the fluke. In more juvenile stages, both the suckers and the somatic muscles are important in the migratory movements from the gut lumen, through the gut wall, across the peritoneal cavity and through the liver parenchyma to the bile duct.

Little is known about the fine structural organization of the muscle cells in *F. hepatica*. The morphology of the cells is believed to be similar throughout the body of the fluke, in both the somatic and sucker musculature, and to be identical in the NEJ and the adult fluke (Bennett and Threadgold, 1973). Essentially, the muscle is of the invertebrate smooth type, with the muscle cell body separated from the contractile portion of the cell but joined to it by a cytoplasmic connection. The cell body contains the nucleus, which is surrounded by a narrow layer of cytoplasm. The latter is packed with α- and β-glycogen; free ribosomes are also present, together with mitochondria that have few cristae (Bennett and Threadgold, 1973). The contractile region of the muscle cell contains thick and thin myofilaments, with thin being more numerous. Up to 12 thin myofilaments surround each thick filament, but there is no regular ratio between them (unpublished observations; also Ishii and Sano, 1980). The thick filaments have tapered ends and the thin filaments show evidence of branching (Kumar *et al.*, 2003). Immunostaining for actin has been localized to the thin myofilaments within the muscle fibres of the somatic muscle layers (Stitt *et al.*, 1992a), whilst actin and myosin have been co-localized to the muscle fibres belonging to the ventral sucker (Kumar *et al.*, 2003). Dense bodies anchor the thin filaments to the sarcolemmal membrane and to the surrounding interstitial material (Bennett and Threadgold, 1973). Native paramyosin-like proteins have been extracted from fluke homogenates (Ishii and Sano, 1980; Cancela *et al.*, 2004). Their molecular masses (97–98 kDa) and amino acid composition are similar to those of

paramyosins from other invertebrates, although the structural pattern of the paracrystal exhibits some differences. The paramyosin is believed to occur in the thick muscle filaments (Ishii and Sano, 1980) and has been localized to the muscle layer beneath the tegument and surrounding the gut (Cancela *et al*., 2004).

The myofilaments are surrounded by a thin layer of cytoplasm, which contains a number of mitochondria, glycogen granules and a poorly developed system of sarcoplasmic reticulum cisternae that lies beneath the outer sarcolemma (unpublished observations). Deposits of Ca^{2+}-ATPase activity have been localized to the sarcoplasmic reticulum and the sarcolemma, indicating the presence of Ca^{2+} pumps on these membranes (Skuce, 1987). The pumps may be involved in the control of the movement of Ca^{2+} ions into the cytoplasm of the muscle (across the sarcolemmal membrane and out of the sarcoplasmic reticulum) which will bring about muscle contraction, also in the opposite direction (involving sequestration in the sarcoplasmic reticulum or exit from the cell) to promote muscle relaxation. Indeed, Graham *et al.* (1999) demonstrated that spontaneous contractions of liver fluke muscle strips are dependent on extracellular Ca^{2+} and that cAMP has a regulatory function in motility by opening plasma membrane Ca^{2+} channels. Subsequent electrophysiology experiments confirmed that muscle fibres isolated from the ventral sucker of adult *F. hepatica* express voltage- and Ca^{2+}-sensitive K^+ channels. However, these channels were found to be pharmacologically distinct from equivalent channels in other phyla (notably being refractory to several classical K^+ channel blockers), suggesting that they might make selective targets for novel therapeutic agents (Kumar *et al*., 2004). Further evidence for a regulatory role for Ca^{2+} in fluke neuromuscular signalling comes from RNA interference (RNAi) studies. Silencing of the Ca^{2+}-sensing protein calmodulin reduced the growth of juvenile flukes maintained *in vitro* and resulted in a hyperactivity phenotype that caused increased migration (McCammick *et al*., 2016). It is likely that the increased mobility observed is due to the disturbance of intracellular Ca^{2+} levels; whether this occurs in muscle cells (thus causing abnormal contraction) or in neurones (by interfering with Ca^{2+}-dependent synaptic transmission) remains to be determined.

Genistein, an active component of *Flemingia vestita*, a plant indigenous to Northeast India and historically used to treat helminth infections (Rao, 1981), was shown to have pronounced neuromuscular effects on muscle strips isolated from adult fluke, bringing about gradual loss of spontaneous movement and eventual complete immobilization (Toner *et al.*, 2008). Similar paralysis of *Fasciolopsis buski* induced by genistein has been linked to the neurotoxic effects of elevated levels of nitric oxide (NO) and its biosynthetic enzyme, nitric oxide synthase (NOS) (Kar *et al.*, 2002). NADPH diaphorase, an isoform of NOS, has been localized in *Fasciola* fluke tissue sections (Gustafsson *et al*., 2001), suggesting that a similar mechanism of action for genistein may operate in *F. hepatica*.

3.5 Nervous System

Following its excystment in the small intestine, the fluke penetrates through the gut wall into the abdominal cavity. It spends some time wandering within the cavity before locating and penetrating the liver. It then burrows through the liver parenchyma before reaching the bile duct, where sexual maturation takes place. Such a complex migration suggests that the fluke is able to detect specific environmental cues and respond to them with the appropriate behaviour pattern. There is some experimental evidence to support this idea. For example, bile is essential for excystment of the metacercaria, but is harmful to the long-term survival of the fluke (Tielens *et al*., 1981; Sukhdeo *et al*., 1988). Indeed, it has been suggested that bile provides a negative chemotactic stimulus for the fluke to leave the gut and enter the abdominal cavity (Tielens *et al.*, 1981). Different bile salts have different effects on the movement of the NEJ: dehydrocholic acid increases activity, whereas taurine- and glycine-conjugated chenodeoxycholic acids decrease locomotory cycles (Sukhdeo *et al.*, 1988). The emergence response to the bile salt, glycocholic acid, is dose-dependent, indicative of a receptor-mediated response (Sukhdeo and Mettrick, 1986). Bovine bile has been shown to increase locomotory movements of the NEJ, as do duodenal extracts, although the latter response is lost within 2 days of development

(Sukhdeo and Mettrick, 1986; Sukhdeo *et al.*, 1987; Sukhdeo *et al.*, 1988). In orientation assays, NEJs showed aversion responses to duodenal and liver extracts, high pCO_2 and acid pH, indicating an ability to orient in a directional manner to specific stimuli (Sukhdeo and Mettrick, 1986). However, location of the liver by juvenile flukes is not believed to be the result of any chemotactic or orientation response; rather, a passive or random movement along the body wall, the fluke recognizing the liver only after contact and penetration. It seems that the flukes migrate to the body wall, then crawl along the body wall to the liver, which they penetrate adjacent to the diaphragm (Sukhdeo and Sukhdeo, 2002).

The adult fluke is also able to respond to chemical cues in the form of hormones that will be ingested with the blood meal. For example, cholecystokinin increases the rate of ventral sucker activity *in vitro* (Sukhdeo and Sukhdeo, 1989). Cholecystokinin acts to stimulate bile flow in the host and, in stimulating sucker activity and attachment behaviour, may serve to prevent the flukes from being flushed out of the bile duct *in vivo*. The related peptide, caerulein, inhibits oral sucker activity and the frequency of contraction of the longitudinal muscles; motilin, another gut hormone, inhibits ventral sucker activity and the frequency and amplitude of contractions of the longitudinal muscles (Sukhdeo and Sukhdeo, 1989). The results suggest that the fluke can respond in different ways to individual hormones and can discriminate between closely related peptides.

While the data described above are limited, they do indicate that *F. hepatica* can respond in various ways to a range of 'environmental' cues within its host. As indicated in section 3.2 dealing with the tegument, the fluke possesses a number of different types of sensory receptors, enabling it to respond to these cues. The cues elicit an extensive repertoire of behaviour patterns, the full extent of which we can barely appreciate at present. The release of a particular behaviour pattern at the appropriate time (and in the correct sequence to complete the life cycle) argues for a high degree of nervous coordination and a fairly complex nervous system – the more so when it is remembered that it is not just the metacercaria-to-adult transformation that we are concerned with here. Somatic continuity extends back to the cercarial stage, so the morphological changes, behaviour patterns and changes of environment with their associated cues that are involved are even more complex. Control of the ontogenetic changes resides in the nervous system, so it needs to be correspondingly sophisticated. A sizeable body of work on the neurochemistry, pharmacology and developmental changes in the nervous system suggests that this is the case.

The gross neuroanatomy of *F. hepatica* was established towards the end of the 19th century, on the basis of histological studies at the light microscope level (e.g. Havet, 1900). The central nervous system (CNS) contains a pair of anterior (so-called 'cerebral') ganglia situated either side of the pharynx just posterior to the oral sucker. The ganglia are connected by a transverse commissure that crosses over the dorsal surface of the pharynx (Fig. 3.9). Nerves pass anteriorly from the ganglia to the oral sucker, but the main nerve cords run posteriorly along the body. Three nerve cords arise from each ganglion: ventral, dorsal and lateral, of which the ventral longitudinal nerve cord is the best developed. The peripheral nervous system (PNS) comprises plexuses of cell bodies and nerve fibres beneath the tegument (in association with the subtegumental musculature), in the oral and ventral suckers and in association with the various reproductive organs and ducts.

The fine structure of the anterior ganglia in the adult fluke has been described by Sukhdeo *et al.* (1988a). Their structure is atypical compared with that of other invertebrates in that nerve cell bodies are not confined to the periphery of the ganglia but are scattered within the neuropile as well. Each cell body has a large nucleus and a small amount of cytoplasm. The cytoplasm contains numerous mitochondria, Golgi complexes, endoplasmic reticulum, microtubules and a variety of vesicles (both electron-dense and clear vesicles). Two types of unmyelinated nerve process have been identified in the neuropile: small processes, < 12 μm in diameter, and large or 'giant' processes, with a diameter > 12 μm. The small nerve processes contain four types of vesicle (one electron-lucent and three with electron-dense cores), while the giant processes contain electron-dense vesicles only. The giant nerve processes form the bulk of the nervous tissue in the commissure and longitudinal nerve cords and are characterized by extensive invaginations

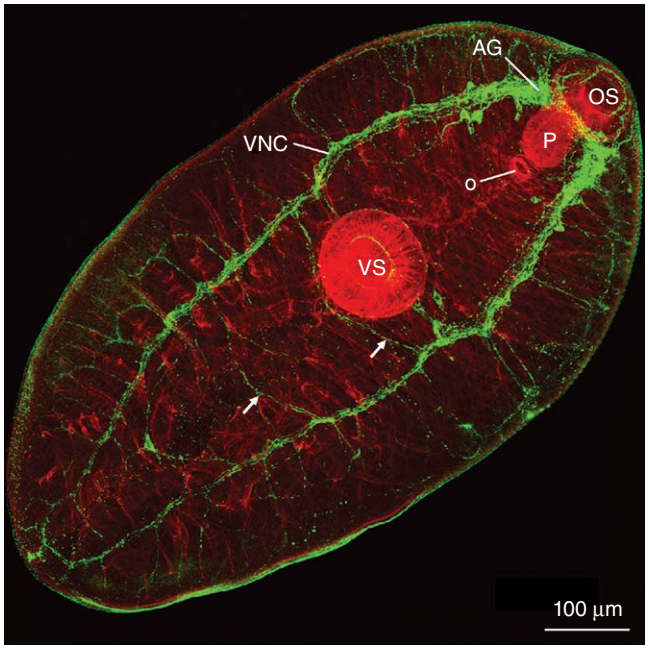

Fig. 3.9. Confocal micrograph showing neuropeptide F immunostaining in 3-week-old *F. hepatica* grown *in vitro*. AG, anterior ganglion; o, oesophagus; OS, oral sucker; P, pharynx; VNC, ventral nerve cord; VS, ventral sucker; arrows, transverse commissures between the nerve cords. Staining and image courtesy of Duncan Wells, Queen's University, Belfast.

of their cell membranes. Two types of synapse occur between the small nerve processes: simple synapses with associated pre- and post-synaptic specializations; and wedge-shaped synapses (or divergent diads) with one pre-synaptic process synapsing on to two post-synaptic processes. No synapses have been observed between giant processes or between the small and giant processes (Sukhdeo *et al.*, 1988a). The invaginations of the giant nerve processes are filled with a second, mesenchyme cell type, whose processes contain a large number of mitochondria and opaque inclusion bodies (Sukhdeo and Sukhdeo, 1994; Sukhdeo *et al.*, 1988a). The mesenchymal cell bodies also contain mitochondria and inclusion bodies, together with Golgi complexes; they occur both around the periphery of the ganglion and within the neuropile. Their processes encircle the ganglion, separating the neuronal processes from the surrounding parenchymal cells, and invaginate into the cell bodies of the giant nerve cells, in addition to their processes. It has been suggested that the mesenchyme cells represent primitive glial-like cells, serving a role in nutrient transfer or acetylcholinesterase production (Sukhdeo and Sukhdeo, 1994). The mesenchyme cells and giant nerve cells only appear in the ganglia following entry of the fluke into the liver; by the time the fluke has reached sexual maturity, these structures occupy up to 60% of the ganglia (Sukhdeo and Sukhdeo, 1990).

The organization of the ganglia undergoes other ontogenetic processes. In 5-day-old flukes, each ganglion is surrounded by a complete rind of cell bodies, one cell thick, that envelops the central neuropile. In intrahepatic juvenile flukes, the integrity of the rind of cell bodies is absent, with the lining of the cell bodies being discontinuous. There is little evidence for any kind of rind in fully mature flukes: cell bodies occur within the neuropile, as well as around it, and mesenchyme cells are also observed within the neuropile (Sukhdeo and Sukhdeo, 1990). The volume of the ganglia increases enormously during development. This is due largely to an increase in the neuropile as a result of the appearance of giant nerve processes and the insinuation of mesenchymal cell elements into the

giant cells and their processes. It is not certain whether the growth of the ganglia is accompanied by a corresponding increase in nerve cell bodies (Sukhdeo and Sukhdeo, 1990). However, the appearance of giant nerve cells suggests that this is likely.

The variety of neuronal vesicles observed in ultrastructural studies suggests that the fluke possesses a number of transmitter molecules. The electron-lucent vesicles probably represent cholinergic vesicles, hence the presence of acetylcholine. Acetylcholine is also indicated by staining for acetylcholinesterase activity, acetylcholinesterase being the enzyme responsible for the inactivation of acetylcholine. Staining for cholinesterase activity in whole-mount preparations has been used to delineate the gross anatomy of the cholinergic component of the nervous system (e.g. Ramisz and Szankowska, 1970). At the electron microscope level, acetylcholinesterase activity has been localized in the cisternae of the endoplasmic reticulum, in the Golgi complex (especially in the *trans* Golgi cisternae where secretory vesicles are budding off) and in vesicles near the Golgi complex; that is, in sites associated with the synthesis, packaging and transport of the enzyme (Sukhdeo *et al.*, 1988b). Acetylcholinesterase is also associated with the outer surface membrane of nerve cell bodies and nerve processes in the neuropile, especially between nerve processes and with synaptic clefts involving presynaptic endings characterized by the presence of clear synaptic (i.e. presumed cholinergic) vesicles – both the simple type of synaptic contact and the wedge-shaped synapse. These are sites where one would expect the enzyme to be situated on the basis of its role in the breakdown of acetylcholine (Sukhdeo *et al.*, 1988b). Pharmacological studies have shown that acetylcholine inhibits the motility of *F. hepatica*, suggesting that it acts as an inhibitory transmitter in the fluke (Holmes and Fairweather, 1984).

The electron-dense neuronal vesicles indicate the presence of aminergic or peptidergic (neurosecretory) transmitters. Among potential aminergic transmitters, 5-hydroxytryptamine (or serotonin), dopamine and noradrenaline (norepinephrine) have been demonstrated in the fluke. The distribution of serotonin has been elucidated by immunocytochemical methods and the synthetic pathways established (Fairweather *et al.*, 1987). It causes a stimulation of fluke motility, suggesting that it is an excitatory transmitter (Holmes and Fairweather, 1984; Tembe *et al.*, 1993). In addition to its role as a neurotransmitter, serotonin serves a wider metabolic role in the regulation of various aspects of carbohydrate metabolism, operating via a cAMP second messenger system (for references, see Fairweather *et al.*, 1987). Dopamine and noradrenaline have also been localized in the nervous system (Bennett and Gianutsos, 1977; Gianutsos and Bennett, 1977) and they exert stimulatory and inhibitory effects, respectively, on fluke motility (Holmes and Fairweather, 1984).

Immunocytochemical studies on the peptidergic component of the nervous system have indicated a potentially greater number of peptidergic than classical neurotransmitters. Indeed, recent bioinformatics analysis, now possible following the release of the *F. hepatica* genome (Cwiklinski *et al.*, 2015b), supports this observation; 36 neuropeptide precursor (npp) genes have been identified in *F. hepatica*, which encode 43 predicted mature peptides, although their functions have not yet been elucidated (McVeigh *et al.*, 2018a). Nevertheless, immunoreactivities to a number of vertebrate peptides have been localized to the nervous system: they are pancreatic polypeptide (PP), peptide tyrosine-tyrosine (PYY), peptide histidine-isoleucine (PHI), gastrin-releasing peptide (GRP), substance P (SP), human chorionic gonadotropin (hCG) and pancreastatin (PST). Immunostaining for the molluscan peptide FMRFamide and the flatworm peptides, neuropeptide F (NPF) and GNFFRFamide, has also been demonstrated (Basch and Gupta, 1988; Gupta and Basch, 1989; Magee *et al.*, 1989, 1991a; Magee, 1990; Marks *et al.*, 1995). These immunofluorescence studies have been reinforced by immunogold labelling studies at the electron microscope level. Labelling for PP and FMRFamide has been localized to electron-dense vesicles in nerve cell bodies and processes within the main elements of the CNS. Double labelling demonstrated apparent co-localization of FMRFamide and PP immunoreactivities in the same dense-cored vesicles, although separate populations of vesicles labelling solely for FMRFamide were also evident (Brownlee *et al.*, 1994). The immunocytochemical evidence for the presence of neuropeptides has been supported by radioimmunoassay data, which have revealed interesting differences in the levels of

peptides in flukes from different hosts (Magee *et al*., 1991a). However, no endogenous peptide has been isolated from *F. hepatica*, so the true identities of the peptides present in the fluke and which bear some relation to peptides present in other organisms are unknown. Having said that, the partial sequence of a PP-like peptide has been resolved (Magee *et al*., 1991b). It is envisaged that further bio-active peptides will be identified through bioinformatics and functional genomics approaches (McVeigh *et al*., 2018a).

Peptide immunoreactivities represent the equivalent of what, in older studies, would have been described as neurosecretions, on the basis of histochemical stains and the presence of large, dense-cored vesicles. The presence of neurosecretory vesicles and cells in *F. hepatica* has been described by a number of workers: Gresson and Threadgold (1964); Grasso (1967a,b); Grasso and Quaglia (1972, 1974); Radlowski (1975); Shyamasundari and Rao (1975). The neurosecretory (peptidergic) component of the nervous system assumes greater significance in the liver fluke and other flatworms because it functions as an endocrine system in the absence of true endocrine and circulatory systems. A gonad-stimulating role for neurosecretions in *F. hepatica* was proposed by Grasso and Quaglia (1972). In support of a morphogenetic role for peptides in the fluke, NPF has been shown to inhibit protein and nucleic acid synthesis, while FMRFamide is stimulatory and GNFFRFamide has no effect (Fairweather *et al*., 1995). The effects of host gut peptides on fluke motility have already been mentioned and it may be that the fluke possesses their equivalents. A number of molluscan, nematode and platyhelminth FMRFamide-related peptides have been examined for their impact on fluke motility. Of the flatworm peptides tested, GYIRFamide had the most excitatory effect, stimulating motility at concentrations as low as 50 μM. RYIRFamide was slightly less potent, while NPF and GNFFRFamide were without effect (Graham *et al*., 1997). Further experiments using isolated muscle fibres, as have been described for *F. hepatica* (Kumar *et al*., 2003, 2004), rather than fluke homogenates or crude muscle strips, will confirm that these signals are specifically transduced within muscle cells. The role of peptides (and serotonin) in egg formation will be discussed in Chapter 4.

In addition to cholinergic, aminergic and peptidergic components of the nervous system, there is evidence for other signalling components. For example, glutamate-like immunoreactivity has been localized in the CNS and PNS of *F. hepatica* (Brownlee and Fairweather, 1996). Further, evidence for the use of the diffusible gaseous neurotransmitter NO comes from localization of NADPH diaphorase in fluke tissue sections (Gustafsson *et al*., 2001) (see section 3.4), although its importance in the *F. hepatica* nervous system has yet to be determined.

Graham *et al*. (2000) published the only report to date on FMRFamide-like peptide (FLP) signalling and found that GYIRFamide appears to signal via a G protein-coupled receptor (GPCR) pathway which results in activation of phospholipase C (PLC) and protein kinase C (PKC). GPCR discovery in *F. hepatica* has been greatly accelerated due to the availability of genome and transcriptome resources (Cwiklinski *et al*., 2015b). Using homology-driven searches of the *F. hepatica* genome, McVeigh *et al*. (2018b) reported the identification of 147 GPCRs, including 135 rhodopsin, five frizzled, three glutamate, two adhesion, one smoothened and one secretin GPCR. Whilst 11 flatworm GPCRs have been deorphanized (i.e. have been matched to their natural ligands), none of them are activated by parasite peptides (McVeigh *et al*., 2018a). However, comparative analysis of the ligand-binding domains of deorphanized rhodopsins with those identified in *F. hepatica* led to the prediction of ligands for 17 receptors activated by acetylcholine, octopamine, 5-HT and neuropeptide F/Y (McVeigh *et al*., 2018b). In line with their role in sensory perception and neuromuscular control/locomotion, *F. hepatica* GPCRs are expressed most highly by NEJs and liver-stage juvenile flukes (McVeigh *et al*., 2018b). Given their expected role in establishing infection, the challenge now is to validate *F. hepatica* GPCRs as targets for novel flukicidal drugs.

3.6 Cytoskeleton

The cytoskeleton can be divided into three components: microtubules (based on tubulin), microfilaments (based on actin) and intermediate filaments. In terms of helminth parasites, intermediate filaments have attracted little

study. However, Tansatit *et al.* (2006) described intermediate filaments in the tegumental cell bodies and throughout the tegumental syncytium (including the spines) of *F. gigantica* using an antibody raised against cytokeratin as a marker. Given the overlap in the immunofluorescence pattern of cytokeratin with that of tubulin and actin the authors suggested that, in fluke, the intermediate filaments act as a scaffold that links microtubules and actin filaments. Most attention has focused on microtubules, because they are the target for benzimidazole anthelmintics (see Chapter 7, this volume).

3.6.1 Microfilaments

Microfilaments play important roles in many cellular processes, including the maintenance of cell shape, movement of single cells (e.g. amoeboid movement), muscle contraction, cytoplasmic streaming, movement of secretory vesicles and cytokinesis (reviewed by Hohmann and Dehghani, 2019). Indirect immunofluorescence techniques using monoclonal and polyclonal anti-actin antibodies have shown that actin is present in the subtegumental and gut musculature of the fluke, as might be expected from its familiar role in muscle contraction. Actin is also present in the vitelline cells, in the spermatogenic cells, in the tegumental cell bodies and their connections with the surface syncytium, and in the tegumental spines (Stitt *et al.*, 1991, 1992a; Tansatit *et al.*, 2006).

Incubation of fluke material in the microfilament inhibitor, cytochalasin B, leads to a number of changes. These include the movement of secretory vesicles in the tegumental and vitelline cells, cell shape and cytokinesis. There is a block in transport of tegumental secretory bodies from the cell body to the base of the tegumental syncytium and from the base of the syncytium to the apical plasma membrane prior to their release (Stitt and Fairweather, 1991). This leads to disruption of the apical plasma membrane, which can be visualized by scanning electron microscopy (Stitt and Fairweather, 1991). Given the important roles that the tegument has and that the integrity of the apical plasma membrane is dependent on the continual movement of secretory vesicles and glycocalyx turnover, such disruption would have serious consequences for the fluke. Treatment with cytochalasin B also leads to disruption of the transport of shell protein globules in the vitelline cells and their aggregation to form shell globule clusters. Furthermore, deposition of shell protein material on the surface of newly formed eggs becomes uneven and abnormal (Stitt and Fairweather, 1991).

Evidence for inhibition of cytokinesis, the final separation phase in cell division, comes principally from studies involving the spermatogenic cells. During spermatogenesis, normal cell division and accompanying cytokinesis is complete until the four-cell stage. Subsequent to this, although mitosis and meiosis continue, cytokinesis is incomplete, resulting in rosettes of eight, 16 and 32 cells (Stitt and Fairweather, 1990). Cytochalasin B treatment leads to formation of bi- and multi-nucleate cells and the typical rosettes of spermatocyte and spermatid cells are replaced by syncytial masses of cells: nuclear divisions continue but not cytoplasmic division. The results are consistent with the distribution of microfilaments in these cells. Moreover, spermatozoon formation becomes abnormal (Stitt *et al.*, 1991).

The combined results of immunostaining with actin antibodies and experiments involving cytochalasin B lend support to the suggestion that microfilaments are involved in a number of processes within the fluke. They include the movement of secretory vesicles in the tegumental and vitelline cells, muscle contraction, the maintenance of cell shape and cytokinesis. These processes are typical of established functions of microfilaments in other cell types. They are disrupted by the flukicides Mirazid and compound alpha, indicating that the actin cytoskeleton is a target for drug action (see Chapter 7 for more detail). Immunoblotting studies of different fluke preparations using a monoclonal actin antibody indicate that there are at least three different forms of actin in the fluke: (i) a 43 kDa form, probably associated with muscle; (ii) a 28 kDa form, not localized to any particular tissue; and (iii) a 15 kDa form in the tegumental spines (Stitt *et al.*, 1992a). The latter result rejects a previous assertion that the spines of *F. hepatica* were not composed of actin (Pearson *et al.*, 1985). This issue has yet to be fully resolved: several studies report that schistosome spines are composed of actin (Cohen *et al.*, 1982; Davis *et al.*, 1985; Abbas and Cain, 1987; Matsumoto *et al.*, 1988;

MacGregor and Shore, 1990), but actin was not detected in the spines of *F. gigantica* (Tansatit *et al*., 2006). The detection of cytokeratin in the spines suggests that this protein, rather than actin, serves to maintain their structural rigidity in *F. gigantica* (Tansatit *et al*., 2006). Interestingly, the Ca^{2+}-sensing protein calmodulin 2 was localized to the spines of adult *F. hepatica*, possibly due to its interaction with scruin, an actin cross-linking protein (Russell *et al*., 2012). Whilst it is not clear how such proteins become embedded within the spines, tegumental granules (circa 0.1–0.2 μm in diameter) have been described within the crystalline matrix of the spines of *Paragonimus westermani* (Fukuda *et al*., 1987). It is speculated that these granules are derived from the tegumental cell cytoplasm and become trapped in the matrix during initial transport and deposit of spine material during early development (Bennett and Threadgold, 1975; Fukuda *et al*., 1987).

3.6.2 Microtubules

Like microfilaments, microtubules are involved in many important cell processes, including the spatial organization and intracellular movements of organelles, the formation of the spindle apparatus and chromosome movements during mitosis and meiosis, the maintenance of cell shape and cell motility (via ciliary and flagellar movement) (reviewed by Hohmann and Dehghani, 2019). Using a monoclonal antibody raised against β-tubulin, tubulin has been localized in the tegumental syncytium, associated cell bodies and the connections between the cells and the surface syncytium (Stitt *et al*., 1992b; Robinson *et al*., 2002, 2003). Immunostaining was also evident in the nerve fibres innervating sensory receptors in the tegument, in the nerve plexus innervating the subtegumental musculature and in the cytoplasmic extensions of the nurse cells within the vitelline follicle (Stitt *et al*., 1992b). Recently, tyrosinated α-tubulin was used as a marker for EVs specifically derived from the gut of *F. hepatica* (Bennett *et al*., 2020a). Immunoblotting of a whole fluke homogenate has shown that fluke tubulin has a molecular mass of approximately 54 kDa, which is consistent with that of tubulin from other helminth parasites and eukaryotes in general (Stitt *et al*., 1992b). Following this initial biochemical characterization, a β-tubulin gene was identified in *F. hepatica* (Robinson *et al*., 2001). This was significant, as it was found to possess a tyrosine at position 200, which has offered a structural explanation for the relative refractoriness of *F. hepatica* to classical (non-triclabendazole) benzimidazoles (Robinson *et al*., 2004); benzimidazole-sensitive nematodes typically have a phenylalanine at this position, but resistant worms have tyrosine (see Fairweather *et al*., 2020 for references). The *F. hepatica* tubulin family has since expanded to five α-tubulin and six β-tubulin isotypes (Ryan *et al*., 2008) and this was confirmed at the genome level (Cwiklinski *et al*., 2015b). Integration of immunofluorescence and quantitative PCR data has revealed that the six β-tubulin isotypes display tissue- and stage-specific expression patterns, with isotype 1 representing the most dominant transcript at the adult stage and with expression largely confined to mature spermatozoa and vitelline follicles (Fuchs *et al*., 2013).

Incubation of fluke material with the microtubule inhibitors colchicine and tubulozole-C results in a number of changes within the fluke. In many respects the changes are similar, but they show some differences and, in general, the changes induced by tubulozole-C are more severe and occur more quickly. Both drugs cause a block in transport of tegumental secretory bodies, but, with colchicine, accumulations of vesicles occur in the cell body (around the Golgi complexes) and at the base of the tegumental syncytium, whereas with tubulozole-C the accumulations occur at the base of the syncytium and in the cytoplasmic connections between the tegumental cells and the surface syncytium (Stitt and Fairweather, 1993). Tubulozole-C also induces: (i) a dramatic coalescence and vacuolation of the GER cisternae and their retraction towards the cell nucleus; (ii) the migration of the Golgi complexes to the periphery of the cell and their gradual disappearance from the cell; and (iii) a change in cell shape. These are classic cell responses to microtubule inhibition, because microtubules are known to be responsible for the organization, cellular distribution and movement of these organelles (Hohmann and Dehghani, 2019). Indeed, tubulozole-C treatment diminished β-tubulin immunofluorescence within the tegumental syncytium of

adult flukes *in vitro* (Robinson *et al.*, 2003). The disruption of the GER and Golgi complexes leads to inhibition of secretory body synthesis and this, together with the block in transport of existing vesicles, has a deleterious effect on the tegumental surface. In the case of tubulozole-C, the damage is so severe that it culminates in the sloughing of the syncytium (Stitt and Fairweather, 1993). In contrast, tubulozole-C caused only minor tegumental disruption in triclabendazole-resistant *F. hepatica* and did not significantly alter the pattern of tubulin immunoreactivity within the tegumental syncytium, suggesting that triclabendazole-resistant flukes are also cross-resistant to tubulozole-C and that both drugs may share the same target molecule (Robinson *et al.*, 2003).

The two inhibitors prevent division of the stem vitelline cells, leading to relatively greater numbers of stem cells but fewer intermediate-type cells (especially the It1-type) in the follicle. Less shell protein is synthesized, resulting in smaller and more loosely packed shell globule clusters, and the movement of the globules to the cell periphery is also blocked. In the mature vitelline cells, the production of 'yolk' globules and glycogen declines and this is accompanied by an increase in autophagy. The nurse cell cytoplasm becomes disrupted, giving the follicle a disorganized appearance (Stitt and Fairweather, 1993). A more dramatic inhibition of mitotic activity by tubulozole-C has been observed in the spermatogenic cells in the testis. The spermatogonial cells are prevented from undergoing cell division and the spermatocyte and spermatid rosettes become disrupted and break apart. With longer periods of time, the tubule becomes almost devoid of cells and some of the spermatozoa that remain show abnormalities in the organization of their microtubules (Stitt and Fairweather, 1992).

As is the case for microfilaments, the combined data from tubulin localization studies and inhibitor experiments indicate that microtubules are involved in a number of important processes in the fluke. They include the movement of secretory vesicles, the spatial organization of cellular organelles, maintenance of cell shape and cell division. These functions are typical of the roles played by microtubules in other cell types. *Fasciola* appears to be more susceptible to the action of tubulozole-C than colchicine, high concentrations (1×10^{-3} M) of the latter being required to bring about any effects. Tubulozole-C is known to be a more potent inhibitor than colchicine (De Brabander *et al.*, 1986). The two inhibitors have different modes of action and the relative insensitivity to colchicine may have a bearing on the limited efficacy of most benzimidazole anthelmintics against *Fasciola*. These drugs are known to act by binding to the colchicine-binding site on microtubules, thus disrupting microtubule-based processes in a variety of helminth parasites. In contrast, *F. hepatica* is susceptible to a benzimidazole derivative, triclabendazole, which lacks activity against other helminths (reviewed by Fairweather *et al.*, 2020). It is possible that triclabendazole acts in a tubulozole-C-like manner and this point is discussed in more detail in Chapter 7.

3.7 Gut

The alimentary tract can be divided into two distinct regions: (i) the foregut, comprising mouth, pharynx and oesophagus; and (ii) the paired intestinal caeca, which end blindly and whose lateral diverticula are highly branched in the adult fluke. Most of the observations on the morphology of the gut are confined to the intestinal caeca of the adult parasite. The epithelial lining of the caeca consists of a continuous single layer of cells of one basic type, although they show considerable variation in fine structure (Robinson and Threadgold, 1975). The differences in structure reflect different functional states as the cells undergo cyclical transformations between absorptive and secretory phases. Neighbouring cells are at different stages in the cycle so that secretion, absorption and digestion are occurring more or less simultaneously and continuously throughout the diverticula. Cells in the secretory phase (group I cells of Robinson and Threadgold, 1975) are characterized by the presence of many dense secretory vesicles, abundant and active Golgi complexes, an extensive network of cisternae of granular endoplasmic reticulum (GER) and numerous mitochondria – features typical of an actively secreting cell (Figs 3.10 and 3.11). The absorptive cells (group II cells of Robinson and Threadgold, 1975) bear much longer and more numerous apical lamellae, between

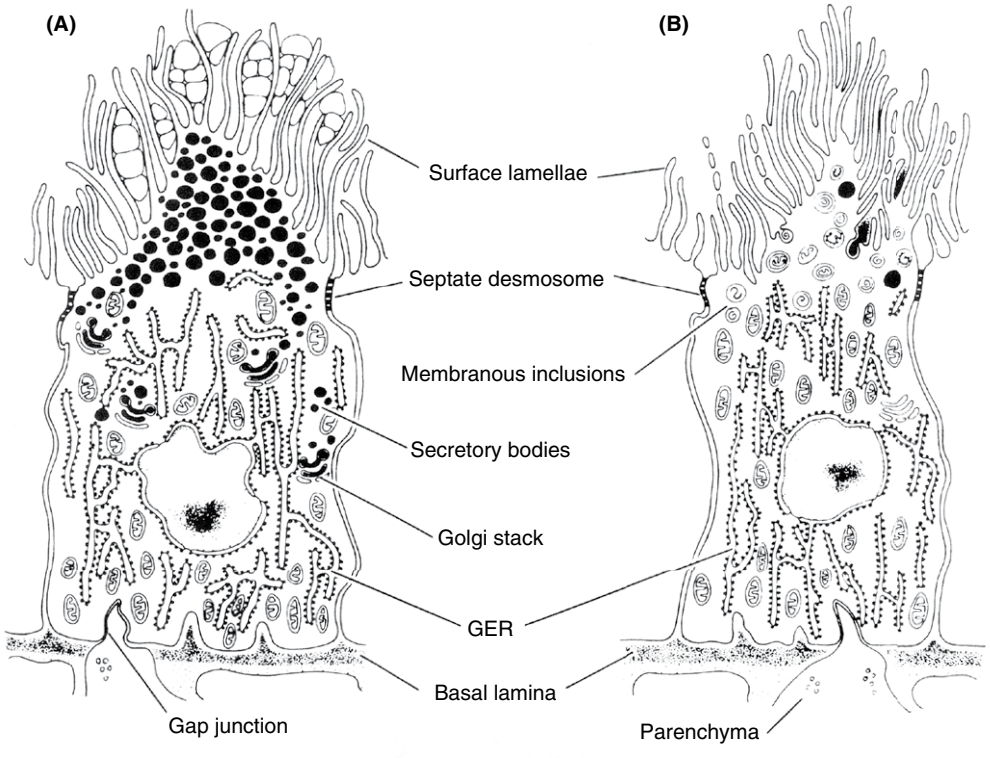

Fig. 3.10. Diagrams showing the fine structure of the gut cell in *Fasciola hepatica* in **(A)** the secretory phase (group I-type cell) and **(B)** the absorptive phase (group II-type cell) of its cycle (Smyth and Halton, 1983; after Robinson and Threadgold, 1975).

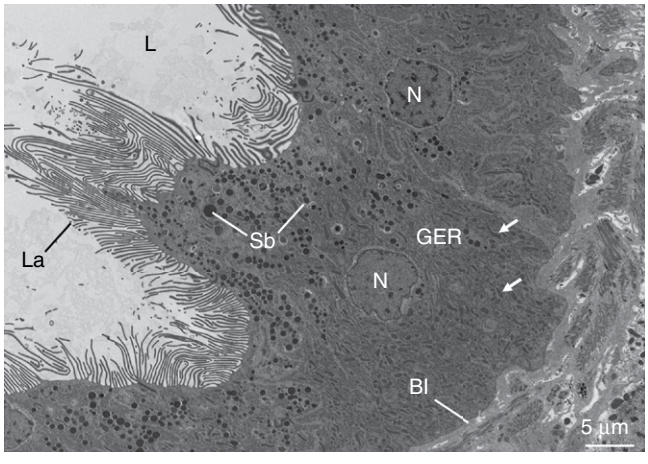

Fig. 3.11. Transmission electron micrograph of a secretory phase gastrodermal cell from adult *Fasciola hepatica*. Secretory bodies (Sb) are densely packed and accumulate towards the apical region of the cell. Granular endoplasmic reticulum (GER) and mitochondria (arrows) dominate the cytoplasm in the basal region of the cell. Bl, basal lamina; L, gut lumen; La, lamellae; N, nucleus.

which lie spherical bodies and membranous whorls. The lamellae are broad, sheet-like structures, with a flat or ruffled surface, that project into the lumen of the gut (see Figs 7, 8, 17 and 18 in Meaney *et al.*, 2005). The group II cells possess few and largely inactive Golgi complexes, show a lack of secretory vesicles and contain numerous cytoplasmic bodies (indicative of endocytosis and autophagy). The main gut caeca are lined by a third form of the cell (group III cells of Robinson and Threadgold, 1975). The group III cells show some signs of secretory and autophagic activity, but their main function appears to be associated with the movement of material back and forth within the lumen of the main caeca and their diverticula and with the mixing of the released secretion with this material; this role is aided by the subepithelial musculature, which is more developed along the main caeca than the diverticula (Robinson and Threadgold, 1975).

The gut is also a site of EV formation, along with the tegument, protonephridial system and parenchyma. The gut EVs are of a larger size than those produced by the tegument, which were discussed in section 3.2.2: the two subpopulations are designated 15K and 120K EVs, respectively (Cwiklinski *et al.*, 2015a; Bennett *et al.*, 2020a). Gut-derived EVs are formed by an SMase-independent mechanism and differ further from tegumental EVs with respect to the cargo molecules they contain; for example, they contain the zymogens of cathepsin L peptidases (Bennett *et al.*, 2020a). Immunolabelling studies have localized EVs to the gastrodermis and specifically to the secretory vesicles, with a concentration towards the apices of the gastrodermal cells (Cwiklinski *et al.*, 2015a; Bennett *et al.*, 2020a). Ligation experiments, involving the tying of ligatures around the oral sucker or excretory pore, or both, were designed to determine the relative importance of different sites for EV release and the results showed that EVs were principally released from the gut (Bennett *et al.*, 2020a). Treatment with the SMase inhibitor GW4869 caused a significant reduction in release of 120K EVs, but had only a minor impact on the release of 15K EVs, indicating that the latter form independently of SMase activity (Bennett *et al.*, 2020a). The inhibitor induced a greater level of autophagy in the gastrodermal cells, along with some vacuolation (Bennett *et al.*, 2020a). The combined data support the idea that disruption of EV formation and release is a valid target for therapeutic intervention.

Development of the fluke gut in the mouse host was described by Dawes (1962). Initially, in the NEJs, the caeca are short and show signs of elongation in the first day following excystment. Lateral diverticula begin to develop in day 3 pi, following invasion of the liver. By day 8 pi, there are 13 diverticula on each side of the body and they are club-shaped. Secondary and tertiary branches are present by day 11 pi, the intestine displaying much of the complexity of the fully developed system (Dawes, 1962). In the NEJ, the gut cells are filled with secretory vesicles produced and stored during the metacercarial phase. The cells appear to be specialized for secretion only, not undergoing the cycles of secretion and absorption seen in the adult fluke. A dramatic reduction in numbers of secretory vesicles is evident post-excystment, leading to the suggestion that they contain hydrolytic enzymes for use in excystment, penetration through the gut wall, migration and penetration of the liver capsule (Bennett and Threadgold, 1973) (see discussion of cathepsin proteases below). Secretory activity, as evidenced by the presence of Golgi complexes, begins during day 1 pi. The development of apical lamellae indicates limited absorptive capacity, mainly of small molecules in solution (Bennett, 1975c). A second, smaller type of secretory vesicle appears in day 3 pi following penetration of the liver capsule and the cells start to assume a more adult-like morphology, but true cyclical activity is not evident until 2 weeks pi (Bennett, 1975c).

Secretory activity in the gut cells of the adult fluke has been studied by pulse-chase autoradiography, involving tritiated amino acids (tyrosine, methionine, leucine and phenylalanine) (Hanna, 1975). Initially (following a 0–10 min chase period), the radioactive labels were incorporated into protein being synthesized by the GER in the base of the cell. After a 20 min chase period, label had moved, via the transition vesicles, into the Golgi cisternae and was associated with the secretory vesicles packaged by the Golgi complex. In longer chase periods (30–45 min), label was predominantly located in the apical region of the cell, being associated with the secretory vesicles, with material being released

at the apical surface and with the lamellae. Little activity was detected in material following a 60 min chase period, indicating that the entire process of synthesis, transport and release of secretory proteins takes less than 1 h (Hanna, 1975). The results also suggest that the entry of label occurs across the basal and lateral membranes, rather than the apical membrane, and probably via the parenchymal cells and their inpushings into the gut cells, as described previously.

As is typical of digenetic trematodes, digestion in *F. hepatica* is predominantly an extracellular process, taking place in the caecal lumen before being completed intracellularly. The eccrine release of secretions by the gastrodermal cells is associated with disruption of the apical plasma membrane and lamellae. This membranous material, together with any substances adsorbed on to its glycocalyx or trapped between the lamellae, is then endocytosed and gives rise to cytoplasmic bodies in the cell. The bodies are reactive for acid phosphatase, which is derived from the ingested lamellar membrane, and develop into secondary lysosomes. Their contents undergo further enzymic breakdown and the soluble products diffuse into the surrounding cytoplasm; the indigestible remnants accumulate in residual bodies that are extruded from the cell. The diet of the fluke changes during its migration within the mammalian host. Initially, during the gut penetration phase, the gut is predominantly secretory and any ingestion of tissue debris resulting from penetration is of minor importance. Feeding on abdominal viscera is probably limited and insufficient for growth and development, since flukes that become lost in the abdominal cavity remain stunted (Dawes, 1963). Following entry into the liver, the juvenile fluke feeds largely on hepatic cells together with some ingestion of blood. The adult fluke, resident in the bile duct, is primarily a blood-feeder, although it ingests hyperplastic bile duct epithelium as well.

Much progress has been made since the first edition of this book in identifying the cathepsin-type cysteine proteases produced by *F. hepatica*. The fluke produces a range of cathepsins, which are expressed at different stages during its development in the mammalian host (Collins *et al.*, 2004; Cancela *et al.*, 2008; Robinson *et al.*, 2008a,b, 2009; Stack *et al.*, 2008; Smooker *et al.*, 2010; Norbury *et al.*, 2011; Cwiklinski *et al.*, 2019). Cathepsin B-type enzymes are more important in the earlier stages, while cathepsin L (especially cathepsin L1) predominates in the adult. Cathepsins are produced and stored as inactive zymogens in the gastrodermal cells, becoming activated in the acidic environment of the gut lumen following their release.

As regards their roles, the cathepsin enzymes produced by the metacercariae most likely function in the excystment process; they are stored in this dormant stage and released following activation during excystment. They are also likely to assist the NEJs in penetrating through the gut wall into the peritoneal cavity and the subsequent penetration of the liver capsule following a period of migration within the cavity. B-type cathepsins predominate and will be released to digest host tissues (Cancela *et al.*, 2008; Beckham *et al.*, 2009; Robinson *et al.*, 2009; Smooker *et al.*, 2010; Cwiklinski *et al.*, 2019). At this stage, the gut in the NEJs has only a secretory role – it does not acquire a dual secretory/absorptive role until the fluke is inside the liver (Bennett and Threadgold, 1973). Cathepsins L and B can also cleave immunoglobulin (IgG), so have a role in immune evasion (Carmona *et al.*, 1993; Wilson *et al.*, 1998; Berasain *et al.*, 2000; Smooker *et al.*, 2010; Cwiklinski *et al.*, 2019).

The cathepsins produced by immature flukes will be involved in their migration through the liver parenchyma prior to penetration of the bile duct wall into the duct lumen, and also in immune avoidance and feeding. Immature flukes possess a range of B- and L-type cathepsins, which have the ability to: (i) degrade collagen and other interstitial matrix proteins (fibronectin, laminin) to facilitate their burrowing activities through the liver tissue and passage through the wall of the bile ducts (Stack *et al.*, 2008; Corvo *et al.*, 2009; Robinson *et al.*, 2011; Mebius *et al.*, 2018; Cwiklinski *et al.*, 2019); (ii) degrade IgG to assist in immune evasion (Corvo *et al.*, 2009; Cwiklinski *et al.*, 2019); and (iii) cleave haemoglobin, for feeding on blood (Mebius *et al.*, 2018; Cwiklinski *et al.*, 2019). By contrast, adult flukes in the bile ducts are in an immunologically privileged environment, so the main focus is feeding on blood, to supply the basic materials for the production of enormously high numbers of eggs. Substrates for the cathepsin L enzymes include haemoglobin, fibrinogen and fibrin, and this enables flukes to feed on blood for prolonged periods of time (Mebius *et al.*, 2018; Cwiklinski *et al.*, 2019).

A number of techniques have been used to determine the roles of cathepsins, including the use of cathepsin inhibitors and RNA_i knockdown experiments. The latter have shown that silencing of either cathepsin B or L in the NEJ reduces gut penetration, indicating that both are required for this process (McGonigle *et al*., 2008). Silencing also induces abnormal locomotory behaviour. Treatment of NEJs with the cathepsin L inhibitor, C34, reduced their ability to penetrate gut sacs *in vitro* and decreased their motility, leading to their death within a week (Ferraro *et al*., 2016). In a separate study, incubation of NEJs in culture medium containing cathepsin B or L inhibitors led to a decrease in motility and viability, suggesting that the enzymes are important for parasite survival (Beckham *et al*., 2009). The cathepsin B inhibitor, CA 074Me, was more potent than the cathepsin L5 inhibitor, E-64-d (Beckham *et al*., 2009).

Inhibitors have been shown to evoke an anti-fecundity effect *in vivo*, with reduced egg output and decreased egg development and viability. Recovered flukes were smaller in size and less well developed than normal (Alcalá-Canto *et al*., 2006, 2007). Incorporation of cathepsin inhibitors into the excystment medium significantly reduced excystment of metacercariae, suggesting that cathepsins are involved in the process of cyst rupture (Robinson *et al*., 2009).

The localization of cathepsin enzymes in *F. hepatica* has been studied by a number of techniques. Incubation of NEJs in a fluorescently labelled cathepsin B-specific inhibitor and examination with a confocal microscope showed that the cathepsin was localized to the gut of the NEJ (Beckham *et al*., 2009). An immunocytochemical study, carried out with confocal microscopy and using an antibody to cathepsin L1, localized the cathepsin to the gastrodermal epithelial cells throughout the gut of adult *F. hepatica* (Collins *et al*., 2004). A complementary immunogold labelling investigation, utilizing antibodies to both the mature cathepsin L1 and its propeptide, showed that the enzyme was confined to the secretory vesicles within the gastrodermal cells (Collins *et al*., 2004). A similar immunogold labelling result was obtained by Smith *et al*. (1993). A separate immunolocalization study localized cathepsin L3 and cathepsin B2 to the gut of NEJs (Cwiklinski *et al*., 2018). *In situ* hybridization studies involving cathepsin L proteases have demonstrated that the enzyme is expressed by gastrodermal cells in adult flukes (Dalton *et al*., 2003).

While the predominant location of cathepsins in *F. hepatica* is in the gastrodermal cells, the enzymes have also been demonstrated in other tissues within the fluke, for example cathepsin L in the Mehlis' gland (Wijffels *et al*., 1994). In the related fluke *F. gigantica*, cathepsin B mRNA has been localized by *in situ* hybridization in a number of tissues of the reproductive system, namely the Mehlis' gland, prostate gland, vitelline glands, testis and eggs (Meemon *et al*., 2004). This suggests that they may play roles in egg production.

The antibody in the coproantigen ELISA assay likely recognizes a cathepsin-type enzyme, as immunolabelling is restricted to the gastrodermal cells of the fluke (Flanagan *et al*., 2011; Muiño *et al*., 2011; Kajugu *et al*., 2012, 2015; Gordon *et al*., 2013).

Cathepsin enzymes are important targets for both drug development and vaccine development (see Chapters 7 and 12, respectively).

3.8 Excretory System

The excretory system of *F. hepatica* is of the protonephridial type and consists of flame cells that are connected to fine tubules. In turn, the tubules lead into primary ascending or descending ducts, which feed into the main (paired ascending, then descending) ducts which drain into a single bladder that opens posteriorly via a median excretory pore. The terminal flame cell contains a large oval or kidney-shaped nucleus, mitochondria, a few cisternae of GER and vesicles (Pantelouris and Threadgold, 1963; Gallagher and Threadgold, 1967). It bears a bunch of 120–135 hexagonally arranged cilia that form the 'flame' and project into the lumen of the associated tubule (Pantelouris and Threadgold, 1963) (Figs 3.12 and 3.13). The beating of the flame creates a movement of fluid within the system. It has been suggested that the axial filaments of individual cilia within the flame are organized in such a way as to enable alternate rows of cilia to beat in opposing directions, resulting in equal propulsion for both directions of the stroke (Smyth and Halton, 1983). The cilia are anchored in the cytoplasm of the cell by means of basal bodies, and fibres from the basal

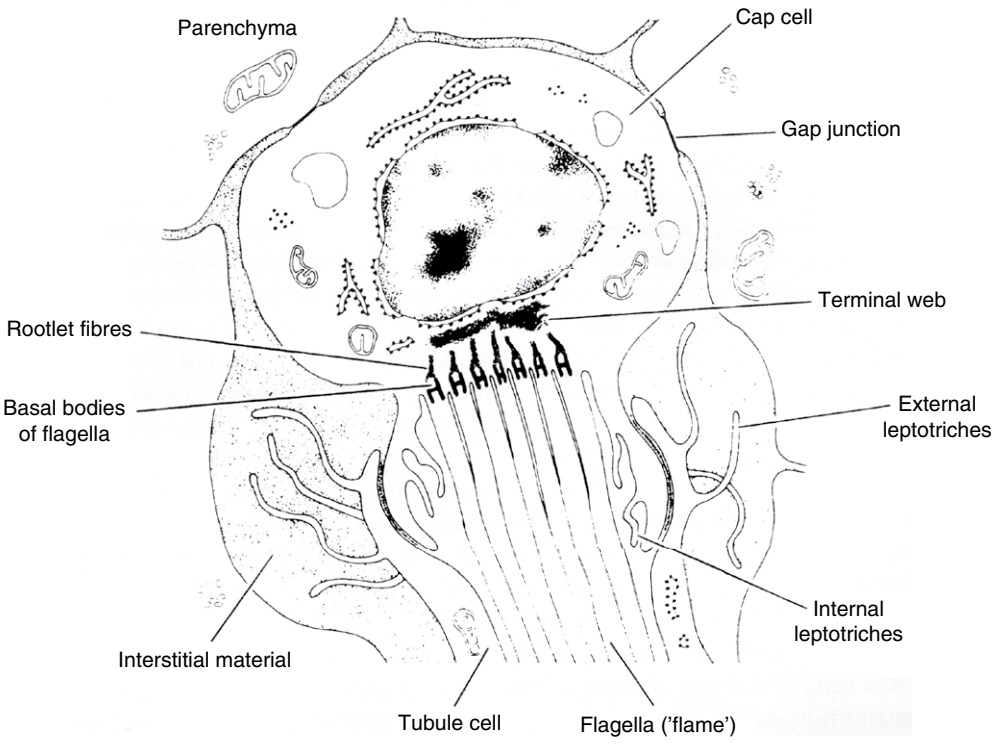

Fig. 3.12. Diagram showing the fine structural organization of a flame cell and associated structures in *Fasciola hepatica* (Smyth and Halton, 1983).

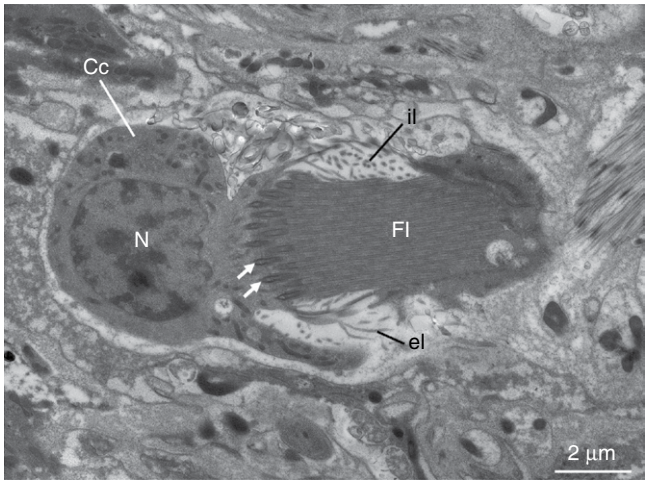

Fig. 3.13. Transmission electron micrograph of a flame cell from adult *Fasciola hepatica*. Cc, cap cell; el, external leptotriches; Fl, 'flame' (flagella); il, internal leptrotriches; N, nucleus; arrows, rootlet fibres.

bodies and their rootlets penetrate more deeply into the cell to form an extensive network (Pantelouris and Threadgold, 1963).

The proximal part of the tubule leading away from the flame cell is composed partly of the cytoplasm of the flame cell and partly of the cytoplasm of the tubule cell. The two cells are connected by interdigitations of ribs of cytoplasm, which are connected to each other by fibrous elements. The ribs occurring internally to the sheet originate from the flame cell and those occurring externally arise from the tubule cell (Pantelouris and Threadgold, 1963; Gallagher and Threadgold, 1967). Ultrafiltration across the fibrous sheet is believed to occur in the spaces between the ribs. Additional microvillus-like projections, termed internal leptotriches, extend from the flame cell cytoplasm into the lumen around the flame. Similar structures, termed external leptotriches, extend from the tubule cell into the surrounding interstitial material (Pantelouris and Threadgold, 1963; Gallagher and Threadgold, 1967) (Figs 3.12 and 3.13). The leptotriches presumably serve a structural role. While the lumen of the proximal region of the small tubule leading from the flame cell is intracellular, that of more distal regions of the tubule and of the primary ascending and descending ducts is intercellular. The lining is formed of one or two cells surrounding the lumen and joined to themselves or adjacent cells by septate desmosomes. The cytoplasm contains some mitochondria and a few smooth-membraned inclusions and the luminal plasma membrane is thrown into short irregular microvilli (Pantelouris and Threadgold, 1963; Gallagher and Threadgold, 1967; Bennett and Threadgold, 1973; Bennett, 1977). Some of the cells bear groups of cilia in the NEJs (Bennett and Threadgold, 1973) but the cilia are absent in the adult (Bennett and Threadgold, 1973; Bennett, 1977). The function of the cilia may be to assist fluid movement against capillary drag, as the ascending ducts of the juvenile are half the diameter of adult ducts. Another possibility is that the cilia specifically help to pump fluid through the excretory system of the metacercaria while enclosed in the cyst (Bennett, 1977).

The lining of the main collecting ducts and bladder is syncytial, the cytoplasm containing mitochondria, ribosomes, GER, Golgi complexes, β-glycogen granules and large lipid droplets (Fig. 3.14). The apical surface bears long, narrow lamellae and the basal plasma membrane forms invaginations (Gallagher and Threadgold, 1967; Bennett and Threadgold, 1973). The lining of the excretory bladder has a morphology similar to that of the ducts, although the lamellae may be longer. Near the excretory pore is a long septate desmosome connecting the bladder

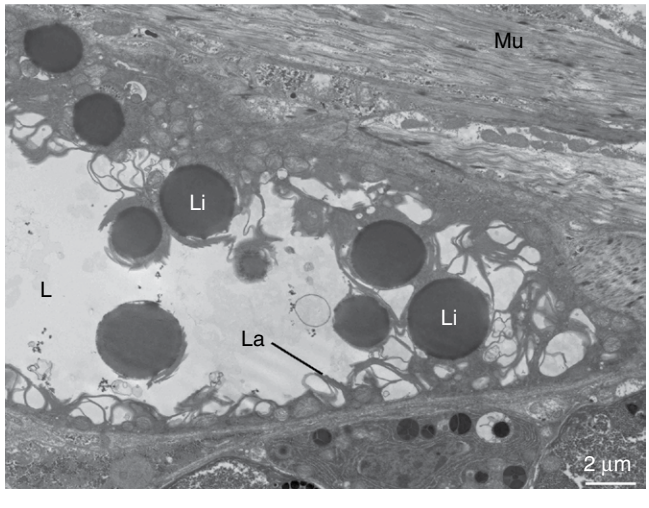

Fig. 3.14. Transmission electron micrograph showing a section through an excretory duct from adult *Fasciola hepatica*. Electron dense lipid droplets (Li) are prominent within the duct lumen (L). Note the numerous lamellae (La) that project from the duct lining. Mu, muscle.

wall to the distal tegument, which appears to line the pore opening itself (Bennett and Threadgold, 1973).

The primary ascending ducts are not present in the NEJ but arise and grow early during development, as the posterior region of the body grows (Bennett, 1977). The number of flame cells doubles during the first 10 days of development in the mouse host, again mainly in the posterior region of the body (Kawana, 1940) (Fig. 3.15). A 'reserve bladder' is not present in the metacercaria but begins to develop 3 days after feeding in the juvenile (Kawana, 1940). The lumen of the main descending duct and bladder of the NEJ is filled with large round concretions, built up of concentric rings of granular or fibrous material, but they disappear within 24 h (Bennett and Threadgold, 1973; Bennett, 1977). The concretions resemble calcareous corpuscles of cestodes and may serve roles in carbon dioxide fixation, in buffering of excretory fluids and in concentrating metabolic waste during the metacercarial stage when removal of waste products would be difficult (Bennett and Threadgold, 1973; Bennett, 1977). Lipid droplets appear in the syncytial lining and lumen of the main ducts and bladder by 12 h pi, suggesting that the excretory system comes into operation very quickly, ejecting the concretions and becoming involved in lipid metabolism (Bennett, 1977). The number of lamellae lining the main ducts and bladder increases in the 12 h following excystment, as do the number and length of the invaginations of the basal plasma membrane. It is evident, then, that there are a number of gross changes in the excretory system that take place during the early stages of development in the final host, but the basic ultrastructure and functioning of the adult system is considered to be established by 12 h pi (Bennett, 1977).

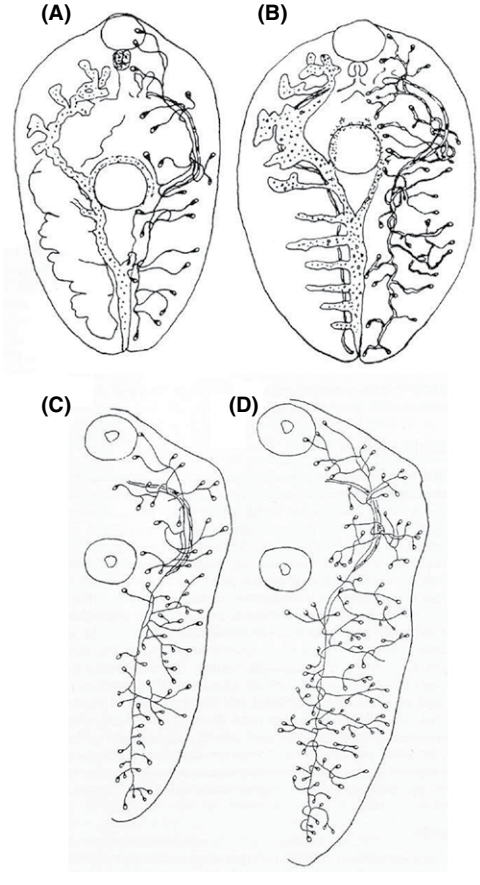

Fig. 3.15. Diagram showing the development of the excretory system of *Fasciola hepatica* in the mouse host: **(A)** 5 days post-infection; **(B)** 10 days post-infection; **(C)** 11 days post-infection; and **(D)** 12 days post-infection.

The excretory system is surrounded by interstitial material. In places around the circumference of the flame cell and along the ducts, the layer is penetrated by processes from the parenchymal cells. Junctional complexes form at the points of contact between the excretory and parenchymal cells, as previously described (Gallagher and Threadgold, 1967). Acid and alkaline phosphatase activity is associated with these complexes, the enzyme being located on the parenchymal side of the complex (Threadgold, 1968; Fujino *et al*., 1983). This observation supports the idea that the complexes are sites where intercellular exchange of excretory products takes place, emphasizing the role of the parenchyma as a transport system (Threadgold and Gallagher, 1966; Gallagher and Threadgold, 1967; Pankao *et al*., 2006). Deposits of phosphatase activity are present within and between the surface lamellae and are associated with the basal invaginations of the syncytial epithelium. Enzyme activity appears to be confined to the smaller and medium-sized ducts of the system, perhaps being related to the resorptive and excretory functions of these ducts, as compared with the purely excretory function of the large ducts (Threadgold, 1968). Moreover, it is indicative of

transport activity, an activity that is aided by the amplification of the apical and basal plasma membranes. Pinocytotic activity has been observed on the luminal surfaces of the flame cells, tubules and primary ducts of juvenile flukes, but does not occur in the adult. The process may be of benefit to the rapidly growing juveniles by increasing the resorption of potentially metabolizable molecules from the fluid drawn into the system by the flame cells (Bennett, 1977).

The fluid in the lumen of the excretory system contains nitrogenous compounds, such as ammonia and urea, and several amino acids, including proline, alanine and histidine (Lutz and Siddiqi, 1971). Both neutral (including cholesterol and its esters) and polar lipids are excreted (Burren *et al.*, 1967), being released from the syncytial epithelium by an apocrine mechanism (Bennett, 1977). Recently, the excretory system has been suggested as a route of export for EVs from adult fluke (de la Torre-Escudero *et al.*, 2019; Bennett *et al.*, 2020a). Initial immunocytochemistry experiments (using an antibody raised against the *F. hepatica* EV marker CD63) stained distinct vesicular structures within 'Y-branched' ducts that are characteristic of the excretory system (Pantelouris and Threadgold, 1963). Intriguingly, the ducts appeared to converge upon the gastrodermis, suggesting that at least some of their contents may drain towards, and exit via, the gut lumen; this is an unexplored morphological scenario that is also considered possible in free-living planarians (Rink *et al.*, 2011). Further immunocytochemical analysis localized a wider panel of *F. hepatica* EV marker proteins (including TSG101, ALIX, Ral-A, SMPD2 and DM9) to the excretory duct epithelium of adult flukes (Bennett *et al.*, 2020a). The antibodies did not, however, label the lipid droplets within the lumen of the excretory ducts (or their proteinaceous lamellar coat). Indeed, TEM shows that the lipid droplets observed by Burren *et al.* (1967) are morphologically distinct (Marcilla *et al.*, 2012; Cwiklinski *et al.*, 2015a; Roig *et al.*, 2018) and not likely to be a component of the EVs isolated from parasite culture supernatants by ultracentrifugation.

The anti-ALIX antibody labelled a cell putatively identified as a flame cell, based on the morphology of the tuft, its connection to a large duct and its distribution in the parenchyma (Bennett, 2019). The capacity for *F. hepatica* flame cells to secrete vesicles is supported by the work of Gallagher and Threadgold (1967), who found them in the duct lumen proximal to the flame cell apex. Studies in cestodes indicate that vesicles in the excretory duct lumen are derived from the ciliated tuft of the flame cell (Valverde-Islas *et al.*, 2011), although this mechanism has not been investigated in liver fluke. Regardless of the cellular origin of the vesicles (i.e. flame cells or excretory duct epithelial cells), mechanical blockage (by ligation with surgical suture) of the excretory pore reduced the release of small EVs from adult flukes by up to 50% during *in vitro* culture (Bennett *et al.*, 2020a), which provides further evidence of a secretory role for this system.

In conclusion, the excretory system of *Fasciola* appears to play a role in the regulation of body-fluid composition and in the excretion of waste materials. Recent evidence suggests that it also acts as a conduit for the trafficking of EVs within parasite tissues (potentially receiving EVs that have been produced in other tissues, such as the parenchymal cells) or exporting them extracorporeally via final discharge from the gut lumen or excretory pore. However, the connectivity and functional relationships with other fluke tissues require further investigation. In contrast to the tegument, which has some control over its osmotic state (Threadgold and Brennan, 1978), the excretory system shows little evidence of any response to ionic or osmotic stress. Rather, it acts as an osmoconformer, being in osmotic balance with its host's body fluids (Knox and Pantelouris, 1966; Siddiqi and Lutz, 1966).

3.9 *In vitro* Models of Fluke Infection

Despite some advances, one of the major gaps that remain regarding our knowledge of *Fasciola* ultrastructure (and of the basic biology of the parasite in general) has been created by the tendency to concentrate on the 'adult' (bile duct) and 'juvenile' (liver parenchyma) stages, to the exclusion of other, earlier stages *before* the fluke reaches the liver. After all, the overall development of the fluke, from the metacercaria to the adult, accompanies a complex migration from the gut lumen through the gut wall, into the peritoneal cavity, penetration into the liver and final location in the bile duct. Thus, it has to accommodate a number of changes of habitat.

The fluke must be able to detect the appropriate stimuli and respond to them by altering not just behaviour but also diet, respiratory metabolism and morphology. The developmental sequence takes several weeks and the changes involved are subtle and gradual. So, there is a need to examine the early stages that are critical in enabling the fluke to become established in the mammalian host. In this section we review the progress made in the development of *in vitro* models of infection and how these have been used to investigate the early stages of *Fasciola* development.

Access to good numbers of viable cysts is critical for the study of the early/invasive stages *in vitro*. After considerable research effort we now have efficient and reliable protocols for *in vitro* excystment of *Fasciola* metacercariae (Dixon, 1964; Hanna and Jura, 1976; Tielens *et al*., 1981; McGonigle *et al*., 2008; Robinson *et al*., 2009). Whilst protocols vary, the key factors for successful excystment appear to be availability of CO_2 and to a lesser extent bile (which may serve to make the cyst wall more permeable to CO_2) (Hanna and Jura, 1976). In some protocols whole bile has been replaced with individual bile salts such as taurocholic acid (Tielens *et al*., 1981; Robinson *et al*., 2009) or tauroglycocholic acid (Hanna and Jura, 1976; McVeigh *et al*., 2014; McCusker *et al*., 2016; Cwiklinski *et al*., 2018). Excystment of *F. gigantica* metacercariae was significantly reduced (60% reduction for *F. gigantica* and 91% for *F. hepatica*) when the reducing agent L-cysteine was omitted from the excystment medium (Hanna *et al*., 1975; Robinson *et al*., 2009). The requirement for reducing conditions implied a role for thiol-dependent cysteine peptidases in the excystment process (the activity of these enzymes typically being enhanced by reducing agents). Indeed, the level of excystment was significantly reduced when the broad-range cysteine protease inhibitor E-64 (98% reduction) or the cathepsin-specific inhibitor Z-Phe-Ala-CHN_2 (99% reduction) was added to the excystment medium (Robinson *et al*., 2009).

Excystment of *Fasciola* metacercaria and subsequent *in vitro* culture of NEJs, typically for a few hours (usually in mammalian cell culture media with addition of supplements – see below), has been a useful approach for the collection of excretory/secretory (ES) products for identification by mass spectrometry-based proteomics techniques (de la Torre-Escudero and Robinson, 2020). Such axenic cultures have limited value for the study of host–parasite interactions given the absence of host tissue or host-derived soluble factors. Consequently, the 'gut loop' model of early fluke infection has seen a resurgence since it was first described in the 1970s. The gut loop model was first described for use with *F. gigantica* by Hanna *et al*. (1975) and represents a simple, yet powerful, technique for studying gut penetration by NEJs *in vitro*. The technique involves addition of NEJs into the lumen of a length of mouse small intestine that has been ligated with thread/surgical suture at one end. Subsequent ligation of the other end of the loop creates a 'bag', which is then submerged, and maintained, in culture media at 37°C for a suitable period of time. The gut loop mimics the early stage of infection of the mammalian host. NEJs that have penetrated the mucosa, and successfully traversed the gut wall, can be collected from the culture medium on the other side. Up to 78% penetration of *F. gigantica* NEJs was reported by Hanna *et al*. (1975) with penetration efficiency waning distally along the gut in the order duodenum/upper small intestine (78% penetration), middle small intestine (68%), lower small intestine (40%) and colon (19%). Kawano *et al*. (1992) found that *F. hepatica* NEJs could readily traverse many regions of the intestine of mice, rats and rabbits but that chicken intestine could not be penetrated. The *in vitro* (rodent) gut loop model has been modified by many groups (e.g. Garcia-Campos *et al*., 2016b, 2017) (Fig. 3.16) and has been variously used to determine the efficacy of anthelmintic compounds against migrating NEJs (Ferraro *et al*., 2016), to investigate the phenotypic effects of silencing cathepsins B and L in *F. hepatica* NEJs by RNAi (McGonigle *et al*., 2008) and to investigate the effect of gut penetration on the tegumental ultrastructure and the cytochemical properties of the glycocalyx of *F. gigantica* NEJs (Hanna *et al*., 2019a,b). The gut loop technique has also been adapted as an *ex vivo* model of gut penetration (Burden *et al*., 1981; Van Milligen *et al*., 1998a,b). However, it is ethically more difficult to justify the use of *ex vivo* models over *in vitro* alternatives (Garcia-Campos *et al*., 2016b).

To date, co-culture of flukes with mammalian cells *in vitro* has been limited, although *F. gigantica* NEJs have been maintained for up to

60 days on bovine spleen cell monolayers (Hanna *et al.*, 1975). There have also been varying levels of short-term success when culture medium was supplemented with sera and/or red blood cells (Davies and Smyth, 1978; Smith and Clegg, 1981). More recently, a cell-free *in vitro* culture model was described by McCusker *et al.* (2016) in which *F. hepatica* NEJs survived in media, supplemented with 50% chicken serum, for up to 29 weeks (Fig. 3.17). Importantly, the cultured flukes underwent significant growth and development, evidenced by the appearance of branched gut caecae, normal progression of T0/T1/T2 bodies in the tegumental syncytium and the formation of early reproductive structures, making this a promising model for studying the development of the early intra-mammalian life-cycle stages.

3.10 Conclusions and Future Perspectives

We are now firmly in the post-genomic era with regard to *Fasciola* research. The availability of '-omics' datasets, particularly those derived from distinct life-cycle stages, are allowing researchers to probe fluke development in detail not previously possible (e.g. Robinson *et al.*, 2009; Cwiklinski *et al.*, 2015b, 2018). The considerable number of ultrastructural studies, the majority of which were conducted in the 1960s and 1970s, are still highly relevant today. Indeed, in many cases knowledge of the fine organization of parasite cells/tissues is helping to interpret molecular data, for example unravelling the multiple pathways of EV biogenesis and release from the *F. hepatica* gut and tegument (de la Torre-Escudero *et al.*, 2016). The challenge now is to integrate molecular data with knowledge of fluke structure to provide a 'moving picture' of fluke development at both cellular and molecular levels. An important next step will be the detailed characterization of *Fasciola* neoblast-like cells (McCusker *et al.*, 2016; Cwiklinski *et al.*, 2018) and their role in the early development of organ systems. The continued use of gene silencing approaches, such as RNAi (reviewed by McVeigh *et al.*, 2018a), and the development of genome editing approaches, such as CRISPR/Cas-9 knockout as tools for investigating gene function in trematodes (Arunsan *et al.*, 2019; Ittiprasert *et al.*, 2019), will undoubtedly facilitate this process.

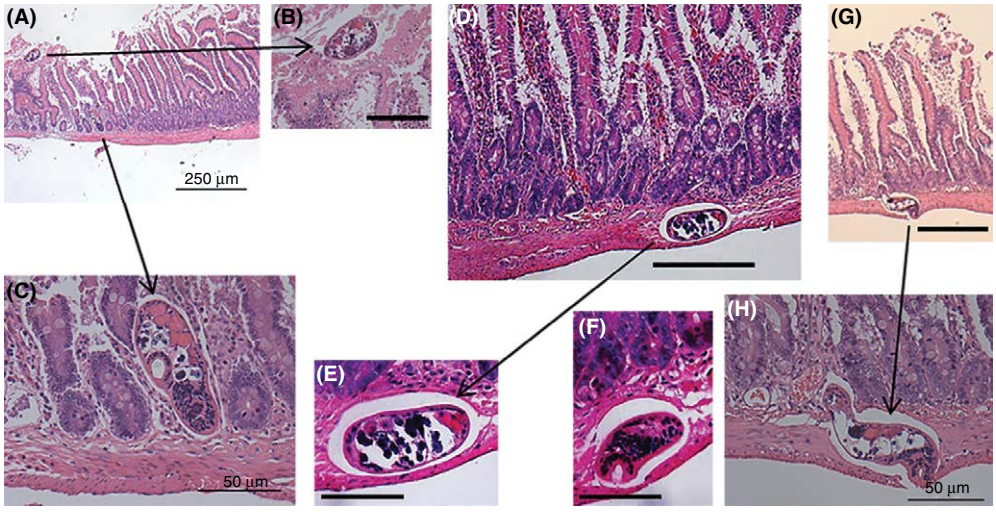

Fig. 3.16. *In vitro* penetration of rat distal jejunum by *Fasciola hepatica* newly excysted juveniles (NEJs). Samples of jejunum were fixed and sections examined every 50 µm of tissue for the presence of NEJs in the mucosa **(A, B, C)** and in the serosa **(D, E, F, G, H)** at 60 min (A, B, C), 90 min (D, E), 120 min (F) and at 150 min (G, H) post NEJ inoculation. Scale bar = 250 µm (A, G). Scale bar = 50 µm (B, C, D, E, F, H) (Garcia-Campos *et al.*, 2016b).

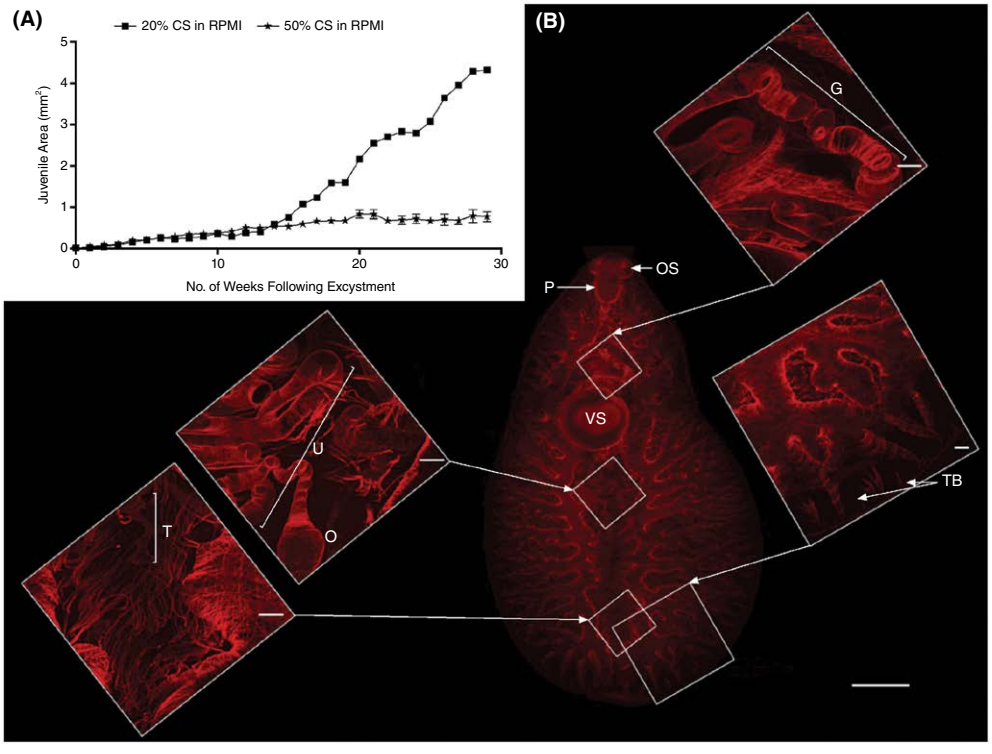

Fig. 3.17. (A) Growth and development of juvenile *Fasciola hepatica* maintained *in vitro* in the presence of chicken serum (CS) over 29 weeks. **(B)** Confocal microscope images of these flukes show the presence of various structural features, including the oral sucker (OS), pharynx (P), gonopore tubing (G), ventral sucker (VS), uterine tubing (U), ootype (O), testes tubing (T) and tertiary branching of digestive caeca (TB). Scale bars: 500 µm, main image; 50 µm, smaller images (McCusker *et al*., 2016).

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4 The Reproductive System of *Fasciola hepatica*

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4.1 Introduction

A very large proportion of the body in adult liver flukes is occupied by components of the reproductive system, in particular the testes, the vitelline follicles and the egg-packed uterus. Correspondingly, the output of eggs is immense, having been estimated at 25,000 per fluke per day, with one egg formed every 3.46 seconds (Happich and Boray, 1969). Each egg comprises a single oocyte, with approximately 30 accompanying vitelline cells, the latter having roles in shell formation and nutrition of the developing embryo. Spermatogenesis and vitellogenesis present by far the greatest demands for energy metabolism and for supply of micronutrient molecules, requirements that are met by digestion of host blood in the gut, with subsequent transfer of amino acids, monosaccharides, nucleotides, etc. across the gastrodermis. Not surprisingly, the high metabolic activity and rapid rate of cellular differentiation and turnover make the tissues of the reproductive system particularly sensitive to shortages in nutrient supply and exogenous stressors such as anthelmintics (Fairweather *et al.*, 2020). This is evidenced by the rapid development of histological and cytological abnormalities in the testes and vitelline follicles, and by failure of normal egg formation soon after administration of effective flukicidal therapy to the host (McConville *et al.*, 2010, 2012; Toner *et al.*, 2011a,b; Hanna, 2015). Histological and ultrastructural examination of the reproductive structures of drug-treated flukes can therefore provide valuable information about anthelmintic efficacy and mode of action, as well as signalling the development of parasite resistance to particular flukicidal drugs. With a possible change in emphasis in fluke control towards prevention of pasture contamination by eggs released from hosts harbouring infections of adult flukes (Hanna *et al.*, 2015; Fairweather *et al.*, 2020), the ovicidal potential of existing or putative new fasciolicidal drugs may become increasingly relevant.

The ultrastructure of most of the principal constituents of the reproductive system of *Fasciola hepatica* was elucidated prior to 1999 and was reviewed by Fairweather *et al.* (1999) in the first edition of this book. The details of structure and function of the testis, ovary and vitelline follicles, of egg formation in the ootype, and of early growth and development of juvenile migrating flukes are still relevant, and as current research places increased emphasis on molecular analysis (e.g. proteomics), it has become important to link the findings from these approaches to the established ultrastructure of the adult fluke. For this reason, much of the ultrastructural detail and functional morphology

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presented in the first edition is recapped here. There have, of course, been significant developments since 1999 that have contributed to our understanding of function related to structure, particularly in relation to growth and development of the reproductive system, vitelline cell activity and embryogenesis (Robinson *et al.*, 2001; Moxon *et al.*, 2010; McCusker *et al.*, 2016). Where possible, these findings have been integrated with the pre-existing structural information, but it is evident that significant gaps exist in our understanding of the development and function of the reproductive system of liver flukes, and of embryogenesis in the developing egg, at the molecular level. Appreciation that different clonal isolates of *F. hepatica* can display variations, not only in sensitivity to anthelmintics, but also in testis morphology (Fairweather, 2011), has led to recognition of the possibility of parthenogenetic strategies of reproduction under some circumstances (Fletcher *et al.*, 2004). This has particular implications for the development of epidemiological models of the emergence and spread of anthelmintic resistance in liver flukes and, therefore, is discussed in detail here.

4.2 Arrangement of Gonads, Accessory Organs and Ducts

The reproductive system of *F. hepatica* is hermaphrodite and the relative positions of the main reproductive structures are readily visualized in carmine-stained and cleared preparations of whole flukes (Hanna *et al.*, 2006; Hanna, 2015). In the male system, the two testes, which are much branched, lie one behind the other and occupy much of the space in the middle of the body (Fig. 4.1). A vas deferens leads forwards from each testis and the two vasa deferentia unite to form the seminal vesicle, which lies within the cirrus sac. The ejaculatory duct leads forward through the protrusible cirrus, opening to the exterior at the common genital pore, which is medially situated between the oral and ventral suckers. The ejaculatory duct is surrounded by cells of the prostate gland.

In the female system, the vitelline glands, which produce the precursor protein for eggshell formation, are composed of numerous follicles and are extensive, lying in a broad band along the posterior and lateral margins of the body. A single duct collecting vitelline cells from the vitelline tissue on each side of the body runs transversely to merge with the duct from the opposite side, forming the vitelline reservoir, which is located at the posterior margin of the Mehlis' gland complex (Fig. 4.1).

The single ovary is situated in front of the testes and on the left (as viewed from the ventral surface). Like the testes, the ovary is branched,

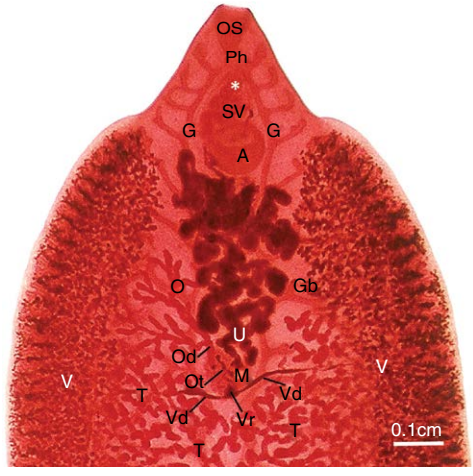

Fig. 4.1. *Fasciola hepatica*. Carmine-stained whole mount of a liver fluke. Behind the oral sucker (OS) the two main caeca of the gut (G) bifurcate from the pharynx (Ph), and lateral branches of the gut (Gb) lie dorsal to the reproductive structures throughout the body. The position of the common gonopore is marked (*), while the seminal vesicle (SV) is located anterior to the ventral sucker (or acetabulum, A). The two testes (T) are highly branched, and these branches occupy the medial portion of the body, with the very numerous vitelline follicles (V) distributed in two broad lateral zones. The two main vitelline ducts (Vd), one from each side, supply the vitelline reservoir (Vr) at the caudal pole of the Mehlis' gland complex (M). The single dendritic ovary (O), on the left side of the fluke, delivers oocytes via the oviduct (Od) to the ootype (Ot), where each oocyte associates with approximately 30 vitelline cells, and an eggshell is formed around the cell mass. Completed eggs pass into the proximal coils of the uterus (U) and are stored there temporarily while being shifted forward towards the gonopore by the new eggs entering from behind (Hanna, 2015).

but the branches are thicker. A short oviduct leads from the ovary towards the midline, close to where the ducts from the right and left vitelline glands converge to form the vitelline reservoir (Fig. 4.1). A short duct from the vitelline reservoir joins the oviduct to give rise to the common ovovitelline duct and this leads into the egg-producing chamber, or ootype. The ootype is surrounded by cells of the Mehlis' gland. It merges with the proximal coils of the convoluted uterus, which runs towards the common genital pore, from whence the eggs are released to the exterior. As in many digeneans, the distal end of the uterus is muscular and serves as an ovijector and a vagina; this structure is termed the metraterm.

The organization of the reproductive system in *Fasciola* spp. is closely similar to that seen in most other digenetic trematodes studied to date, with the exception of the Schistosomatidae, which are dioecious. Informative and comprehensive reviews of the structure and function of the reproductive organs throughout the Digenea have been provided by Peoples and Fried (2014) and Carbonell and Fried (2019).

4.3 Development of the Reproductive System

The development of the fluke reproductive system in the mouse host has been described in detail by Dawes (1962) and only the most significant features will be repeated here. The genital rudiment is present in the metacercaria and newly excysted juveniles (NEJs). It has an hourglass shape: the anterior portion gives rise to the cirrus, cirrus pouch and terminal part of the uterus; the posterior region forms the gonads, the Mehlis' gland complex and related ducts; and the intervening region forms the main parts of the uterus and vas deferens. By 22 h pi, the posterior end of the rudiment has become U-shaped, the two limbs developing into the testes. The rudiment grows larger in the next 2 days and shows signs of separation; separation is complete by day 8 pi and the testes are separated from the rudiment of the ovary and the Mehlis' gland complex. At this stage, the anterior end of the genital rudiment shows some indication of duality, the larger portion representing the rudiment of the cirrus pouch. By day 11 pi, the testes are lobed and the ovarian rudiment is growing out from the rudiment of the Mehlis' gland complex. The testes are much branched by day 13 pi and the ovarian rudiment is more elongate and shows three or four branches. The Mehlis' gland complex stands out sharply and in front of it the uterus displays slight folds. The rudiment of the cirrus, which is clearly developed at day 11 pi, becomes more prominent at day 18 pi and the branching of the testes is more extensive. The testes approach the follicular stage by day 21 pi; at this stage the ovary is longer and its outgrowths more branched, the cirrus forms an S-shaped bend and the uterus is longer and more folded. These trends continue through day 24 pi to day 28 pi when the lateral fields of the body are filled with vitelline follicles, although the vitelline cells are immature. Full maturity is reached by day 37 pi in the mouse (although it may be attained earlier) and several hundred eggs are present in the uterus. Secretory activity in the vitelline cells appears to be switched on a few days before maturity.

In a recent important study of the *in vitro* development of *F. hepatica* NEJs it was shown that media containing 50% chicken serum in RPMI promoted the survival of juvenile flukes for up to 29 weeks (McCusker *et al*., 2016). This long-term maintenance was dependent upon fluke growth, which was supported by increased proliferation of cells resembling 'neoblast' stem cells. Growth led to dramatic morphological changes in the juveniles, including the development of the digestive tract, reproductive organs and the tegument, towards more adult-like forms. The pluripotent 'neoblast' cells, which show the typical morphology of neoblasts described in other flatworms (round cell, large nucleus, prominent nucleolus, scant cytoplasm), appear to arise within the parenchyma below the tegument and muscle layers and subsequently migrate towards differentiated tissues within the body, where they contribute to growth of somatic structures such as tegument and gut, as well as to development of the reproductive structures.

The final maturation of the reproductive organs appears to occur rapidly, once the immature flukes move from the hepatic parenchyma of the definitive host into the bile ducts. In a study involving whole-mount stained preparations

and light and electron immunocytochemistry with an antibody to vitelline protein B (an eggshell precursor protein synthesized by *F. hepatica*), no vitelline cells could be identified in flukes recovered from the liver parenchyma of rats, but follicles were present in flukes at the earliest time of recovery from the bile duct, namely, 5 weeks and 3 days pi. The vitellaria in these flukes formed a row of small follicles on either side of the body. By 6 weeks and 3 days pi the follicles resembled those in the adult fluke and eggs were present in the uterus (Robinson *et al.*, 2001).

In a study of the effects of parasite intraspecific interactions on fluke size and reproductive development in two isolates of *F. hepatica* grown experimentally in rats, it was found that the body size and degree of testicular development achieved by individual flukes was positively influenced by the presence of two or three coinhabiting mature flukes in the main bile duct. It was concluded that stimulation of testis development may be due to physical contact with coinhabiting flukes or may be pheromonal in nature (Hanna *et al.*, 2011). The role of intraspecific influences on the later stages of development and maturation of flukes in the definitive host merits further investigation.

4.4 Male Reproductive System

4.4.1 Testes and spermatogenesis

The most complete account of spermatogenesis and the fine structure of the mature spermatozoon of *F. hepatica* has been provided by Stitt and Fairweather (1990). The following description is based on this account; other references will only be included where they illustrate specific points not in that account. During spermatogenesis, the primary spermatogonium undergoes three mitotic and two meiotic divisions to give rise to 32 spermatids, which become morphologically transformed into spermatozoa. Cell development begins at the periphery of the tubules and, as cells proceed through spermatogenesis, the later stages become increasingly located towards the centre of the lumen of the tubule. The primary spermatogonia are highly basophilic and have a high nucleus–cytoplasmic ratio. The cytoplasm is packed with free ribosomes and contains many small mitochondria, typically grouped at one pole of the cell. Sections through primary spermatogonia that are nearing the end of interphase often contain a centriole pair, located close to the nucleus.

The secondary spermatogonia differ little from the primary spermatogonia. They divide to produce four tertiary spermatogonia, which lie grouped together but are not joined (Fig. 4.2). However, their inner faces begin to push out conical protrusions towards the centre of the four-cell cluster. Mitochondria move into and accumulate within the protrusions. The protrusions display a strong reaction for actin, suggesting that microfilaments are involved in their formation, a process that bears some analogy to the development of growth cones in other cell types, particularly nerve cells (Stitt *et al.*, 1991).

Mitosis of the tertiary spermatogonia results in a rosette of eight primary spermatocytes, joined together by means of cytoplasmic bridges connecting a central cytoplasm or cytophore (Fig. 4.3). The latter is presumably formed as a result of fusion of the conical processes observed in the tertiary spermatogonia and subsequent incomplete cytokinesis of the cells. Actin filaments are concentrated in the cytophore region of this and later rosette stages, as might be expected from the established role of actin in the cytokinesis phase of cell division (Stitt *et al.*, 1991). The nuclei of the primary spermatocytes contain one or more synaptonemal complex, reflecting the pairing of homologous chromosomes at zygotene during meiotic prophase (Fig. 4.4). Golgi complexes and long cisternae of granular endoplasmic reticulum (GER) are present in the cytoplasm of these cells. The first meiotic division of the primary spermatocytes produces a rosette of 16 secondary spermatocytes, which appears to be a short-lived stage. Upon completion of meiosis, there are 32 fusiform spermatid cells, still connected by a fairly large cytophore.

It is at this stage that spermiogenesis begins and each cell undergoes a number of morphological changes during its development into a mature spermatozoon. Differentiation of the spermatid commences with elongation of the cell and its nucleus. A number of nuclear pores appear along the length of the nuclear envelope as it elongates. The chromatin becomes increasingly condensed and granular, the granules

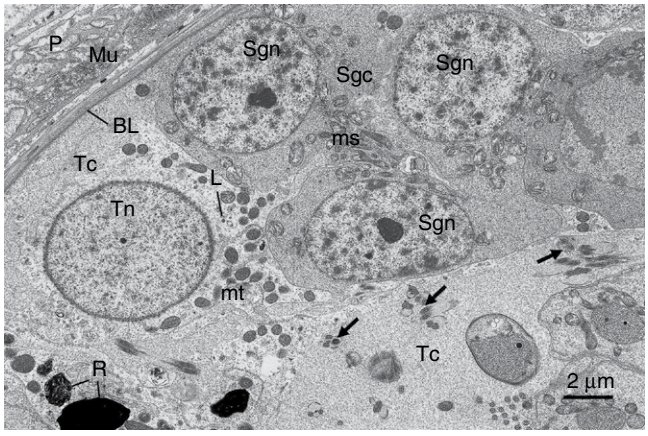

Fig. 4.2. Transmission electron micrograph of the testis of a liver fluke showing a cluster of spermatogonia at the periphery of a testis tubule. The nuclei of the spermatogonia (Sgn) are typically rather heterochromatic, while the cytoplasm (Sgc) is scant and densely packed with ribosomes. Mitochondria (ms) are often grouped towards one pole of the cell. Also present at the periphery of the tubule are nuclei of the sustentacular tissue (Tn). These are euchromatic and characterized by a dense nuclear envelope. The ramifying cytoplasm of the sustentacular tissue (Tc), likely syncytial in organization, extends to envelop the germinal line cells and encloses profiles of maturing spermatozoa (arrows). Mitochondria (mt), lysosomes (L) and residual bodies (R) are also visible. The tubule wall is bounded by a basal lamina (BL), underlain by extensions of the parenchyma (P) and muscle cells (Mu).

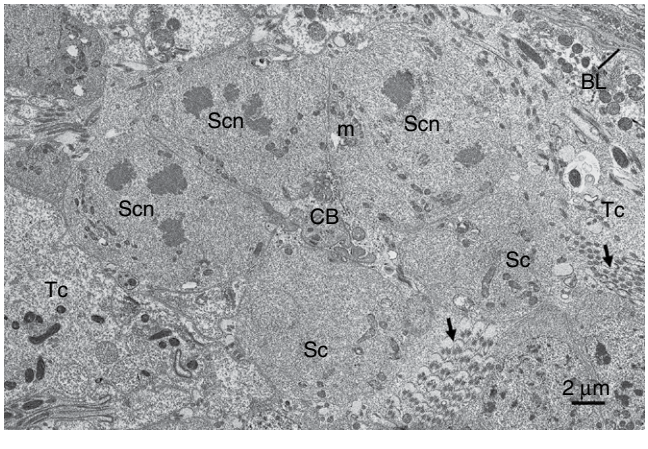

Fig. 4.3. Transmission electron micrograph of liver fluke testis showing part of an 8-cell rosette of primary spermatocytes. Following mitosis, the chromatin in the nuclei (Scn) is still partially condensed, while the cytoplasm (Sc) of the cells remains connected by cytoplasmic bridges (CB) due to incomplete cytokinesis. Although less dense than in spermatogonia, the cytoplasm is packed with ribosomes and contains abundant mitochondria (m). The rosette is enveloped by cytoplasmic extensions of the sustentacular tissue (Tc), which also encloses developing spermatozoa (arrows). The basal lamina (BL) marks the periphery of the testis tubule.

eventually forming lamellar-like structures, lying along the longitudinal axis of the nucleus. The lamellae become tightly folded into a reticulum of scroll-like structures, which appear as a honeycomb structure when cut transversely (Fig. 4.5). Accompanying the nuclear changes is the formation of the zone-of-differentiation at the distal end of the cell with respect to the

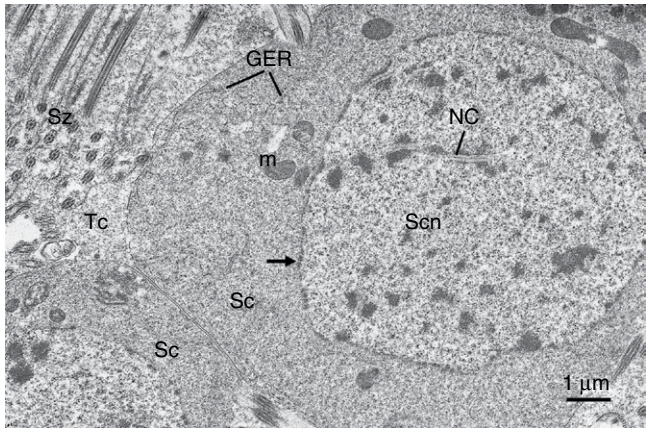

Fig. 4.4. Transmission electron micrograph of liver fluke testis showing a primary spermatocyte, the nucleus of which (Scn) features a number of synaptonemal complexes (NC). These represent the pairing of homologous chromosomes at zygotene during meiotic prophase. Numerous nuclear pores are evident in the nuclear envelope (arrowed), and the abundant cytoplasm (Sc) contains many ribosomes together with strands of granular endoplasmic reticulum (GER) and mitochondria (m). The spermatocytes are surrounded by sustentacular tissue (Tc), embedded within which are maturing spermatozoa (Sz).

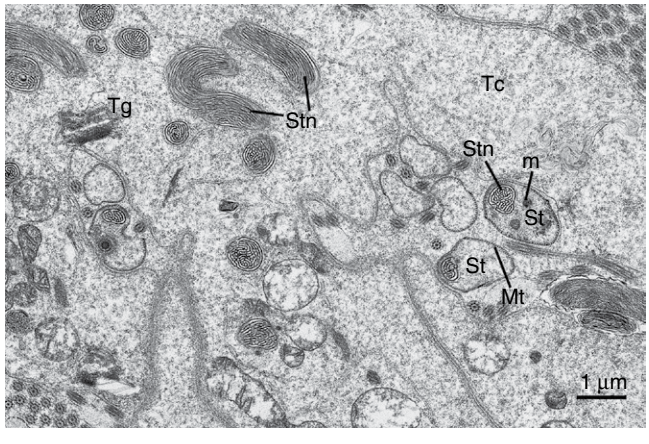

Fig. 4.5. Transmission electron micrograph of liver fluke testis showing early stages in the differentiation of spermatids, with condensation of the chromatin in the spermatid nuclei (Stn) to form lamellar structures which become tightly folded into a scroll-like reticulum. Within each developing spermatid (St) a pair of elongated mitochondria (m) flank the nucleus, and a zone of cortical microtubules (Mt) underlies the limiting plasma membrane, but the exact appearance of the profile depends on the level at which the spermatid was sectioned. Developing spermatids are surrounded by sustentacular tissue (Tc), within which occur ribosomes, strands of GER and Golgi fields (Tg).

cytophore. Initially, this takes the form of a conical projection from the cell, the plasma membrane of which is underpinned by a single row of microtubules. It contains a pair of centrioles, which divide to form four such structures, two of which come together to form the 'central body' (Fig. 4.6). The two remaining centrioles lie on either side of the central body and become basal bodies for the development of the axonemes. The central body together with the basal bodies probably represent a microtubule-organizing centre, the basal bodies being responsible for the formation

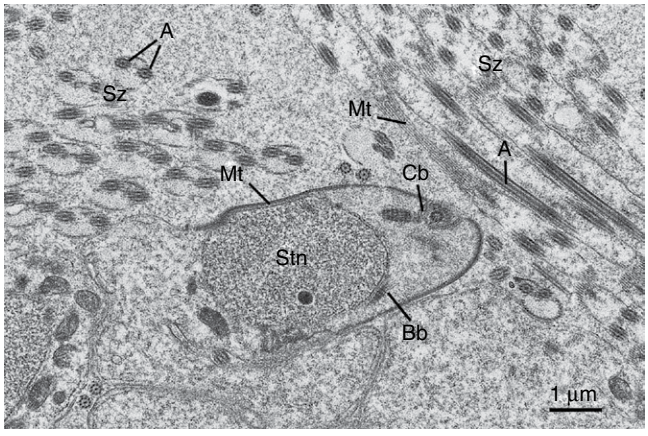

Fig. 4.6. Transmission electron micrograph of liver fluke testis showing the zone of differentiation at the distal end of a developing spermatid. This takes the form of a conical projection from the cell into which the spermatid nucleus (Stn) will eventually migrate. The plasma membrane is underpinned by a single row of microtubules (Mt), organized by a pair of centrioles that form the 'central body' (Cb). Two remaining centrioles that come to lie on either side of the central body become basal bodies (Bb) for the development of the axonemes. Profiles of maturing spermatozoa (Sz) in the vicinity feature paired axonemes (A) and cortical microtubules, sectioned in various orientations.

of the axonemes, while the central body may be responsible for the cortical microtubules that provide support for the zone of differentiation as it elongates to form the median process. Initially, the axonemes develop at right angles to the central body but later rotate through 90 degrees to lie parallel to the median process. The next stage is characterized by the migration of the nucleus into the median process: it migrates to the distal end of the process and, as it does so, the two axonemes and median process fuse together to form a single structure. Along with the nucleus, two mitochondria (formed by fusion of a larger number) migrate into the median process. The fully developed spermatozoon is pinched off from the residual cytoplasm at a point marked by a groove-like collar at the proximal end of the median process and is released into the lumen of the testis tubule.

Living spermatozoa are very long, thread-like structures, approximately 400 µm in length. They exhibit active undulatory movements, especially along the anterior and middle regions, whereas the posterior (nuclear) region is relatively immobile.

The structure of the mature spermatozoon has been reconstructed from transverse sections along its length (Fig. 4.7). It possesses two axonemes that are slightly staggered, so that only one axoneme is present at each end of the spermatozoon. The axonemes have a '9+1' pattern typical of flatworm sperm, the central element being a rod-like element rather than a microtubule. The anterior region of the spermatozoon (which is proximal in relation to its formation from the zone of differentiation) contains the first of the two mitochondria. The two mitochondria are separated by a short space, the second mitochondrion occupying the middle region (Fig. 4.8) and overlapping slightly with the nucleus, which resides in the posterior (i.e. distal) region of the spermatozoon. Cortical microtubules lie beneath the outer plasma membrane, principally in the anterior and middle regions, being scarce or absent in the posterior region. In the region posterior to the nucleus, dynein side-arms are missing from the outer doublet microtubules of the axonemes and this, together with the lack of cortical microtubules, may explain why this region of the sperm is immobile. Very little, if any, glycogen has been observed in the spermatozoon of *F. hepatica*, although in separate radiolabelling experiments incorporation of [^{3}H]glucose into glycogen granules in the spermatozoon has been demonstrated (Hanna, 1976b).

Further details on the process of spermiogenesis and the ultrastructure of the spermatozoon

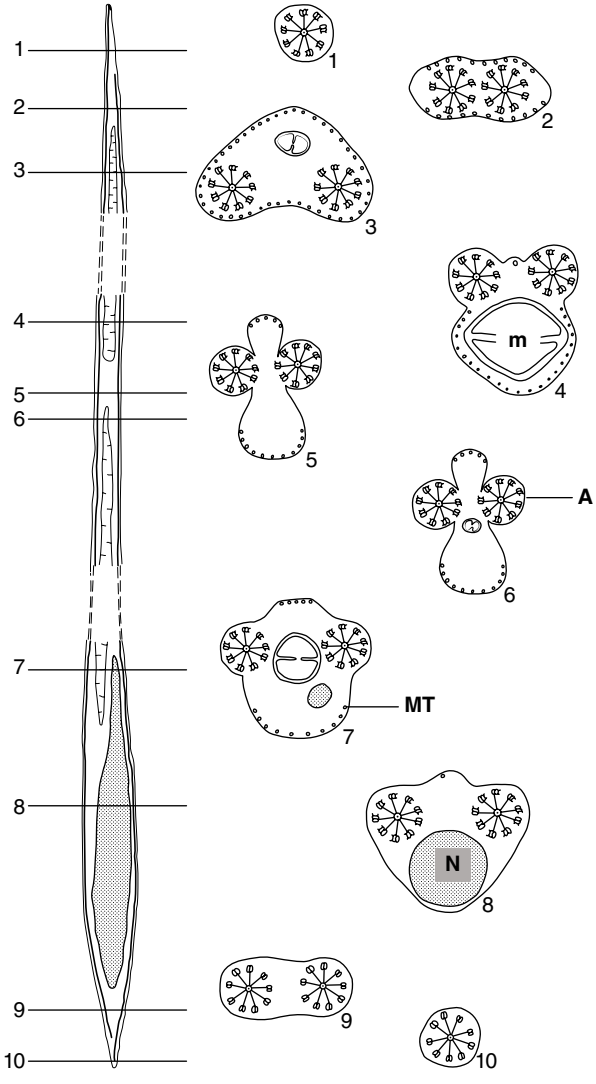

Fig. 4.7. Diagrammatic interpretation of the structure of the mature spermatozoon of *Fasciola hepatica,* derived from successive transverse sections (1–10) along its length. A, axoneme; m, mitochondrion; MT, cortical microtubule; N, nucleus (Stitt and Fairweather, 1990).

in *F. hepatica* and *F. gigantica* have been contributed by Ndiaye *et al*. (2003, 2004), using scanning and transmission electron microscopy. In both species, dorsolateral cytoplasmic expansions, external ornamentation of the plasma membrane and spine-like bodies have been described in the spermatozoon.

For processes so heavily dependent upon microtubule action, it is scarcely surprising that spermatogenesis and spermiogenesis are disrupted by microtubule inhibitors such as tubulozole-C. Mitosis is inhibited and this leads to a sharp decline in the number of cells in the testis tubule. The spermatocyte and spermatid rosettes become disrupted and the cytophore regions become fragmented. Abnormalities of the microtubular organization of the spermatozoa were also observed (Stitt and Fairweather, 1992).

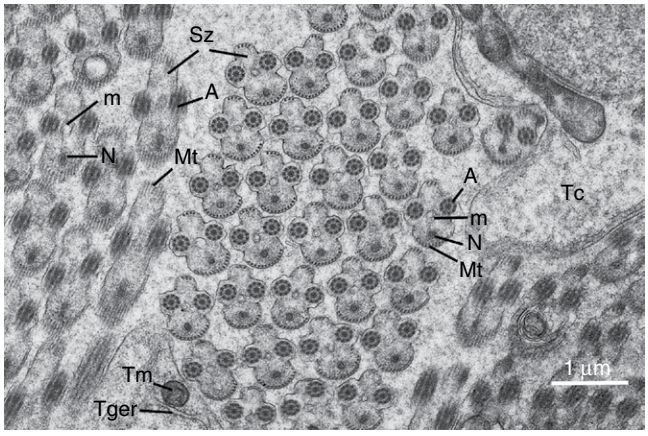

Fig. 4.8. Transmission electron micrograph of liver fluke testis showing several clusters of spermatozoa (Sz) maturing within the sustentacular cytoplasm (Tc), which also contains mitochondria (m) and strands of granular endoplasmic reticulum (Tger). Each profile of a spermatozoon features paired axonemes (A), a mitochondrion (m), nucleus (N) and cortical microtubules (Mt), but the exact appearance depends on the position of the plane of section (compare with Fig. 4.7).

Cytokinesis is the final, separation phase of cell division and is dependent on microfilament action. Treatment with the microfilament inhibitor, cytochalasin B, leads to severe disruption of spermatogenesis. Bi- and multinucleate cells increase in frequency with time and the typical rosettes of spermatocyte and spermatid cells are replaced by syncytial masses of cells, since nuclear, but not cytoplasmic, divisions continue. Spermatozoon formation becomes increasingly abnormal, the spermatozoa containing variable numbers of axonemes and an altered distribution of peripheral microtubules (Stitt *et al*., 1991).

Ndiaye *et al*. (2003) analysed the distribution pattern of tubulin in the microtubular cytoskeleton of *F. hepatica* by means of monoclonal anti-tubulins (anti-α-tubulin, anti-β-tubulin, anti-α-acetylated tubulin and anti-α-tyrosinated tubulin). These anti-tubulins labelled axonemal and cortical microtubules but not the central core of the '9+1' axoneme. Scanning and transmission electron microscopy revealed a helical pattern of axonemes around the sperm body.

In addition to cells of the germinal line that take part directly in spermatozoon production, there exist, at the periphery of each testis tubule, a number of large euchromatic nuclei, each bearing a prominent nucleolus. Surrounding these nuclei is a layer of cytoplasm, extensions of which partially envelop and probably support the germinal line cells and embedded in which there are maturing spermatozoa (Fig. 4.2). Since no lateral cell boundaries have been identified, it is likely that this sustentacular tissue has a syncytial organization (Hanna *et al*., 2012). Its cytoplasmic features are consistent with a role in protein/glycoprotein synthesis (through a GER–Golgi-mediated mechanism) and intracellular digestion/heterophagy (through a lysosomal system). The sustentacular tissue probably serves to scavenge effete cells and cytoplasmic debris, to recycle useful molecules, to promote spermatozoon maturation and to aid osmoregulation within the tubules. Certain protein-containing macromolecules synthesized by the sustentacular tissue may contribute to the seminiferous fluid, or have pheromonal activity (Hanna *et al*., 2012). In the sustentacular tissue of certain flukes with dysfunctional spermatogenesis, there was increased heterophagic and cytolytic scavenging activity (Hanna *et al*., 2012), while in flukes exposed to anthelmintics such as triclabendazole, which inhibit microtubule formation and thus disrupt spermatogenesis, there is increased heterophagic scavenging activity in the sustentacular tissue (McConville *et al*., 2010; Toner *et al*., 2011a; Hanna *et al*., 2013).

Karyotyping studies on a range of *F. hepatica* samples by Fletcher *et al*. (2004) identified a

triploid isolate which was effectively aspermic, rendering it necessarily asexually reproducing. Histological studies on the testis of this laboratory-maintained Cullompton isolate by Hanna *et al*. (2008) verified that there was arrested spermatogenesis, with no stages later than primary spermatocytes present in the testis tubules. No mature spermatozoa were present and the presence of numerous eosinophilic apoptotic bodies and nuclear fragments suggested that meiotic division was anomalous and incomplete. However, a high proportion of the eggs collected from these flukes embryonated normally, despite being unfertilized, and the miracidia produced were capable of promoting the laboratory-based life cycle.

Considering also that there is an extensive presence of asexually reproducing diploid and triploid *Fasciola* in Asia (Terasaki *et al*., 1982, 2000) and that hybrids between *F. hepatica* and *F. gigantica*, in certain areas of the Far East where the two species co-exist, exhibit abnormal gametogenesis, diploidy, triploidy, mixoploidy and parthenogenesis (reviewed by Mas-Coma and Bargues, 1997), it is likely that facultative gynogenesis is widespread in this parasite. This has important implications for the population genetics and evolution of *Fasciola*, especially in relation to the development and spread of drug resistance, and should be considered in the mathematical modelling of this process (Fletcher *et al*., 2004; Hanna *et al*., 2008). A possible mechanism of facultative parthenogenesis in liver flukes is considered below.

4.4.2 Accessory ducts and glands

The seminal vesicle consists of two spherical bodies, filled with mature spermatozoa, and the two lobes lie within the cirrus sac. The epithelium comprises a single layer of squamous to cuboidal cells and the apical plasma membrane either bears thin lamellae or is invaginated to form deep, pit-like structures. Both the pits and bulbous endings of the lamellae contain spermatozoa. The cells contain mitochondria, a moderate amount of GER and Golgi complexes which produce lucid secretory vesicles. Projections from the parenchymal cells penetrate into the base of the epithelial cells with which they form typical junctional complexes. While the majority of the spermatozoa in the seminal vesicle lie in the lumen, many lie close to the apical surface or are enclosed within the lamellae or pits. This may be indicative of a nutritive and/or maturation function, the lucid secretory bodies perhaps supplying the appropriate substances (Threadgold, 1975a).

The ejaculatory duct is a small duct that takes a sinuous course through the cirrus sac. The epithelium is composed of cuboidal to columnar cells, although it may be a syncytium. The situation is complicated by the presence of the openings of the prostate gland cells; the terminal parts of the ducts of the latter penetrate between/through the cells/syncytium to open into the duct lumen and are connected to the epithelium by septate desmosomes. The apical plasma membrane of the epithelium is extended to form long, thin lamellae which may branch and unite to form a complex system of projections. The amplification of the apical surface is indicative of a role for the duct in absorption or reabsorption of the breakdown products of the prostate gland secretions or other fluids. The cytoplasm contains mitochondria, a small amount of GER and Golgi complexes which synthesize a dense ovoid secretion. Extensions from parenchymal cells form junctional complexes with the basal plasma membrane (Threadgold, 1975a).

Both the cirrus sac and cirrus are covered by a thin modified tegument; that of the cirrus is especially so, which may be related to its need to evaginate, as the thick tegument covering the general body surface would hinder such a process. There are some differences between the tegument of the body surface and of the cirrus and cirrus sac, but they are small. For example, there are fewer basal invaginations in the cirrus and cirrus sac, and fewer mitochondria; the cirrus sac has very few spines whereas the cirrus has many; and type 2 secretory bodies predominate over T1 bodies in the cirrus sac, while there is a normal ratio of T1 to T2 bodies in the cirrus. Processes from the parenchymal cells form junctional complexes with the tegumental cells and their connections to the surface syncytium (Threadgold, 1975a) (see Chapter 3 for description of tegumental structure).

The prostate gland consists of numerous unicellular glands (Fig. 4.9) grouped around the

ejaculatory duct. The individual cells show a high level of secretory activity, containing an extensive system of GER cisternae and numerous Golgi complexes – up to 18 complexes have been observed in one section of a cell (Fig. 4.10). The Golgi complexes form large, electron-lucid secretory vesicles whose content is carbohydrate rich. Moderate numbers of mitochondria occur within the cell. The plasma membrane is invaginated to form deep invaginations, which extend almost to the nucleus at times. Running parallel to the invaginations are cisternae of GER, which lack ribosomes on the side facing the plasma membrane. They are the equivalent of subsurface

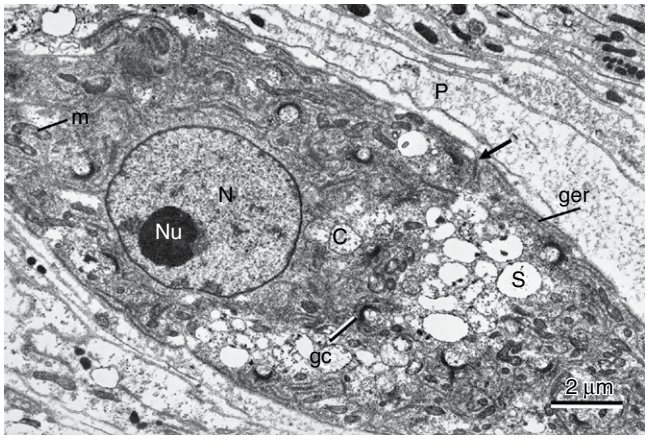

Fig. 4.9. Transmission electron micrograph of a prostate gland cell of *Fasciola hepatica* showing the general cell shape and interrelationship of all organelles. N, nucleus; Nu, nucleolus; m, mitochondrion, C, condensing vacuole; S, secretory vesicles; gc, Golgi complex; ger, granular endoplasmic reticulum; P, parenchyma. The large arrow indicates the origin of a plasma membrane invagination (Threadgold, 1975b).

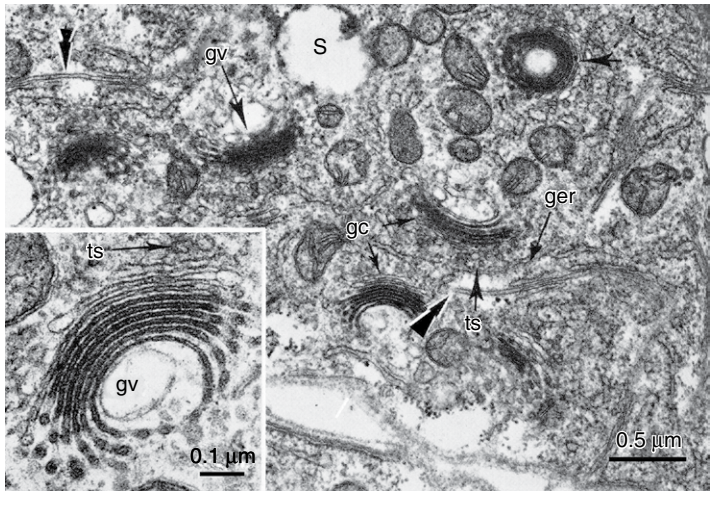

Fig. 4.10. Transmission electron micrograph of the peripheral area of a prostate gland cell of *Fasciola hepatica* showing numerous curved Golgi complexes (gc) with associated vesicles (gv) and secretory vesicles (S). The single arrow indicates a Golgi complex sectioned parallel to its forming–maturing face axis and the double arrows indicate plasma membrane–GER complexes. ts, transitional vesicles between the GER and the forming face of the Golgi complex; ger, granular endoplasmic cisternum (Threadgold, 1975b).

cisternae observed in other cell types (e.g. Rosenbluth, 1962). This arrangement of cisternae is believed to facilitate the passage of precursor molecules into the cell. The necks or ducts of the gland cells are long and run in a random fashion through the parenchyma towards the ejaculatory duct. Close to the latter, the gland ducts are supported by a single ring of peripheral microtubules which probably serve to resist the compressive forces of the muscle layers, interstitial material and ejaculatory duct epithelium around them. The ducts from the gland cells pass through the epithelium of the ejaculatory duct to release their secretion into its lumen and are connected to the surrounding epithelium by septate desmosomes. The terminal region of the gland duct within the epithelium is swollen and appears to serve as a storage site for the prostate secretions (Fig. 4.11). While the precise role of the secretion is not known, a number of suggestions have been put forward, including stimulation of spermatozoa previously stored in a quiescent state in the seminal vesicle and as an extra energy source for the spermatozoa in addition to the glycogen stored within the spermatozoon (Threadgold, 1975b).

4.5 Female Reproductive System

4.5.1 Ovary and oogenesis

The ultrastructure of the ovary in flukes collected from field-infected sheep and from rats infected with *F. hepatica* of the Cullompton and Oberon isolates (for isolate provenance, see Fairweather, 2011), was examined by Hanna *et al.* (2016). The following description is based on that account. The electron microscope findings are broadly consistent with previous accounts by Björkman and Thorsell (1964) and Gresson (1964) but offer the improved resolution possible with a modern transmission electron microscope and improved fixation methods. They were augmented by light microscope immunocytochemical labelling of synaptonemal proteins.

Each tubule of the dendritic ovary is bounded by a basal lamina and surrounded by muscle cells. Internally, at the periphery, tissues of both the somatic and germinal lineage occur, the former represented by an interstitial tissue (termed 'peripheral cells' by Björkman and Thorsell, 1964), which is likely to be syncytial, while oogonia, early and late primary oocytes comprise the germinal cell population. The spheroidal nuclei of

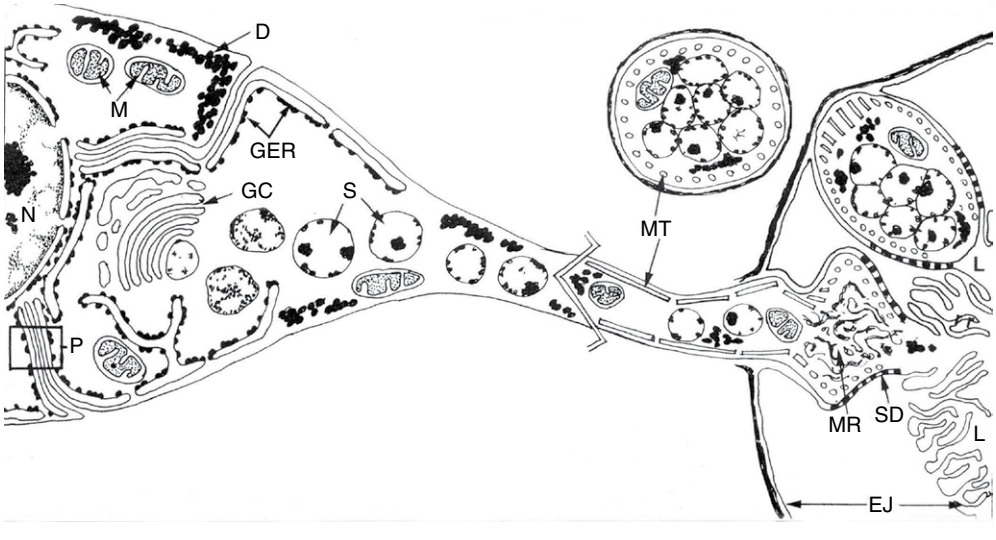

Fig. 4.11. Diagram of part of a typical prostate cell of *Fasciola hepatica* and its relationship to the ejaculatory duct. N, nucleus; M, mitochondrion; GC, Golgi complex; GER, granular endoplasmic reticulum; D, dense material; S, secretory vesicles; P, plasma membrane–GER complex; MT, microtubules; MR, membrane remnants; SD, septate desmosomes; EJ, ejaculatory duct epithelium; L, lumen of duct (Threadgold, 1975b).

the interstitial tissue have a characteristically dense nuclear envelope, and the cytoplasm ramifies prolifically, with multiple extensions enveloping the adjacent germinal line cells and lining the basal lamina, with which the limiting membrane of the interstitial tissue is in intimate contact (Fig. 4.12). The presence of very numerous mitochondria, together with Golgi fields and extensively developed smooth endoplasmic reticulum containing fibrillary material, are consistent with roles in physical and metabolic support for the developing germ line cells, osmoregulation, secretory activity and heterophagic 'scavenging' activity.

The oogonia, which are discontinuously distributed at the periphery of the ovarian tubules, bear spheroidal or oval heterochromatic nuclei 6–8 μm in diameter, each usually with a single nucleolus. The cytoplasm, characterized by very numerous ribosomes and polyribosomes, but with few strands of GER consistent with active transcription and translation of cytoplasmic proteins, forms a thin layer round the nucleus and has numerous pseudopod-like extensions into the surrounding cytoplasm of the interstitial tissue (Fig. 4.13). Sometimes the oogonia are seen in mitotic division, and the daughter cells of such division events may both have the features of oogonia, or one may be an oogonium while the other presents characteristics of an early oocyte (Fig. 4.14). Clearly, mitotic activity in the oogonia provides the source of oocytes for further differentiation, while maintaining the population of germinal-line cells.

Early primary oocytes, which occur mainly in the peripheral zone of the ovarian tubules, are distinguished from the oogonia by having rather larger nuclei (7–9 μm in diameter) that are relatively euchromatic and contain occasional synaptonemal complexes, adjacent to which the chromatin often appears dense. Each nucleus also contains a large dense nucleolus, and nuclear pores are numerous and conspicuous (Fig. 4.15), a feature also noted by Björkman and Thorsell (1964) and Gresson (1964). Synaptonemal complexes represent sites of pairing (synapsis) between homologous chromosomes and appear at the zygotene stage of meiotic prophase, completing formation at the ensuing pachytene stage and disappearing, due to depolymerization of the proteinaceous component, at diplotene stage. Both oogonia and oocytes are capable of synthesis of synaptonemal proteins, as evidenced by the presence of numerous ribosomes and polyribosomes in the cytoplasm and by the distribution of immunocytochemical labelling for

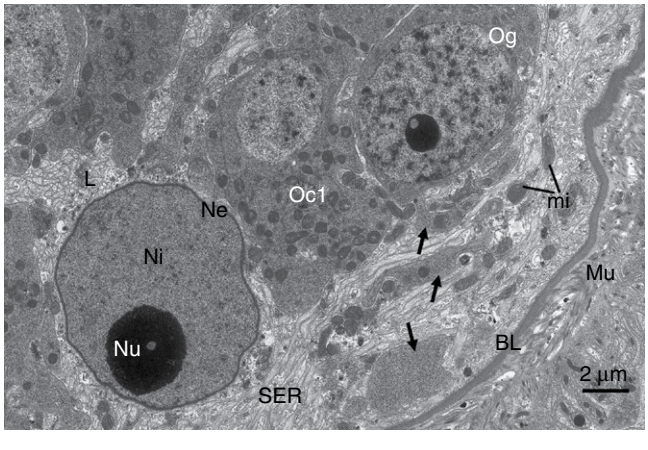

Fig. 4.12. Transmission electron micrograph of the peripheral zone of an ovarian tubule of *Fasciola hepatica*. A euchromatic nucleus of the interstitial tissue (Ni) displays a dense nuclear envelope (Ne) and a large nucleolus (Nu). The cytoplasm ramifies extensively to surround oogonia (Og) and early primary oocytes (Oc1), and directly abuts the basal lamina (BL). Pseudopod-like extensions of cytoplasm from the oogonia and oocytes are embedded in the interstitial cytoplasm (black arrows), which also contains dense mitochondria (mi), an extensively developed network of smooth endoplasmic reticulum cisternae (SER) and lysosomes (L). A layer of muscle cells (Mu) surrounds the tubule (Hanna *et al*., 2016).

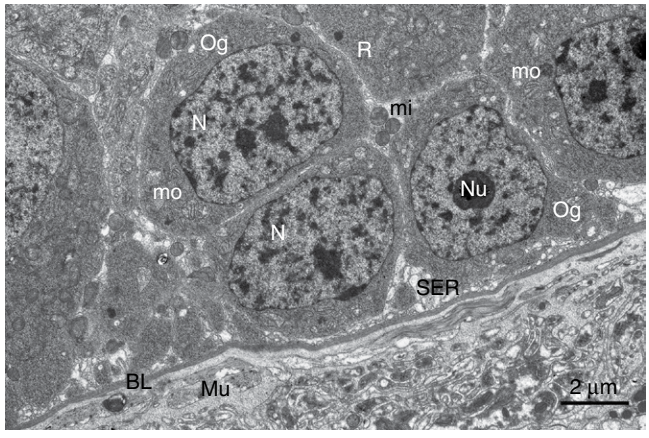

Fig. 4.13. Transmission electron micrograph of the ovary of *Fasciola hepatica.* Several oogonia (Og) are lying close to the basal lamina (BL) of the tubule wall. The nucleus of each oogonium (N) is heterochromatic and bears a conspicuous nucleolus (Nu). The cytoplasm contains numerous ribosomes (R) and mitochondria (mo), the latter often clustered near one pole of the cell. Each oogonium is surrounded by a thin layer of interstitial cytoplasm, which contains clusters of mitochondria (mi) and cisternae of smooth endoplasmic reticulum (SER). Muscle cells (Mu) surround the tubule (Hanna *et al*., 2016).

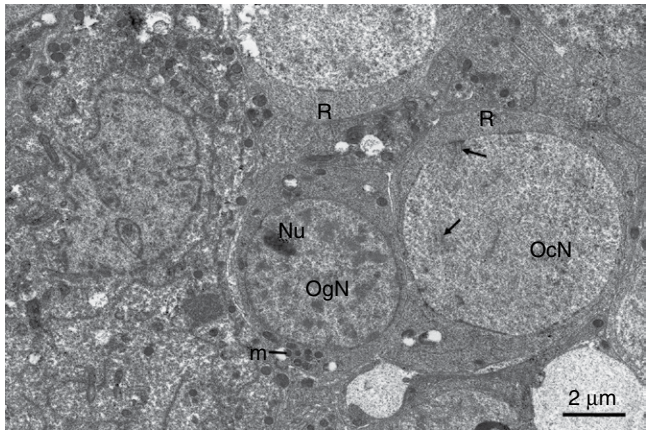

Fig. 4.14. Transmission electron micrograph of an oogonium and an early primary oocyte, daughter cells following mitotic division of an oogonium at the periphery of an ovarian tubule of *Fasciola hepatica*. The nucleus of the oogonium (OgN) is heterochromatic, bearing a nucleolus (Nu), while that of the oocyte (OcN) is relatively euchromatic and features several synaptonemal complexes (arrows). The cytoplasm of the cells contains many ribosomes (R) and dense mitochondria (m) (Hanna *et al*., 2016).

synaptonemal protein monomers SCP1 and SCP3 (Fig. 4.16) (Hanna *et al*., 2016). Once translated in the cytoplasm, the protein monomers move to the nucleoplasm of the oocytes via the pores in the nuclear envelope for assembly of polymerized synaptonemal complexes. The cytoplasm of early oocytes is more abundant than that of oogonia, and contains many mitochondria, often clustered towards one pole of the cell. The pseudopod-like cytoplasmic extensions into the interstitial cytoplasm are fewer and smaller than in the oogonia.

The nuclear and cytoplasmic volume of the oocytes increases progressively as they are displaced from the periphery towards the central zone of the ovarian tubule, where the late primary

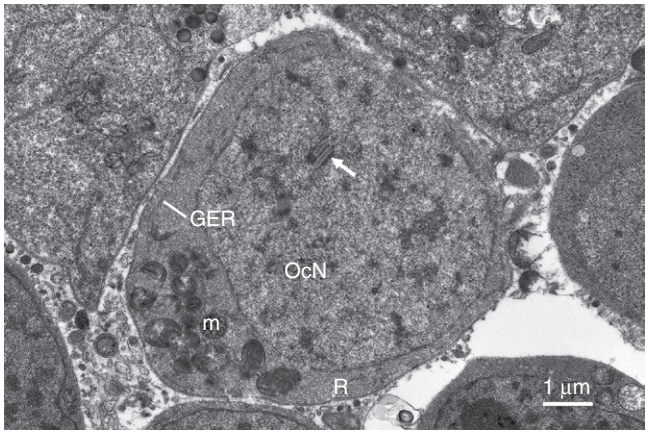

Fig. 4.15. Transmission electron micrograph of an early primary oocyte in the ovary of *Fasciola hepatica* showing synaptonemal complexes (arrowed) within the nucleoplasm (OcN). The cytoplasm contains ribosomes and polyribosomes (R), occasional strands of granular endoplasmic reticulum (GER) and mitochondria (m), the latter clustered at one pole of the cell (Hanna *et al*., 2016).

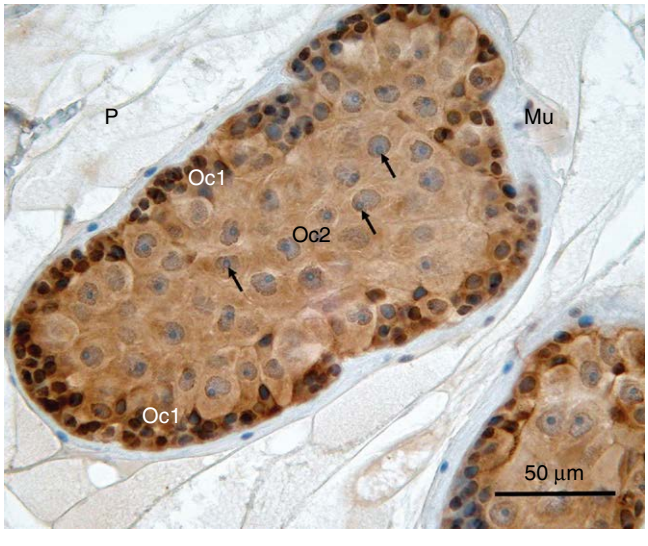

Fig. 4.16. Immunocytochemical labelling of synaptonemal protein on sections of the ovary of *Fasciola hepatica*. Brown-coloured positive labelling is strongest over nuclei and cytoplasm of cells in the peripheral zone of the ovarian tubule, where the early prophase oocytes are concentrated (Oc1). In the central zone of the tubule, labelling is less intense than that seen at the periphery. It is present over the cytoplasm and nuclei of the late prophase oocytes (Oc2), and in these the nucleoli are visible (arrowed). The parenchyma (P) and the muscle layer (Mu) are unlabelled (Hanna *et al*., 2016).

oocytes are often packed tightly together, each assuming a rather polygonal outline with a maximum diameter of 30 µm (Fig. 4.16). The nucleoplasm remains euchromatic, often less electron dense than the cytoplasm, and each nucleus contains one or two conspicuous nucleoli. Synaptonemal complexes are much less evident than in the early oocytes and few microvilli project from the smooth surface into the interstitial cytoplasm, if any attenuated strands of the latter remain.

A feature of the peripheral cytoplasm in late primary oocytes, not seen in the earlier stages, is the presence of numerous spherical membrane-bound secretory bodies concentrated beneath the plasma membrane (Fig. 4.17). These are strongly osmiophilic and PAS-positive, indicating a polysaccharide nature (Govaert, 1960; Björkman and Thorsell, 1964). They are likely to be equivalent to the cortical granules seen in the oocytes of other invertebrates and in mammalian oocytes, where they are most often associated with the prevention of polyspermy after fertilization (Longo, 1987).

Having reached the final stages of meiotic prophase 1, further development in the oocytes is arrested (Gresson, 1964) as they are propelled from the ovarian tubule towards the oviduct by the pressure of cells developing in the peripheral zone, possibly aided by muscular peristalsis in the tubule wall. Metaphase of meiosis 1 occurs only after each individual oocyte has been incorporated in an egg in the ootype and has entered the proximal coils of the uterus (Govaert, 1960; see later).

4.5.2 Vitelline cells

A large proportion of the body is taken over for vitelline cell formation, the vitelline follicles occupying the lateral margins of the fluke. Fine ducts from the individual follicles lead into the two main longitudinal vitelline ducts on each side of the body. The latter feed into the two main lateral vitelline ducts that unite medially to form the vitelline reservoir; from the reservoir, a short duct enters the ootype, uniting with the oviduct to form the common ovovitelline duct before it does so. The vitelline follicles contain a cluster of cells at different stages of development. The cells synthesize the shell protein material involved in egg formation and also provide the developing embryo with nutrients in the form of glycogen and 'yolk' material.

The fine structure of the vitelline cells of *F. hepatica* has been described by Irwin and Threadgold (1970) and the developmental sequence divided into two major phases: growth and cell synthesis (Threadgold, 1982). Shell protein is produced at first, followed later by glycogen synthesis and storage. Although development is a continuous process, four distinct cell types have been identified as representative of the different stages in the developmental sequence: they have been designated the stem (S) cell, the intermediate types 1 and 2 (It1 and It2) and the mature (M) cell (Threadgold, 1982) (Fig. 4.18). The stem cells occupy a peripheral position in the follicle and give rise to the other stages by mitosis,

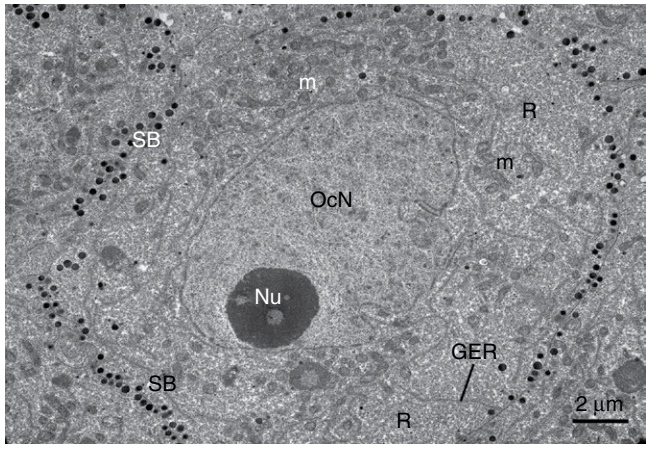

Fig. 4.17. Transmission electron micrograph of a late primary oocyte of *Fasciola hepatica* featuring an ovoidal but deeply indented euchromatic nucleus (OcN) with a conspicuous nucleolus (Nu). Synaptonemal complexes are not evident. The cytoplasm contains mitochondria (m), ribosomes and polyribosomes (R) and strands of granular endoplasmic reticulum (GER). Dense secretory bodies (SB) are concentrated below the apical plasma membrane. The oocyte is closely surrounded by other late primary oocytes, with no intervening interstitial cytoplasm.

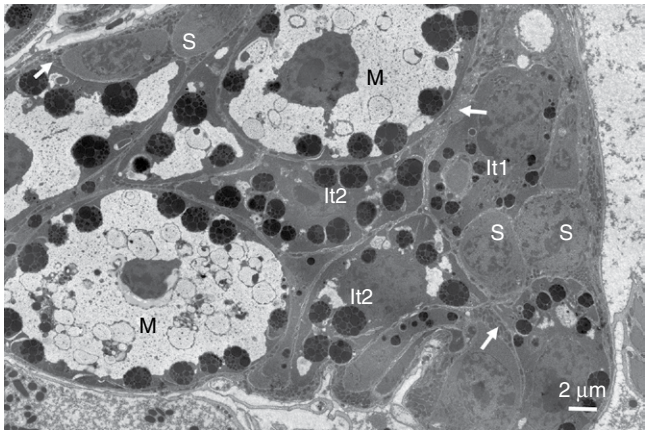

Fig. 4.18. Transmission electron micrograph of a vitelline follicle of *Fasciola hepatica* containing a heterogeneous population of vitelline cells. Present are stem cells (S), intermediate type 1 (It1) and intermediate type 2 (It2) cells and mature (M) vitelline cells. Cytoplasmic extensions of the nurse cells (arrowed) containing numerous dense mitochondria envelop the various vitelline cells.

one daughter cell remaining as a stem cell and the other undergoing development. Each stem cell contains numerous free ribosomes and mitochondria, but no GER or globules of shell protein material. The It1 cell contains cisternae of GER, numerous single shell protein globules and a few small shell globule clusters at the periphery of the cell. The It2 cell contains an extensive system of GER cisternae, many large shell globule clusters and a few single protein globules. The shell protein material in the intermediate cells is packaged by Golgi complexes, but the rapid turnover of secretory material by these cells results in the Golgi complex being reduced to a rather diffuse system of cisternae. The mature cells occupy a central position within the follicle. In these cells the GER cisternae are confined to the perinuclear region or the extreme cell periphery. The numerous shell globule clusters lie at the cell periphery, while the intermediate zone of the cell is filled with glycogen granules, together with a number of heterophagosomes (the 'yolk' globules) (Threadgold, 1982).

At a late stage in maturation of the vitelline cells, another particular type of carbohydrate-containing secretory body, termed the glycan vesicle, is produced. Cytochemical analysis and the binding of lectins with specificities for a variety of sugar residues revealed that the glycan vesicles contain neutral, glycogen-like polysaccharides composed of glucosyl/mannosyl residues. While the glycogen and yolk globules appear to provide nutrients for the developing embryo, the glycan vesicles are retained until late embryogensis and participate in the egg hatching process (Schmidt, 1998).

A fifth cell type, the nurse cell, is also present in the vitelline follicle and occupies a peripheral position within it. Long cytoplasmic processes arise from the cell body and ramify between and around the developing vitelline cells, being connected to them and to the surrounding parenchymal cells by junctional complexes (Fig. 4.19). It was suggested that the nurse cell is involved in the uptake of precursor molecules (such as amino acids and sugars) from the surrounding parenchyma and their distribution to the developing vitelline cells (Irwin and Threadgold, 1970).

While the preceding description of the vitelline follicles refers to *F. hepatica*, a very similar process for production of eggshell protein, glycogen and glycan vesicles has been described for *F. gigantica* by Meepool *et al*. (2006).

A series of pulse-chase autoradiographic experiments involving tritiated amino acids and monosaccharides has been carried out to examine shell protein and glycogen synthesis by the vitelline cells in *F. hepatica* (Hanna, 1976a). Following short chase periods (0–20 min), amino acid labels were confined to the It1 cells in which shell protein globules were beginning to appear. Silver grains were present over the GER, then

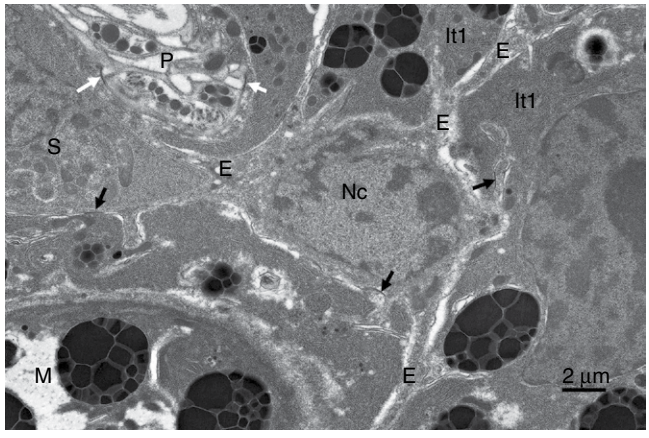

Fig. 4.19. Transmission electron micrograph of a vitelline follicle of *Fasciola hepatica* showing the nucleus of a nurse cell (Nc), with cytoplasmic extensions (E) enveloping vitelline cells (S, stem cell; It1, intermediate type 1 cell; M, mature cell) in the vicinity. The nurse cell processes are connected by numerous dense junctional complexes to protrusions of parenchymal cells (P) at the periphery (white arrows) and to the limiting plasma membrane of the vitelline cells (black arrows).

shell protein globules. After longer chase periods (45–60 min), labels were mainly associated with shell protein globules and shell globule clusters and with It2, not It1, cells (Hanna, 1976a). The experiments show that the amino acids (tyrosine, leucine, phenylalanine, not methionine) become incorporated into shell protein material and follow the typical secretory pathway from GER to Golgi complex via transition vesicles and from Golgi complex to secretory vesicle (that is, shell protein globule). The heaviest labelling was obtained with [^{3}H] tyrosine, which is not surprising given that di-tyrosine links are responsible for the cross-linking reactions involved in shell formation. The experiments also show that the vitelline cells undergo some development during the time scale of the experiment, but do not give a precise indication of the lifespan of the cell, from stem to mature cell. Egg production data suggest that one vitelline cell is produced by the fluke every 0.115 s (see below), but the time for one cell to complete its development sequence is clearly much longer than this. Labelling with tritiated monosaccharides was confined to the mature cells, in which glycogen synthesis was occurring and was associated with the glycogen deposits and the 'yolk' globules (Hanna, 1976a). The latter are formed by engulfing portions of cytoplasm containing mitochondria and glycogen granules (Irwin and Threadgold, 1970).

The descriptive account of vitelline cell development has been extended by use of stereological techniques to provide quantitative data on the changes in the whole cell and individual organelles that occur during development (Threadgold, 1982). For example, the volume of the M cell is eight times that of the S cell. The most significant growth of the nucleus occurs between the It1 and It2 cell stages, while that of the nucleolus occurs at an earlier phase, between the S cell and It1 cell stage. The nuclear changes can be linked to the activation of new genes and transcription of new mRNAs that are required for shell protein synthesis and other metabolic activities of the cells. Nucleolar changes are undoubtedly associated with ribosome production required for formation of the GER cisternae and for the synthesis of other proteins during development. The GER increases in volume some 16 times and its surface area 25 times between the stem cell and mature cell. However, these figures mask the real extent of membrane production by this system, since it is transferred to the shell protein globules via transition vesicles and the Golgi complex. In reality, when the surface area of the shell protein globules is added to that for GER, the increase in GER-derived membranes is 42, not 25, times. The quantity of shell protein globules increases steadily during development, although the greatest

rise occurs between the It1 and It2 cell stages, and the globules eventually contribute about 21% to cell volume of the M cell. Not unexpectedly, the main phase of glycogen and yolk formation takes place between the It2 and M cell stages and these two inclusions contribute approximately 20% and 14%, respectively, to final cell volume. The mitochondrial data show some interesting patterns. On the one hand, the volume and surface area of individual mitochondria and their cristae peak at the It1 cell stage, suggesting that the capacity for energy production is potentially at its greatest at this stage. However, one would expect that energy supplies would continue to rise beyond this stage to cope with the demands of shell protein synthesis and glycogen and 'yolk' formation, for example. This is borne out when the data for total numbers of mitochondria per cell and the total volume and surface area of mitochondria and their cristae are considered. The peak for these parameters occurs at the It2, not It1, stage, and even at the M cell stage the values are up to three times those in the S cell stage (Threadgold, 1982). The results from the stereological study show that this kind of analysis can provide significant information on changes in individual organelles that is not given by more qualitative methods. Moreover, it can provide an insight into the pattern of development and interrelationships between particular organelles during the cell cycle. Unfortunately, stereology has not found any wider use on *F. hepatica* or other flatworm parasites; it could be used, for example, to determine changes in vitelline and other cells resulting from drug action.

Vitelline cells have been shown to be susceptible to the action of drugs known to disrupt specific processes within the cell. For example, monensin, which is a sodium ionophore used to selectively block the intracellular transport of secretory products at the level of the Golgi complex (causing an osmotic dilation of the cisternae), does not interfere with their initial synthesis (for references, see Skuce and Fairweather, 1988). Incubation of whole flukes and tissue slices in monensin (1×10^{-6} M) leads to vacuolation of the Golgi complexes in the intermediate cells that are actively engaged in the synthesis of shell protein material. In addition, there is a block in the normal migration of the shell protein globules to the periphery of the cell; the shell globule clusters becoming very loosely packed and empty; and distended single globules accumulate in the perinuclear region of the cell (Skuce and Fairweather, 1988). In these respects, then, the effect of monensin on the vitelline cells follows its classical pattern established with a variety of cell types. However, there is no upstream accumulation of secretory material in the GER, as has been observed in other cells.

Treatment of fluke material with the microtubule inhibitors, tubulozole-C and colchicine, prevented division of the stem cells and this led to a change in the population of cells within the follicle, with relatively more stem cells than normal, but fewer intermediate-type cells, especially It1-type cells (Stitt and Fairweather, 1993). Inhibition of mitosis by the inhibitors is expected because they are known to prevent the formation of the spindle apparatus during cell division (Geuens *et al*., 1985). The nurse cell cytoplasm, which contains microtubules (Stitt *et al*., 1992), became fragmented but only after changes in the vitelline cells, indicating that the cellular effects are the direct consequence of microtubule inhibition. In the intermediate (It1 and It2)-type cells, less shell protein material was synthesized, leading to loosely packed shell globule clusters, and movement of globules from the perinuclear region to the cell periphery was disrupted, a characteristic feature of microtubule inhibition. Less glycogen and fewer 'yolk' globules were produced by the mature cells (Stitt and Fairweather, 1993).

The microfilament inhibitor, cytochalasin B, disrupted the movement of shell protein globules and their formation into clusters, suggesting an additional role for microfilaments in these processes. Binucleate stem cells were observed, indicating incomplete cytokinesis or separation of the cells following mitosis, and the eggs produced by treated flukes were abnormal with an uneven coating of eggshell material (Stitt and Fairweather, 1993).

It is evident, then, that the vitelline cells display a high level of secretory activity and undergo a rapid turnover. These two features render them particularly susceptible to the action of drugs such as anthelmintics. Consequently, they represent a good model cellular system for determining the morphological effects of anthelmintics in 'mode of action'-type

studies. The results of such studies are discussed elsewhere in this volume (Chapter 7).

4.5.3 Ootype/Mehlis' gland complex

The ootype is the site where egg formation takes place. It is lined by a single layer of epithelial cells, rests on a basal lamina and is surrounded by a layer of muscle (Threadgold and Irwin, 1970). Beyond this, no detailed description of the epithelial lining is available. The ootype is surrounded by the Mehlis' gland and the fine structure of this gland has been described by Threadgold and Irwin (1970). It consists of two types of secretory cell, designated the S1 and S2 types. The cells are arranged radially around the ootype, with the S2 cells relatively close to the ootype and the S1 cells situated further away (Fig. 4.20). Duct-like extensions of the cells converge on the ootype, passing through the muscle layers and epithelial cells to release their secretions into the lumen of the ootype (Fig. 4.20). The extensions are lined by microtubules and are anchored to the ootype epithelium by septate desmosomes. The microtubules provide support,

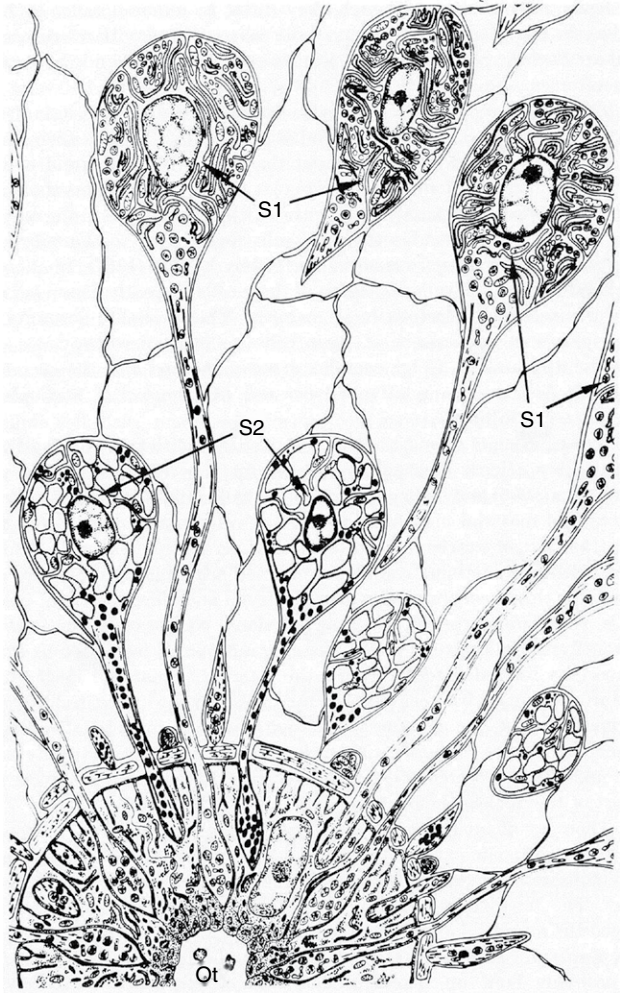

Fig. 4.20. Diagram of the Mehlis' gland of *Fasciola hepatica* showing the relationship of the S1 and S2 cell types to each other and to the ootype (Ot) (Threadgold and Irwin, 1970).

enabling the ducts to resist the compressive forces exerted by the muscular contractions of the ootype wall that assist the movement of ova and vitelline cells through the ootype. Extensions of interstitial material project into the gland cells' cytoplasm and may provide mechanical support for the cells, but no junctional complexes have been observed between the Mehlis' gland cells and the parenchymal cells.

As might be expected of actively secreting cells, the S1 cells contain an extensive network of GER cisternae, numerous mitochondria and many Golgi complexes. The latter produce sausage-shaped secretory bodies which have a filamentous content radiating from the central core (Fig. 4.21). The bodies undergo some morphological changes as they migrate from the cell body to the ootype. The terminal regions of the cell extensions within the ootype epithelium are swollen and this may indicate that the secretory bodies can be stored before their release into the lumen (Fig. 4.22). The S1 cells described by Threadgold and Irwin (1970) correspond to the 'mucous' cells described by Gönnert (1962).

The S2 cells are filled with distended cisternae of GER containing relatively electron-lucid material. This may account for the opalescent or 'serous' appearance of the cells in light microscopy; the S2 cells described by Threadgold and Irwin (1970) correspond to the 'serous' cells described by Gönnert (1962) and the 'large cells' described by Thorsell and Björkman (1965). Numerous mitochondria and Golgi complexes are present, the latter producing dense secretory bodies that are spherical with a crystalline or packed fibrous appearance (Fig. 4.23). Unlike the S1 secretory bodies, the S2 secretory bodies do not change shape or form as they move towards the ootype. The secretion contained within the secretory bodies dissociates immediately on entering the lumen of the ootype.

The cells of the Mehlis' gland give a positive staining reaction with the periodic acid–Schiff (PAS) technique, indicating the secretion of a mucopolysaccharide or mucoprotein (Johri and Smyth, 1956; Rao, 1959; Clegg, 1965). It has been suggested that the mucous secretion contains lipoprotein and that the lipoprotein may form some kind of template on which eggshell material is deposited (Clegg, 1965), although the latter idea now appears unlikely (see section 4.6.1 below). A number of functions of the Mehlis' gland have been put forward. They include: lubrication of the uterus for the passage of eggs; activation of sperm; release of shell protein material from the vitelline cells; and a role in the tanning process, in addition to the template idea just mentioned (Threadgold and Irwin, 1970; Smyth and Halton, 1983). A more recent idea on the role of the Mehlis' gland secretions in egg formation is discussed below.

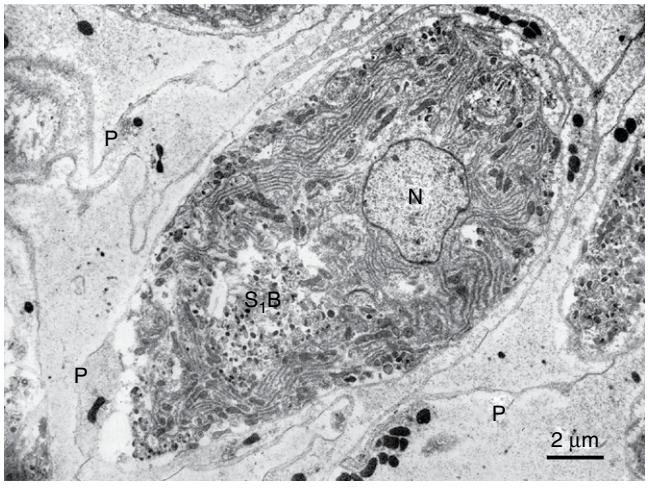

Fig. 4.21. Transmission electron micrograph of a Mehlis' S1 type secretory cell of *Fasciola hepatica* showing nucleus (N) and secretory bodies (S_1B). P, parenchymal cells (Threadgold and Irwin, 1970).

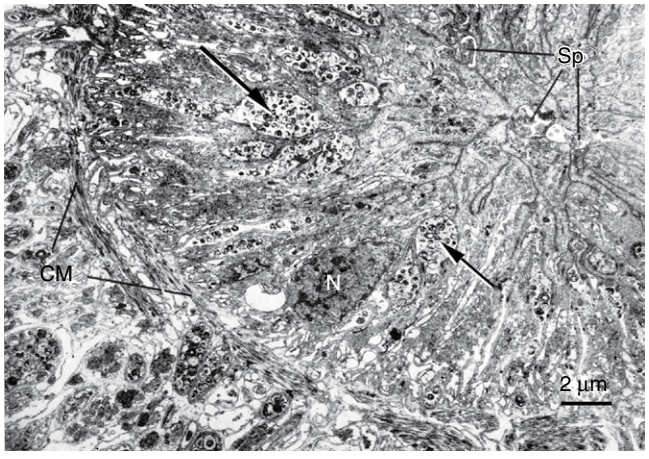

Fig. 4.22. Transmission electron micrograph showing a segment of the ootype of *Fasciola hepatica* with circular muscle (CM) surrounding it. N, nucleus of an epithelial cell of the ootype; S1 secretory bodies (arrowed) are held short of the ootype lumen, which contains spermatozoa (Sp) (Threadgold and Irwin, 1970).

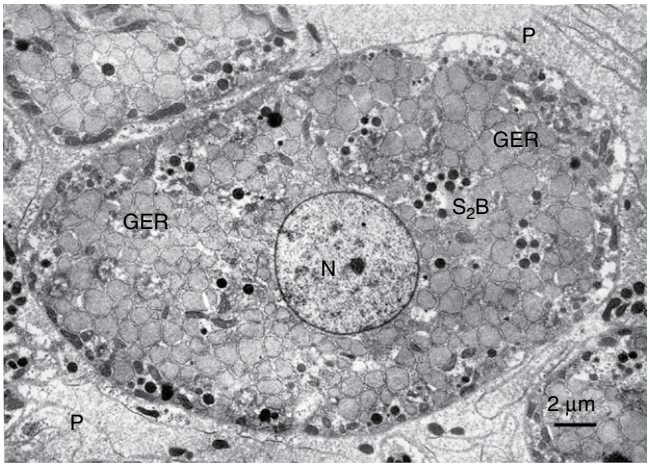

Fig. 4.23. Transmission electron micrograph of a Mehlis' S2 type secretory cell of *Fasciola hepatica* showing distended GER cisternae in the cytoplasm (GER) and dense secretory bodies (S_2B). N, nucleus; P, parenchymal cells (Threadgold and Irwin, 1970).

4.6 The Egg

4.6.1 Egg formation

Egg production figures for *F. hepatica* are impressively high: in a light infection in sheep (up to 50 flukes), each fluke produces an average of 25,000 eggs per day (equivalent to the release of 1,250,000 eggs per day by the sheep) (Happich and Boray, 1969). This equates to the production of one egg every 3.46 s. Each egg requires approximately 30 vitelline cells (Stephenson, 1947; Rao, 1959). Therefore, the fluke has to produce one vitelline cell every 0.115 s in order to maintain this output. The vitelline cells pass from the vitelline reservoir into the common ovovitelline duct and on into the ootype, together with a single oocyte from the oviduct. Within the ootype, the vitelline cells release the contents of the shell globule clusters and the

globules coalesce to form the eggshell around the cell mass.

The eggshell is formed of a sclerotin or quinone-tanned protein. Quinone tanning involves the conversion of DOPA (3,4-dihydroxyphenyl-L-alanine) residues to *o*-quinones by the action of phenol oxidase (phenolase or catechol oxidase). The cross-linking of DOPA-containing proteins forms a very stable, tough, waterproof, resistant and protective capsule around the embryo. The phenol oxidase enzyme needs to be activated (by enzyme action) before it triggers the tanning process. The activating enzyme is presumed to be a protease-type enzyme, because phenol oxidases in other organisms are known to be activated by proteolytic cleavage (for references see Wells and Cordingley, 1991). A model for eggshell formation in schistosomes makes the assumption that the three components – the eggshell protein, the phenol oxidase 'tanning' enzyme and its activating enzyme – are packaged together in the same membrane-bound vesicle (namely, the shell globule cluster) in the vitelline cells (Wells and Cordingley, 1991). In *F. hepatica*, three different eggshell precursors have been identified and designated as vitelline proteins (vp) A, B and C, with sizes of 70, 31 and 17 kDa, respectively (Waite and Rice-Ficht, 1987, 1989, 1992; Zurita *et al*., 1987, 1989; Rice-Ficht *et al*., 1992). Each form exhibits heterogeneity due to variations in amino acid sequence and post-translational modification. The precursors are rich in DOPA residues, formed from tyrosine residues during co- or post-translation modification. Precursor vpB is a particularly heterogeneous, though closely related, group of proteins (Waite and Rice-Ficht, 1992) and represents 6–7% of total protein in the fluke (Waite and Rice-Ficht, 1987). Moreover, up to seven copies of vpB genes may be present in the genomic DNA of *F. hepatica* (Rice-Ficht *et al*., 1992). These observations highlight the emphasis placed on reproductive activities (and egg production in particular) within the overall energy budget of the fluke. Also, they suggest that the eggshell is a very complex heteropolymer of proteins, a feature common to other eggshell protein families (see Rice-Ficht *et al*., 1992). Immunocytochemical studies utilizing an antibody to vpB have localized the protein to the shell protein globules in the vitelline cells (Rice-Ficht *et al*., 1992; Robinson *et al*., 2001). Synthesis of the protein by these cells has been confirmed by use of *in situ* hybridization techniques (Zurita *et al*., 1989; Rice-Ficht *et al*., 1992).

Phenol oxidase activity has been demonstrated in the vitelline cells of *F. hepatica* (Johri and Smyth, 1956). The enzyme is inhibited by DDC (diethyldithiocarbamic acid), a copper chelator and phenol oxidase inhibitor; inhibition can be reversed by copper sulfate and enzyme activity is stimulated by copper sulfate alone. The results indicate that phenol oxidase is a copper-containing protein (Mansour, 1958). So, two of the three components required for eggshell formation in *F. hepatica* have been localized to the vitelline cells. The third component – the putative phenol oxidase activating enzyme – has yet to be identified in *F. hepatica* or any other digenetic trematode and its site of synthesis remains to be elucidated.

The model for eggshell formation in schistosomes suggests that the release of shell protein material from the vitelline cells is a calcium-dependent process and that the fusion and tanning of the shell protein globules require alkaline conditions (Wells and Cordingley, 1991). Experiments with *F. hepatica* indicate that a similar mechanism operates in *F. hepatica*. Thus, incubation in the calcium ionophore lasalocid induces premature release of the shell globule clusters from the mature vitelline cells but does not cause any precocious tanning of the shell protein material (Colhoun *et al*., 1998). Treatment with either monensin or ammonium chloride, which both serve to increase the pH of membrane-bound acidic compartments within cells, leads to fusion of the shell protein globules within the clusters and premature tanning of the shell protein material. The rise in pH serves to abolish the charges on the surface of the globules that prevent their fusion within the clusters. The changes induced by monensin and ammonium chloride can be prevented by DDC (Colhoun *et al*., 1998).

Translating these results into what happens within the ootype, it can be envisaged that the vitelline cells are induced to exocytose the contents of the shell globule clusters via a calcium-dependent process, although the trigger remains to be identified. The free shell protein globules meet an 'interface' between two liquids of different viscosities and at different pHs: the fluid around the vitelline cells at acid pH and the

more viscous Mehlis' gland secretion at an alkaline pH. On contact with the Mehlis' gland secretion, the surface charges on the shell protein globules are lost and the globules can coalesce to form a uniform layer along the interface. The phenol oxidase and its activating enzyme will be released from the vitelline cells along with the shell protein globules and, following their activation, will trigger the cross-linking reactions between DOPA residues in the shell protein precursors that lead to eggshell formation. The mixing movements of the ootype, brought about by contractions of the muscle in the ootype wall, will aid the process. The concept of an interface rather than a lipoprotein template (as suggested by Clegg, 1965) fits in better with the electron microscope observations of Irwin and Threadgold (1972) (Fig. 4.24) and with the schistosome model (Wells and Cordingley, 1991). According to this model, which may be applicable to other trematodes as well, the secretions from the Mehlis' gland may play a number of novel roles in egg formation, including: (i) provision of the alkaline conditions required for fusion of the shell protein globules; (ii) triggering the release of the shell protein globules; and (iii) activation of the tanning enzyme.

As indicated above, the rate of egg production in *F. hepatica* is extremely rapid: one egg every 3.46 s. The combining of one ovum and

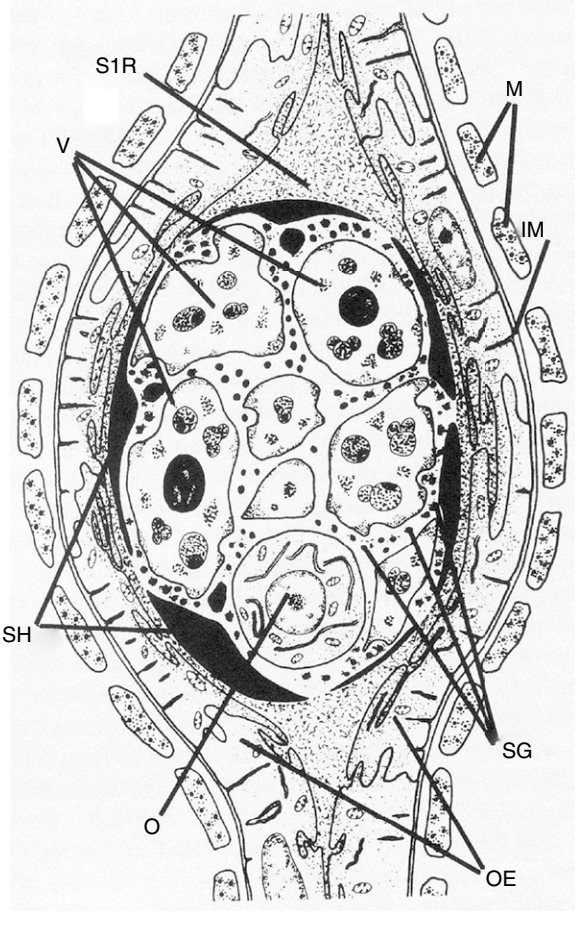

Fig. 4.24. Diagram representing a longitudinal section through the ootype of *Fasciola hepatica* containing a developing egg. IM, interstitial material; M, muscle block; O, ovum; OE, ootype epithelium; SG, shell protein globule; SH, accumulating eggshell; S1R, residue of secretion from the S1 type of Mehlis' gland cell; V, vitelline cell (Irwin and Threadgold, 1972).

30 or more vitelline cells to form an egg in such a short interval of time argues for a sophisticated and highly synchronized mechanism of egg production involving a complex sequence of muscular contractions and relaxations in the proximal portions of the female reproductive tract. Two groups of peptidergic nerve cells have been observed at the junction of the vitelline and ovovitelline ducts and at the entrance of the uterus from the ootype (Magee *et al*., 1989); these sites correspond to nerve plexuses I and II described by Gönnert (1962) (Fig. 4.25). The cells may control the entry of ova (or secondary oocytes) and vitelline cells into the ootype and exit of newly formed eggs from the ootype into the uterus via the uterine valve (Magee *et al*., 1989). Other peptidergic cells (together with cells immunoreactive for 5-hydroxytryptamine) lie among the S2 type of Mehlis' gland cells (Fairweather *et al*., 1987; Magee *et al*., 1989) (Fig. 4.25). Secretions from these cells may exert a paracrine-like influence over the secretory activity of neighbouring Mehlis' gland cells. A plexus of nerve fibres exists within the wall of the ootype and may coordinate the contractions of the ootype musculature, thus controlling the movement of cells through the ootype (Fairweather *et al*., 1987; Magee *et al*., 1989). It seems likely that there is a strong neural influence over egg production, but a full understanding of the mechanisms involved awaits clarification.

The successful formation of a normal shelled egg in the ootype of *F. hepatica* represents the culmination of a complex sequence of cytokinetic and cytological events involving multiple tissues and secretory products as well as neural/peptidergic controlling mechanisms. Drug-induced defects in the function or output of any of these are likely to be reflected in the appearance and integrity of the eggs emerging from the ootype and entering the uterus. Thus, the histological appearance of the uterus and its contents provides an early and sensitive indication of the emergence of malfunctions throughout the female reproductive tract, and informs the assessment of mechanisms of drug action and of fluke resistance to drugs such

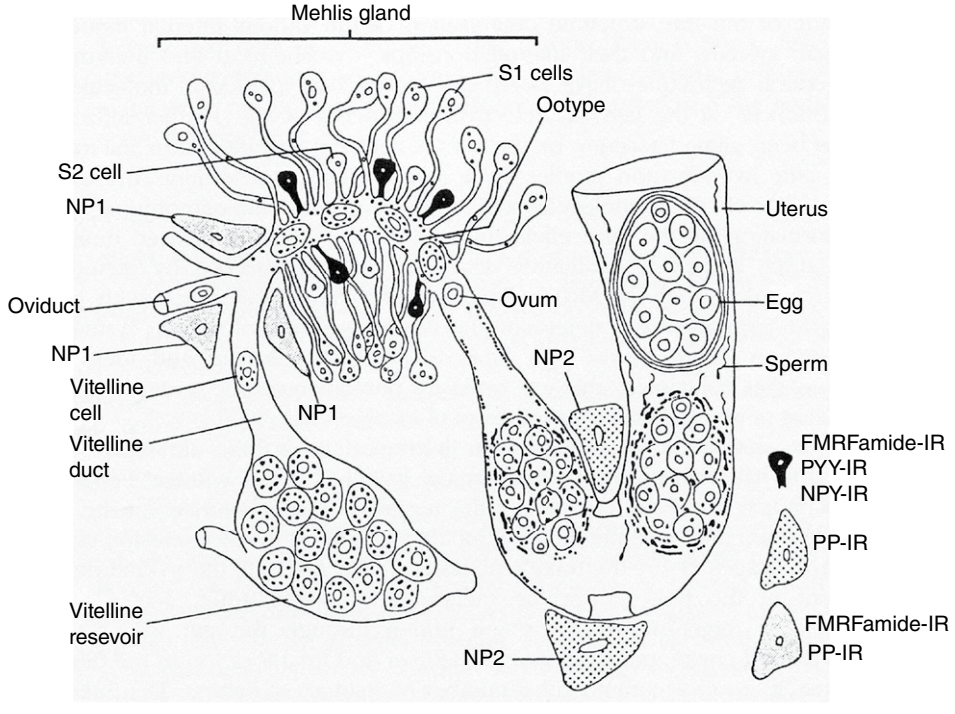

Fig. 4.25. Diagram showing the distribution of PP-, PYY- and FMRFamide-immunoreactive cells in the ootype/Mehlis' gland complex of *Fasciola hepatica*. NP1, cell body in nerve plexus 1; NP2, cell body in nerve plexus 2; S1, S1-type of Mehlis' gland cell; S2, S2-type of Mehlis' gland cell (after Magee *et al*., 1989).

as triclabendazole and its derivatives (Hanna *et al.*, 2010; Toner *et al.*, 2011b; McConville *et al.*, 2012).

The newly formed egg with its semi-soft shell passes from the ootype into the proximal uterus, where fertilization takes place. The eggshell becomes hardened as the egg passes along the uterus. The egg of *F. hepatica* has an oval shape with a smooth surface and is marked at one pole by the presence of the operculum. The operculum is formed by an unusual process: the ovum apparently pushes out pseudopodia towards the eggshell during its process of formation exactly at the point of rupture in the operculum. The result is that the eggshell is weakened at this point and so readily fractures on hatching (Gönnert, 1962). Studies on the solubility and permeability of *F. gigantica* eggs by Balasubramanian *et al.* (2010) have shown that cross-linkages of the opercular cement, the operculum and the quinone-tanned eggshell differ in their permeability and solubility properties.

The glycan vesicles produced at a late stage in the maturation of the vitelline cells are involved in hatching. These vesicles are retained intact within the egg during embryonation in the environment. When hatching of the egg is induced by exposure to light and a slight rise in temperature, the vesicles merge, swell and coalesce into two vacuoles that fill the space between the embryo and the eggshell. The internal hydrostatic pressure rises, probably due to depolymerization of the polysaccharides, causing an osmotically driven water influx. Finally, the operculum of the egg bursts open and the miracidium escapes (Schmidt, 1998).

4.6.2 Parthenogenesis in *Fasciola* spp.?

As previously stated, development of the oocytes leaving the ovary is arrested at meiotic prophase 1 (Meiosis 1). Resumption of meiosis occurs only after each individual oocyte has been incorporated in an egg in the ootype. In the proximal coils of the uterus, oocytes usually display meiotic metaphase chromosome alignments and, in diploid flukes but not in the aspermic Cullompton isolate flukes, a sperm nucleus can sometimes be presumptively identified in light microscope sections using the acid Feulgen staining technique (Hanna *et al.*, 2016) (Fig. 4.26A). After completion of Meiosis 1, the most advanced stage achieved before the eggs are shed to the environment, two pronuclei are visible within each developing oocyte in the distal uterus (Fig. 4.26B). In outcrossing diploid flukes, it is likely that one of these pronuclei contains nuclear components from the sperm, fertilization having occurred before the eggshell hardened, and that syngamy occurs with the female pronucleus to restore the diploid chromosome complement ($2n$=20). In

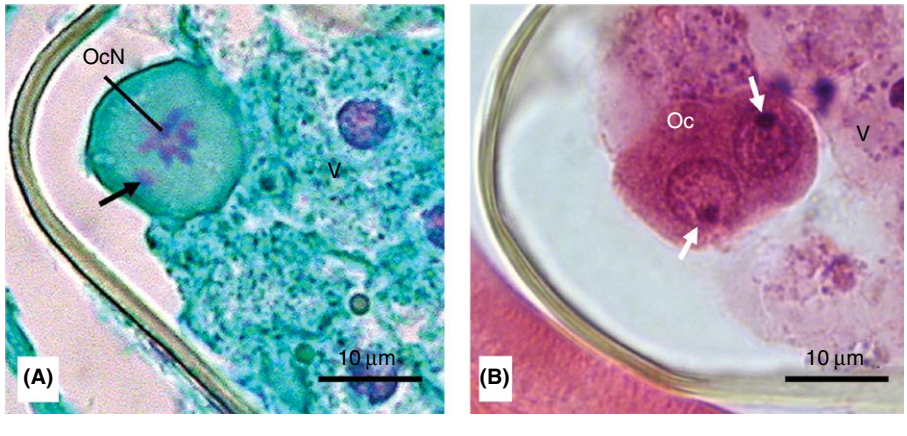

Fig. 4.26. (A) Light micrograph of an acid Feulgen-stained metaphase nucleus (OcN) in the proximal uterus of a liver fluke. An additional peripheral chromatin granule (arrowed) may originate from the spermatozoon. V, vitelline cell nucleus. **(B)** Light micrograph of a Haematoxylin and Eosin-stained oocyte (Oc) of an egg in the metraterm of a liver fluke, showing two pronuclei. Each pronucleus bears a conspicuous nucleolus (arrowed). V, vitelline cell (Hanna *et al.*, 2016).

the aspermic Cullompton flukes, where fertilization does not occur, the two pronuclei that appear may result from the completion of Meiosis 1 without elimination of a polar body (Sanderson, 1953). Fusion of these daughter pronuclei might restore the triploid complement (3*n*=30) characteristic of Cullompton isolate flukes according to Fletcher *et al*. 2004) and ensure success in the subsequent cleavage division (Meiosis 2). This model, illustrated in Fig. 4.27, permits the possibility of facultative parthenogenesis as a reproductive strategy in *F. hepatica* while accommodating the survival and proliferation of triploid (and other polyploid) isolates,

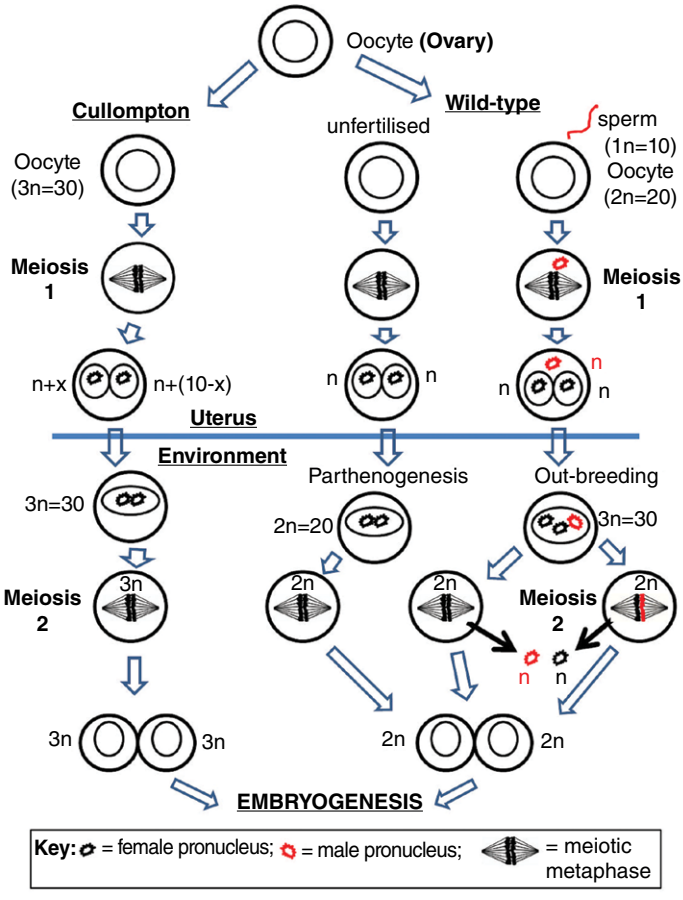

Fig. 4.27. Diagram summarizing the proposed mechanisms for out-breeding and parthenogenesis in wild-type and in aspermic Cullompton-isolate *F. hepatica* eggs. Oocytes in the ovary of all flukes undergo chromosome synapsis, recombination and chiasmata formation during prophase. In the uterus, the triploid Cullompton oocytes form unequal daughter nuclei following meiotic division 1 (reduction division). Wild-type oocytes form two haploid daughter nuclei after Meiosis 1, and incorporate a haploid chromosome complement from a spermatozoon if fertilization occurs in the ootype. Subsequent development in the environment involves re-fusion of the daughter nuclei, restoring the triploid chromosome number in Cullompton eggs. In wild-type oocytes that are not fertilized, the 2*n* chromosome number is also restored by fusion of the daughter pronuclei. In fertilized eggs, the haploid spermatozoon chromosome complement or a haploid daughter nucleus is eliminated, while the two remaining haploid pronuclei fuse to restore the 2*n* chromosome number. The second meiotic division (cleavage division) results in 2 × 3*n* parthenogenetic daughter cells in the Cullompton eggs, or 2 × 2*n* daughter cells in the wild-type eggs, which will be parthenogenetic or out-bred depending on whether or not a male pronucleus contributed to the spindle in the cleavage division.

which appears to be a feature of the Fasciolidae in those areas of the world where *F. hepatica* and *F. gigantica* often co-infect ruminants, and may hybridize (see, for example, Agatsuma *et al.*, 1994; Terasaki *et al.*, 2000; Periago *et al.*, 2008; Afshan *et al.*, 2014; Beesley *et al.*, 2015). Possibly the same mechanism of parthenogenesis might operate in diploid *F. hepatica* if sperm is not available, for example in small infections where flukes may be physically isolated, or where a large proportion of the population has been removed by chemotherapy, leaving only isolated drug-resistant individuals (Hanna *et al.*, 2016).

In an experiment where rats were infected with small numbers or single flukes of the Cullompton (aspermic, 3*n*) and/or Oberon (sperm-producing, 2*n*) isolates, Cullompton flukes, as expected, had no spermatozoa in their uteri when present alone or with other Cullompton flukes. However, even when present in multiple infections with Oberon flukes, cross-insemination appeared to be a rare and limited event, suggesting that failure to produce viable spermatozoa may preclude copulation or sperm transfer from a virile partner. By contrast, the Oberon flukes almost always had spermatozoa in the uterus when present with other sperm-producing flukes, but when present as single-fluke infections, or with Cullompton flukes as the only co-inhabitants, the majority had no spermatozoa present in the uterus, with only 33% showing evidence of self-insemination. The findings provide further evidence that fertilization (self- or cross-) is not essential for the production of viable eggs in *F. hepatica* (Hanna *et al.*, 2011). On the other hand, in a recent survey, all flukes examined from naturally infected sheep and cattle were diploid and contained sperm (Beesley *et al.*, 2015). Thus, parthenogenesis probably occurs infrequently, and perhaps only in hosts harbouring low worm burdens. Further studies, in which morphological and molecular analyses are carried out in tandem, would be of value in clarifying the contribution of parthenogenesis in the reproductive repertoire of *Fasciola* spp.

4.6.3 Embryogenesis

Investigation of the morphological changes occurring during embryogenesis of the miracidium in the developing egg of *Fasciola* spp. has been impeded by the impermeability of the eggshell to penetration by fixatives and embedding resins for electron microscopy. However, molecular studies on incubated eggs have enabled the identification of certain proteins which, in addition to their involvement in development, are sometimes recognized as antigens by the definitive host, and therefore may be partially expressed during oogenesis. Proteomic analysis has been carried out on eggs of *F. hepatica* incubated for up to 9 days at 30°C in distilled water containing fungizone and gentamycin by Moxon *et al.* (2010). During the embryonation process, antioxidant and chaperone proteins were predominantly expressed and plasticity was recorded in the expression of major components, particularly a prominent 65 kDa protein cluster. This suggests that embryonation of liver fluke eggs is a plastic process, possibly responsive to variations in the ambient conditions experienced by developing eggs. Consistent expression of alpha crystallin domains confirmed the protein to be a member of the alpha crystallin-containing small heat shock protein (AC/sHSP) superfamily. AC/sHSPs are ubiquitous in nature but this is the first time a member of this protein superfamily has been described from *F. hepatica*. The antigenic AC/sHSP was named Fh-HSP35α based on predictions of molecular weight. Interestingly, immunoblotting revealed that the abundant 65 kDa protein cluster is recognized by infection sera from three *F. hepatica*-challenged host species, and it is possible that expression of a secretory peptide facilitates maternal packaging of FhHSP35α during oogenesis (Moxon *et al.*, 2010). Recombinant Fh-HSP35α has potential as an egg-based diagnostic marker for liver fluke infection, and this study indicates the prospective value of further proteomic investigations of the reproductive structures of liver fluke. A 28 kDa egg protein of *Clonorchis sinensis*, the amino acid sequence of which showed 60% homology with the vitelline B precursor protein of *Opisthorchis viverrini* and 33% homology with the vitelline B1 and B2 proteins of *F. hepatica*, also displayed an antigenic reaction with serum samples from cases of clonorchiasis, opisthorchiasis, schistosomiasis and paragonimiasis. This egg protein of *C. sinensis*, termed Cs28, was identified as an antigen common to all the trematode species examined by Lee *et al.* (2005).

4.7 Conclusions and Future Perspectives

While our understanding of the basic functional morphology of the main components of the liver fluke reproductive system has changed little in the past 20 years, technological advances in proteomics and cellular imaging have created challenges and opportunities to integrate pre-existing knowledge with ongoing molecular developments. Thus, topical issues such as drug resistance, development of novel chemotherapeutic agents and identification of putative vaccines can be addressed in relation to potential impact on parasite reproduction, which in some circumstances may present a more realistic approach to control than absolute elimination or prevention of infection. Areas of particular interest include the early differentiation of the reproductive structures in the juvenile fluke as it migrates through the hepatic parenchyma. In this respect, the recognition and tracking of pluripotent neoblasts, highlighted by the *in vitro* studies of McCusker *et al.* (2016), is of particular interest and signals a need for ultrastructural investigation of flukes at sequential stages during *in vivo* growth in the liver. The point of transition between hepatic parenchyma and bile duct lumen is especially relevant, since that change in environment apparently triggers a relatively rapid onset of functionality in the vitelline follicles (Robinson *et al.*, 2001) with likely repercussions on all of the reproductive structures. It is also likely that subtle changes in nutrition at this transition are significant and may be reflected by ultrastructural changes in the tegument, gut and excretory system.

A recurring theme in the ultrastructure of the reproductive system of *F. hepatica* is the occurrence of certain non-germinal 'support' tissues in those organs displaying a high rate of cellular differentiation and turnover, viz. the 'nurse cells' in the vitelline follicles, the 'sustentacular tissue' in the testis and the 'interstitial tissue' in the ovary. While it has been proposed that the primary role of these cells/syncytia is to act as a conduit for inward and outward flux of micronutrients and metabolites to and from the actively metabolizing germinal cells, and to scavenge the cytoplasmic debris and effete cells resulting from cell division and differentiation, it is possible that they have more subtle roles, for example in elaboration of growth factors and hormones to modulate and direct cytokinesis and gametogenesis (Hanna *et al.*, 2011, 2012). It is important, therefore, to examine these elements of the reproductive system (for example, by transcriptome/proteome analysis of microdissected tissue sections) for the identification of potential targets for chemotherapy or immunotherapy.

The exact role of neurosecretory and neural mechanisms in directing egg formation is another area that would benefit from the integration of functional genomics approaches with established structural information (McVeigh *et al.*, 2018), and might offer possibilities of advancement in the search for novel targeted anthelmintics and vaccines. In contrast, proteomics analysis of developing *F. hepatica* eggs (Moxon *et al.*, 2010) has not been preceded by ultrastructural analysis of embryogenesis, due to the technical difficulties imposed by the impermeable shell. In this area, a combination of freeze-fracture technology with appropriate immunolabelling might prove informative in signalling the temporal and spatial distribution of significant gene products during growth and differentiation of the miracidium.

Finally, recognition that parthenogenesis might participate as a reproductive strategy in circumstances where fertilization of the ovum is not possible or fails (Fletcher *et al.*, 2004; Hanna *et al.*, 2016) should perhaps be considered in predictive epidemiological modelling of fluke populations, especially where there is genetic variability in parasite resistance to anthelmintic treatment. Molecular-level analysis of intrauterine and developing eggs is necessary to clarify the mechanisms that might be involved.

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5 Pathology, Pathophysiology and Clinical Aspects

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5.1 Pathology

5.1.1 Introduction

Infections with *Fasciola hepatica* cause an economically important disease in sheep and cattle, but goats, buffalo, horses and pigs are also affected. It is also a pathogen of wildlife that may act as natural reservoirs of the parasite. Fasciolosis is an emerging zoonosis mainly seen in areas of South America, Asia and Africa. In addition, experimental animals such as mice, rats, guinea pigs and rabbits have been used to explore aspects of life history, pathology and immune responses as well as for initial evaluation of vaccine candidates. Since the pathology of fasciolosis due to *F. hepatica* has been mainly studied in sheep, the latter animal has been chosen here for description and is summarized in Table 5.1. Comparisons with pathology in other species are also drawn in the text. The disease in goats is quite similar to that of sheep. In general, the life cycle in experimental models such as rat and mouse is shorter, so the disease process is accelerated.

The disease occurs in two phases: (i) the parenchymal phase during migration of juvenile flukes through the liver parenchyma; and (ii) the biliary phase, which coincides with the adult flukes residing in the bile ducts. While *F. hepatica* has a worldwide distribution, *Fasciola gigantica* is a tropical and subtropical species, mainly found in Asia and Africa (Sharma *et al*., 2011). Where the two species overlap, hybrids known as *Fasciola* 'intermediate forms' can occur and molecular identification of species and hybrids can be achieved using internal transcribed spacers (Sumruayphol *et al*., 2020) (see Chapter 13, this volume). Traditional morphometric analyses have been used to identify the different species: *F. gigantica* is the longer and narrowest form, while intermediate forms are shorter and wider compared with both *F. hepatica* and *F. gigantica* (Sumruayphol *et al*., 2020).

Clinical signs and pathology of fasciolosis depend on a wide variety of factors, such as infective dose, strain of *F. hepatica* and, for the host, its species, age, body condition and health status. In heavy infections in susceptible hosts, such as sheep and goats, sudden deaths may decimate flocks quickly. In these cases, the appearance of a heavily infected liver from an animal with acute terminal fasciolosis is a characteristic finding. However, the most common form of fasciolosis is chronic and subclinical but, even in those cases, important economic losses

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Table 5.1. Development of *F. hepatica* liver pathology and clinical signs of fasciolosis in sheep.

Week pi	Flukes	Gross pathology	Histopathology	Cell infiltrate	Bile ducts	Clinical signs	Eggs, blood	Ref.
1–2	1–1.5 mm. Penetrate capsule and form tracks in parenchyma	Congested left lobe, red spots and occasional white tracks (0.5–1 cm length) with red edges most common on parietal surface of left lobe, red tracks red in parenchyma (Fig. 5.1.1)	Damage limited to subcapsular areas with small haemorrhages, necrotic tracks and infiltration of eosinophils and macrophages to tract regions. Older tracts are surrounded by inflammatory infiltrate while recent ones show little inflammation (Fig. 5.1.3) Subcapsular haemorrhage, degenerate hepatic cells and eosinophils in blood-filled tracts. Eosinophils and macrophages found in adjacent parenchyma	Eosinophils, macrophages	Normal	No clinical effects	No eggs. Blood: normal	[1, 2, 3, 7, 8, 9]
2–3	1.5–2.5 mm. Form tracts in parenchyma. Inflammatory infiltrate surrounding migrating larvae varies from absent to very severe and composed mainly of eosinophils	Occasional red spots and higher number of tortuous tracts (1–2.5 cm in length) some of them with red edges involving mainly the left lobe (Fig. 5.1.2)	Necrotic tracts are found 0.5–1 mm behind migrating larvae, which show little or no parenchymal damage and have inflammatory infiltrate surrounding them. Necrotic tracts are surrounded by infiltration of eosinophils, macrophages, lymphocytes and plasma cells. Older necrotic tracts are surrounded by abundant macrophages, epithelioid cells conforming granulomas (Fig. 5.1.4) Migrating larvae show from very little (Fig. 5.1.5) to heavy (Fig. 5.1.6) infiltrate of eosinophils and macrophages	Eosinophils, macrophages, epithelioid cells, multinucleate giant cells, lymphocytes, plasma cells	Infiltration of eosinophils, macrophages and lymphocytes in portal areas adjacent to necrotic tracks	Few clinical signs. in heavy infections	No eggs. Blood: +/– eosinophilia, +/– hyperglobulinaemia	[1, 2, 3, 4, 9]

4–5	4–5 mm. Migrating in parenchyma	Whitish-yellowish tortuous tracts some of them raised and surrounded by haemorrhage centred on left lobe. Some cases show fibrinous perihepatitis, possible peritonitis	Old tracts with contracting core, some fibrosis, cuff of infiltrate with giant cells, macrophages and lymphocytes predominating. Eosinophils appear in portal regions adjacent to tracts. May be regions of infarction. Fresh haemorrhagic tracts	Eosinophils, macrophages, epithelioid cells, multinucleate giant cells, lymphocytes, plasma cells, fibroblasts	Eosinophilic infiltration in ductules near tracks	Few clinical signs	No eggs. Blood: eosinophilia, hyperglobulinaemia	[1, 2, 3, 4, 9]
6–8	6–10 mm. Localized in left lobe, start to enter ductules	Tracts are blood red (2–3 mm), haemorrhagic surface plaques present on visceral surface of left lobe (predominantly). Fibrinous adhesions to and congestion of local organs. Enlarged hepatic lymph nodes, congestion of liver	Haemorrhage and hepatocyte debris in fresh tracts, now larger with larger flukes. Fibrosis and haemosiderin pigment occur in older tracts. Granulomas with necrotic centre surrounded by macrophages. Infiltrate of lymphocytes plasma cells and eosinophils in the periphery of granulomas and portal areas. Venous thrombosis	Eosinophils, macrophages, epithelioid cells, multinucleate giant cells, lymphocytes, plasma cells, fibroblasts	A few flukes present, duct hyperplasia	ACUTE TYPE I FASCIOLOSIS Sudden death in infections >5000 mc. Ascites, abdominal haemorrhage, icterus, pallor of membranes, weakness	No eggs. Blood: Typically, anaemia, hypoalbuminaemia, eosinophilia	[1, 2, 3, 4]
8–10	10–12 mm. Majority enter bile ducts	Subcapsular haemorrhage on visceral surface. Tracts form grooves on the surface. Fibrinous/gelatinous liver surface	Necrotic cores with giant cells, granulation tissue and lymphocytes forming concentric rings. Portal inflammatory infiltrate of lymphocytes, plasma cells and eosinophils.	Eosinophils, macrophages, epithelioid cells, multinucleate giant cells, lymphocytes, plasma cells, fibroblasts	Flukes in dilated ducts. Bile duct hyperplasia and fibrosis. Mucous cell hyperplasia. Some inflammatory infiltration	ACUTE TYPE II FASCIOLOSIS >1000 flukes cause death, but animals may briefly show pallor, loss of condition and ascites	Eggs +/– in faeces. Blood: Typically, anaemia, hypoalbuminaemia, eosinophilia	[1, 2, 3, 4]

Continued

Table 5.1. Continued.

Week pi	Flukes	Gross pathology	Histopathology	Cell infiltrate	Bile ducts	Clinical signs	Eggs, blood	Ref.
12–40	12–30 mm. Resident in bile ducts	Enlarged liver of lighter than normal colour, rough surface due to healed tracts (Fig. 5.2.1) diffuse cirrhosis. Enlarged bile ducts with thickened but pliable walls contain brown detritus and flukes. Ducts project above the rough visceral liver surface, thinned in places, (Fig. 5.2.2). Gallbladder, hepatic lymph nodes enlarged	Hyperplasia of bile duct, some showing mature flukes causing erosion of the epithelium and severe portal fibrosis with scarce inflammatory infiltrate (Fig. 5.2.3) or severe inflammatory infiltrate leading lymphoid follicles (Fig. 5.2.4), in some hyperplasic bile ducts intraepithelial globule leukocytes are also found (Fig. 5.2.5). Granulomas with necrotic centre, some of them calcified and surrounded by multinucleate giant cells and fibrosis are also found in some areas during the biliary stage (Fig. 5.2.6).	Eosinophils, macrophages, epithelioid cells, multinucleate giant cells, lymphocytes, plasma cells, fibroblasts globule leucocytes	Hyperplastic cholangitis. Mild fibrosis of ductules. Extreme cell hyperplasia and wall thickening of main bile ducts. Necrosis of duct mucosa caused by presence of flukes. Eggs and tarry bile also present. Haemorrhagic epithelium	SUBACUTE FASCIOLOSIS > 800 flukes often acquired over time. Flukes are mostly in bile ducts by 20 weeks. Sheep are lethargic, anaemic and may die. Weight loss CHRONIC FASCIOLOSIS > 200 flukes. Gradual development of bottle jaw and ascites (ventral oedema), emaciation	Many eggs in faeces and bile ducts. Blood: Anaemia (normochromic, normocytic) hypoalbuminaemia, elevated liver enzyme activities. Eosinophilia Many eggs in faeces and bile ducts. Blood: Anaemia, hypoalbuminaemia, elevated liver enzyme activities. Eosinophilia	[1, 2, 3, 4, 5, 6]

Notes: This is a guide to pathology. Animals may simultaneously carry infections at different stages and show a complex of pathologies. The timing and severity will vary between animals, even in experimental conditions when the same dose of metacercariae is administered and animals are maintained in the same conditions. Heavier infections generally result in death at earlier times. Data are based on reports by [1] Ross *et al*. (1967); [2] Dow *et al*. (1968); [3] Boray (1969); [4] Meeusen *et al*. (1995); [5] Martínez-Moreno *et al*. (1999); [6] Pérez *et al*. (2002); [7] Zafra *et al*. (2013a); [8] Zafra *et al*. (2013b); [9] Escamilla *et al*. (2016a). 'Eggs' refers to fluke eggs in faeces.
mc: metacercaria; pi: post-infection

can occur in livestock. Liver pathology is linked to direct effects and the local inflammatory/immune responses to the parasites while they are migrating in the liver or living within the bile ducts. The level of infection also affects the pathology. Heavy burdens cause more severe pathology and earlier death of sheep whereas smaller infections (fewer metacercariae) generally have a longer and less severe course.

Under some environmental and management conditions large simultaneous intakes of metacercariae occur and, therefore, large numbers of juvenile fluke enter the liver at the same time. However, 'trickle' infections, where flukes are acquired over several weeks, are more typical. Both juvenile and older flukes can inflict lesions simultaneously while those caused earlier by older flukes are resolving. As a result, examination of an individual liver from an endemic area could reveal all of the features of pathology described below. In goats (Martínez-Moreno *et al.*, 1999) and sheep (Pérez *et al.*, 2002) trickle infections cause more severe hepatic lesions than a single infection, even when the total dose of metacercariae administered experimentally is the same. Because infections of adult flukes in the bile ducts can persist for years, parasites may be present many years after the source of infection has gone. Detailed descriptions of hepatic lesions have appeared in classical studies such as Ross *et al.* (1967), Sinclair (1968), Dow *et al.* (1968) and Boray (1969), or more recent ones: Meeusen *et al.* (1995), Martínez-Moreno *et al.* (1999), Pérez *et al.* (2002), Zafra *et al.* (2013a,b) and Escamilla *et al.* (2016a). The picture of the disease described below is drawn from both experimental and natural infections.

5.1.2 Prehepatic stages

Newly excysted juveniles (NEJs) penetrate the intestinal mucosa and can be found in the abdominal cavity by 72 h pi (Dow *et al.*, 1968). Experimental studies in mice and rats revealed that intestinal penetration by NEJs was rapid: in naive rats 52% of metacercariae injected into the intestinal lumen passed through the intestinal wall within 6 h (Van Milligen *et al.*, 1998). Intestinal penetration induced very little inflammatory infiltrate in mice (García-Campos *et al.*, 2016) and in naive rats, while in secondarily infected rats an infiltrate of eosinophils was found in the intestinal submucosa, which was associated with partial protection of intestinal penetration (Van Milligen *et al.*, 1998). In goats experimentally infected with *F. hepatica* and sacrificed at 7–9 days post-infection (pi) no microscopic changes were recorded in the duodenal wall and peritoneum, and only occasional small haemorrhages with mild infiltration of eosinophils were found in the lamina propria and submucosa of the duodenum (Zafra *et al.*, 2013a,b). In cattle, *F. hepatica* infection induced increases of mucosal mast cells and eosinophils in the small intestinal mucosa (Wicki *et al.*, 1991). Intestinal penetration is not associated with clinical disease, perhaps because of the very mild intestinal lesions caused by NEJs.

After intestinal penetration, NEJs migrate through the peritoneum towards the hepatic surface (Dow *et al.*, 1968). *In vitro* studies revealed that NEJs crept over the cell culture surface using the ventral sucker, suggesting that this sucker is used during peritoneal migration *in vivo*. The destination of most NEJs is the left hepatic lobe, probably because of its anatomical proximity to the duodenum. Fewer NEJs reach other hepatic lobes. Occasionally NEJs penetrate other organs such as the diaphragm and lung, causing pneumonia and fibrinous pleuritis, particularly in heavy infections (Boray, 1969).

5.1.3 Hepatic stages

Recovery rates of flukes from livers vary from less than 25% to more than 50% in sheep and goats given 150–200 metacercariae (Martínez-Moreno *et al.*, 1999; Pérez *et al.*, 2002; Zafra *et al.*, 2008, 2013a; Mendes *et al.*, 2010a). The liver is such an attractive destination for flukes that, despite the barriers, flukes can find their way to the livers of fetuses in ewes and cows and set up prenatal infections. The life history of penetration, migration and localization into the bile ducts is described in Chapter 1 of this volume. The main features of the pathology of those phases in sheep and goats are summarized in Table 5.1 and photographs appear in Figs 5.1 and 5.2. Individual flukes enter the liver by crossing the Glisson capsule in the visceral or

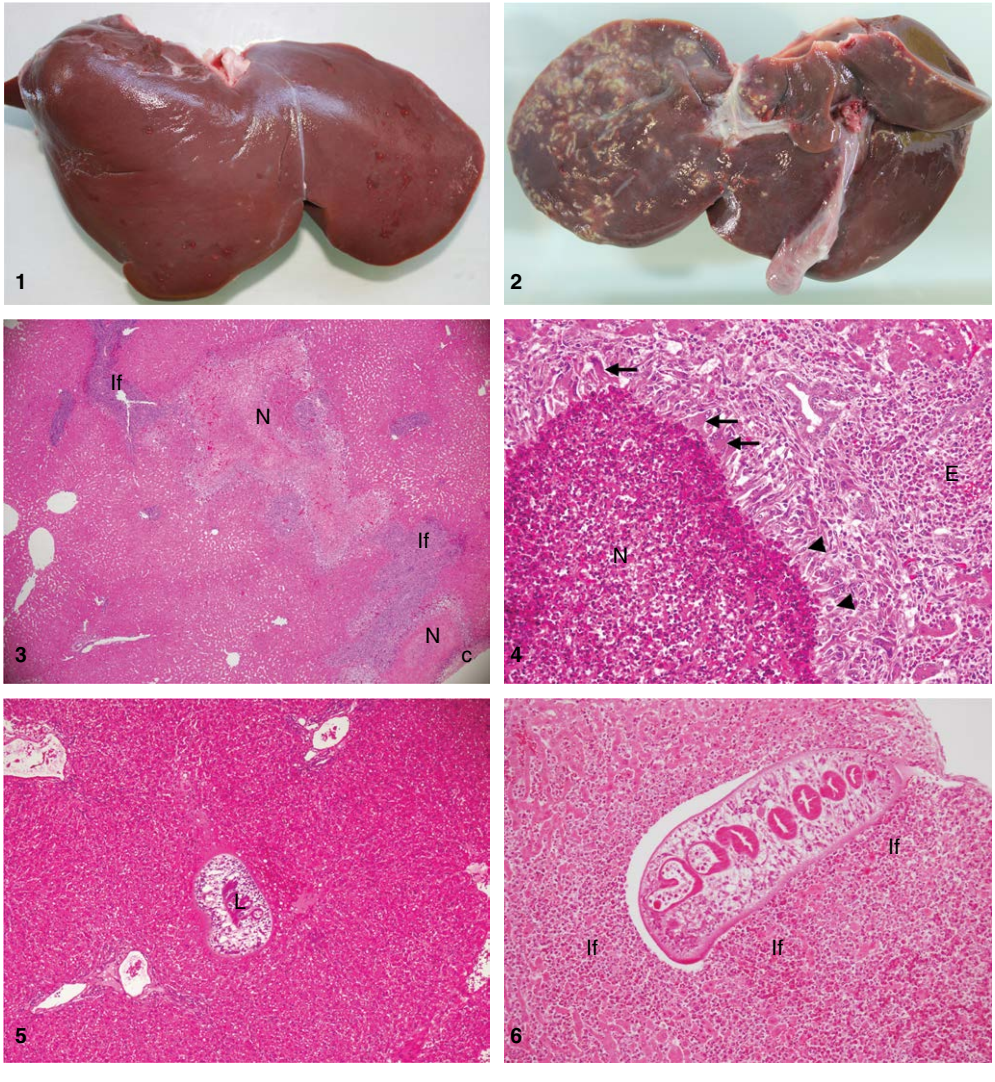

Fig. 5.1. Liver and liver histology showing migratory stages. **(1)** Liver of goat, 8 days post-infection (pi). Red and white spots and whitish short tortuous tracts surrounded by reddish halo involving mainly the left lobe. **(2)** Liver of sheep, 18 days pi. Yellowish-greyish tortuous tracts affecting mainly the left lobe. **(3)** Goat, 8 days pi. Necrotic tract (N) starting in the Glisson capsule (c); note the presence of inflammatory infiltrate (If) in the older areas of the tract while the recent areas show scarce inflammatory infiltrate. H&E, ×25. **(4)** Sheep, 18 days pi. Granuloma with necrotic centre (N) surrounded by a palisade of macrophages (arrowheads) and multinucleate giant cells (arrow) and externally by eosinophils (E). H&E, ×200. **(5)** Sheep, 18 days pi. Migrating larva (L) surrounded by normal hepatic parenchyma with absence of inflammatory reaction or tissue damage. H&E, ×100. **(6)** Sheep, 18 days pi. Migrating larva located in the hepatic parenchyma below the hepatic capsule. The larva is surrounded by abundant inflammatory infiltrate (If) mainly composed of eosinophils. H&E, ×100. H&E, haematoxylin and eosin staining.

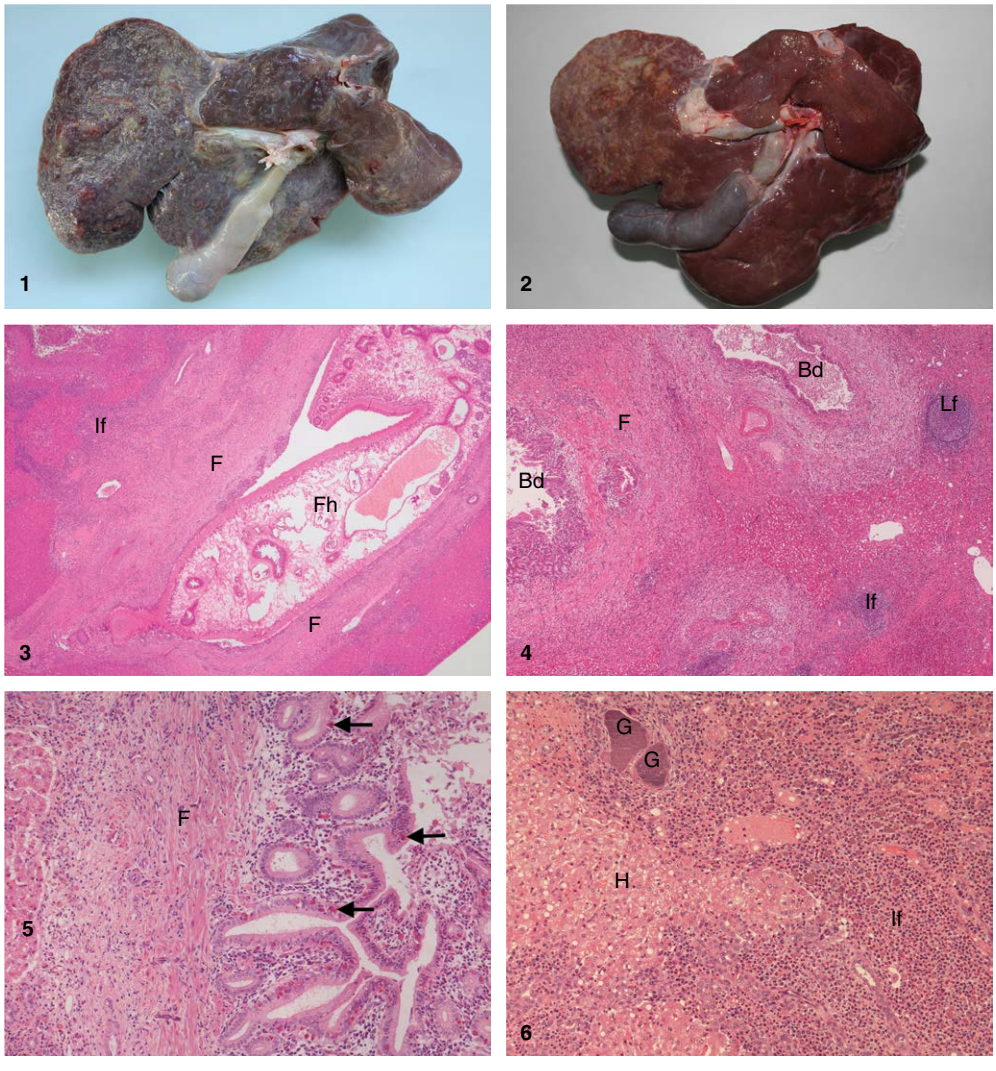

Fig. 5.2. Liver and liver histology showing biliary stages. **(1)** Liver of goat infected with 200 metacercariae (mc), 16 weeks post-infection (pi). Numerous yellowish-greyish scars involving almost all the visceral surface, gallbladder and large bile ducts are enlarged and whitish. **(2)** Liver of sheep infected with 200 mc, 16 weeks pi. Yellowish scars and patches mainly involving the left lobe show atrophy while the right and quadrate lobes are less severely affected and undergo compensatory hypertrophy. **(3)** Sheep, 16 weeks pi. Mature fluke (Fh) within an enlarged bile duct surrounded by marked fibrosis (F) and scarce inflammatory infiltrate (If). H&E, ×25. **(4)** Sheep, 16 weeks pi. Hyperplasic bile ducts (Bd) surrounded by marked fibrosis (F) and inflammatory infiltrate (If) associated with lymphoid follicle (Lf). H&E, ×25. **(5)** Sheep, 16 weeks pi. Hyperplasic bile ducts showing intraepithelial globule leukocytes (arrows) and surrounded by fibrosis (F). H&E, ×100. **(6)** Sheep, 16 wpi. Granulomata (G) with necrotic centres surrounded by macrophages and some multinucleate giant cells, severe inflammatory infiltrate (If) is replacing part of the hepatic parenchyma (H). H&E, ×100. H&E, haematoxylin and eosin staining.

parietal aspect of the liver, with marked predilection for the left lobe.

5.1.4 Other host species

5.1.4.1 Cattle

The course of infection in cattle follows a broadly similar pattern to sheep (Dow *et al.*, 1967). While less severe in adult cattle, calves exposed to > 1000 metacercariae may develop clinical fasciolosis. The disease is characterized by weight loss, anaemia, hypoproteinaemia and, after infection with 10,000 metacercariae, death (Boray, 1969). Immunity develops with age so that adult cattle are more resistant to infection than young cattle. Infected younger animals suffer lower carcass weights and quality than uninfected animals, with the highest difference observed in the 23–30 months age range (da Costa *et al.*, 2019). For *F. gigantica* infection, susceptibility differs with host species, with cattle more susceptible to infection and clinical signs than swamp buffalo (Molina *et al.*, 2005), while Boran cattle are more susceptible than Friesian cattle (Wamae *et al.*, 1998).

There is considerable variation in both infection rates and the severity of disease between individual animals. In the migratory phase, fibrosis tends to be more common in cattle compared with sheep and in severe infections hepatic fibrosis can progress to cirrhosis (Marcos *et al.*, 2007). Fibrotic liver tissue in *F. hepatica*-infected cattle is composed of spindle-shaped myofibroblasts in the peribiliary connective tissues and is positive for vimentin, desmin and α-smooth muscle actin (α-SMA) (Golbar *et al.*, 2013). In severely fibrotic livers, portal branches of bile ducts are thinned or completely terminated. Fine and irregular newly formed bile ducts, not parallel with portal branches, appear (Shirai *et al.*, 2006). Flukes are concentrated in the ventral portion of the liver and tracks are less distinct than in sheep. Many flukes become trapped in the parenchyma and following high-level infections only around 5% of the inoculum reaches the bile ducts (Ross *et al.*, 1966).

Surviving flukes confront a hostile inflammatory reaction. The bile ducts are thickened due to epithelial hypertrophy and subsequent fibrosis of the walls of the duct. As a result, the ducts enlarge up to 3 cm in diameter and become prominent on the surface of the liver. Calcium deposits start to form in the duct walls after 16–20 weeks of infection, leading to the name 'clay pipe' liver. The lumens of the ducts are variously dilated and stenosed and the epithelium shows ulceration and haemorrhage. Due to the host reaction, few flukes reach the bile duct; most flukes are lost by 30–50 weeks after infection and few eggs are passed.

5.1.4.2 Buffalo

Fasciolosis, caused by *F. gigantica*, presents major disease and production problems, particularly in the rice-producing areas of South and South-East Asia (Mahato and Harrison, 2005; Diyana *et al.*, 2020). Twelve- to 15-month-old Murrah-breed buffalo experimentally infected with 1000 metacercariae revealed clinical signs typical of acute fasciolosis, such as apyrexic inappetence, anaemia, poor weight gain, diarrhoea and sub-mandibular and facial oedema, from week 6 to week 17 pi; and at necropsy, hyperplasia of bile duct was the main hepatic lesion (Yadav *et al.*, 1999). In natural infections, low egg burden occurred in animals with no clinical signs and no hepatic biliary changes while high egg burden was correlated with evident clinical symptoms and hepatic and biliary changes (El Damaty *et al.*, 2018). An unusual lesion reported in *F. hepatica*-infected buffalo is glomerulonephritis due to circulating immune complexes (Marques *et al.*, 2004). Aberrant migration of *F. gigantica* in lung and spleen has also been reported in buffalo (Choudary, 1979).

5.1.4.3 Humans

Humans are not a natural host and few flukes develop sufficiently to reach the bile duct, so human disease presents quite differently to fasciolosis in sheep. The presentation of human disease shows marked heterogeneity, depending on the severity of infection and age of host, among other factors (Mas-Coma *et al.*, 2009), so that clinical disease can vary from asymptomatic to a severe, debilitating disease. The migratory phase between 6 and 12 weeks from metacercariae ingestion can cause an acute clinical disease characterized by very high fever, right upper-quadrant

abdominal pain, hepatomegaly and, occasionally, jaundice. A marked peripheral eosinophilia is characteristic of this stage. In addition, some patients show myalgias, urticarial rash, nausea, anorexia and diarrhoea. These symptoms are attributed to the flukes migrating through the liver parenchyma and stimulating inflammatory and immune responses (Mas-Coma *et al.*, 2019). Early extrahepatic manifestations include type III (immune complex deposition) or type IV (IgE) hypersensitivity-mediated responses, causing pneumonitis, vasculitis and myocarditis.

Chronic clinical disease coincides with the biliary-stage adult flukes causing inflammation and hyperplasia of the epithelium that results in cholangitis and cholecystitis, which, combined with the large body of the flukes, is sufficient to cause bile duct obstruction. This phase includes biliary colic, epigastric pain, fatty food intolerance, nausea, jaundice, pruritus and right upper-quadrant abdominal tenderness, among others (Mas-Coma *et al.*, 2019). Prolonged severe infections may lead to chronic hepatobiliary damage with chronic biliary cirrhosis, sclerosing cholangitis and even cholangiocarcinoma (Kim *et al.*, 2005). Liver abscesses (1–30 mm), often containing eosinophils, are also a common finding. Peripheral eosinophilia is not common during the chronic stage.

The presence of ectopic flukes has been reported to cause a variety of lesions in the dorsal spine, lung, heart, blood vessels, skin, brain and eye, leading to a variety of clinical manifestations such as dermatological, neurological and ophthalmological disorders (Mas-Coma *et al.*, 2014; Taghipour *et al.*, 2019).

5.1.4.4 Rabbits

Rabbits have been used as experimental models of fasciolosis and can tolerate infections of up to 75 metacercariae. The liver pathology has been described by Han *et al.* (1999). The migratory phase is characterized by tortuous cavitary tracts that histologically were composed of coagulative necrosis surrounded by a heavy core of eosinophils, leading to eosinophilic granulomas with necrotic centres. Flukes start entering bile ducts at 5 weeks pi, leading to a biliary phase characterized by papillary bile duct hyperplasia and portal fibrosis. Granulomas with necrotic centres surrounded by eosinophils, macrophages and multinucleate giant cells are a common feature in chronic fasciolosis of rabbits (Han *et al.*, 1999).

5.1.4.5 Rats

Thorpe (1967) described the pathology of fasciolosis in rats. Juveniles reach the body cavity within 24–48 h and flukes enter the bile duct at between 4 and 6 weeks. Although the migratory phase is abbreviated, the pathology is similar to that of sheep with necrotic and/or haemorrhagic tracts, granulomas with necrotic centre and cores of macrophages and eosinophils. In the biliary stage, adult flukes reside in the common bile duct, which can reach 20 times its normal size due to the absence of a gallbladder (Isseroff *et al.*, 1977a). Rats develop partial resistance to *F. hepatica* reinfection, which has been attributed to the local response of eosinophils at the intestinal level (Doy *et al.*, 1978). The rat model is considered a useful approach for the immunopathological research of the human chronic fasciolois, as the rat's resistance level, susceptibility and pathology closely mimic chronic disease in humans (Valero *et al.*, 2017).

5.1.4.6 Mice

Although flukes appear to undergo a similar migration in mice as in sheep, the small size of the mouse liver both speeds up and exacerbates the pathology. Juveniles reach the body cavity in under 24 h and first start to appear in the bile duct after 24–30 days (Dawes, 1961). Gross hepatic lesions in mice infected with *F. hepatica* consist of focal sub-capsular whitish areas ranging from 1–5 mm long and 1–3 mm wide, which microscopically are identified as migratory tracts with necrotic areas and inflammatory infiltrate, mainly composed of neutrophils (de Paula *et al.*, 2010). Because of the small size of the mouse liver, even a single fluke may kill the animal due to liver trauma and abdominal haemorrhage during the migratory stage. In such cases flukes can be found in the abdominal cavity at post-mortem. Flukes in the biliary ducts induce duct hyperplasia accompanied by enlargement of the bile duct walls and periportal fibrosis (Masake *et al.*, 1978; de Paula *et al.*, 2010). These flukes can cause ulcers but haemorrhage into the duct does not occur. Feeding

physiology of flukes in the bile duct of mice also appears to differ from the situation in sheep. As a model host, the mouse may be useful to study host–parasite interactions, but it may not be the ideal model for vaccine testing.

5.2 Clinical Aspects

5.2.1 Introduction

Because *F. hepatica* and *F. gigantica* parasites infect a wide range of definitive hosts and the two species may be present separately, together or as hybrids, the clinical presentation is varied. Furthermore, the severity of disease depends on the level of infection, the infective species, the stage of the infections, the nutritional plane of the animals and between animals in a group. Fasciolosis causes significant loss of production especially in grazing ruminants, mainly through reduced weight gain. This section focuses on clinical aspects of disease in production animals. Parallels with human infection with *F. gigantica* have been reported by Kamel *et al.* (2015).

The clinical presentation of acute fasciolosis in sheep and goats can appear as sudden death of stock, often with no warning. These outbreaks may be traced to access to snail habitats in the previous weeks to months, when animals consumed many metacercariae over a short period. Subacute disease, which is slightly more protracted than the acute disease, presents as haemorrhagic anaemia. These forms of acute fasciolosis can be serious and death of up to 25% of a flock is possible. Chronic disease is caused by biliary stages of fluke and is accompanied by anorexia, weight loss, pallor of mucous membranes, ventral oedema and wool break. Sheep die with clinical signs as well as the presence of typical eggs in faeces.

The disease in ovines may also follow grazing fluke-prone areas with long-term intake of metacercariae infection so that parenchymal and biliary stages are present simultaneously along with their clinical effects. In contrast, bovines are inherently more resistant to fluke infection and generally suffer less severe disease. Calves and young buffalo exposed to heavy infections may suffer from acute fasciolosis and death (Sharma *et al.*, 2011) and chronically infected adult cattle can develop anaemia and long-term weight loss. In all host species, fasciolosis may exacerbate disease caused by gastrointestinal parasites such ovine haemonchosis and bovine ostertagiosis. In addition to the direct effects on liver function, fasciolosis can impact several other physiological functions of the host. Impacts on fertility and calcium homeostasis have been reported, most likely as secondary effects of low nutrient intake, blood loss and oxidative stress.

Fluke infection limits herd productivity. Charlier *et al.* (2014) reviewed research on the impact of fasciolosis on a range of production parameters in cattle herds. Given the variability in natural infection rates, the degree of pathology and the types of production systems, impacts on production are variable and the following are examples. Reduced weight gains are in the order of 6–9% and mean carcass weights are 0.7% lower. Negative impacts on carcass composition and value have also been reported. Milk yield can be reduced by between 0.7 and 2 kg/cow/day. Impacts on reproduction are also important, with a reported delay to first oestrus of 39 days and an increased inter-calving interval of 4.7 days.

Fasciolosis caused by *F. gigantica* in buffalo presents as a similar disease as *F. hepatica* in cattle, with some differences. The life cycle of *F. gigantica* is longer than *F. hepatica*, with entry into the bile ducts occurring at around 12 weeks pi (compared with 6–8 weeks) and achieving patency at 13–16 weeks (compared with 10–12 weeks) (Sharma *et al.*, 2011). *F. gigantica* flukes are also larger and, 'fluke for fluke', appear to cause more severe clinical disease than *F. hepatica* (Sharma *et al.*, 2011). For example, buffalo calves suffer a potentially fatal disease, and it has been reported that their feed conversion efficiency falls from 33.4 to 9.6 intake/gain during infection. Hallmarks of *F. gigantica* infection in buffalo include inappetence, anaemia and elevated serum enzyme profiles that parallel those in cattle (Sharma *et al.*, 2011).

5.2.2 Effects on blood components

5.2.2.1 Anaemia

Haemorrhagic anaemia is the most important factor contributing to host morbidity and

mortality in fluke infections. Subclinical infection is associated with lower packed cell volume and haemoglobin of the order of 20% (Sykes *et al.*, 1980). Except in peracute disease where haemorrhage is life-threatening, the blood loss is gradual enough for the animal to adapt and so the anaemia is commonly normochromic and normocytic. Erythropoiesis is increased, leading to a mild reticulocytosis, but is limited in the later stages of the infection when limits of the availability of dietary iron and protein increase. Anaemia is exacerbated in animals on poor-quality diets or suffering anorexia due to additional iron and protein limitations (Sinclair, 1965; Berry and Dargie, 1976; Dargie, 1981). Blood loss is primarily due to blood feeding by the flukes and haemorrhage into the parenchymal tracts, the bile duct and the abdominal cavity as a result of trauma and inflammation caused by flukes. It has been estimated that blood is lost at the rate of 0.2–0.5 ml/day per fluke (Dawes and Hughes, 1964; Jennings, 1976). In addition, considerable amounts of iron are lost and not reabsorbed (see Dargie and Mulligan, 1970). Buffalo that have been artificially infected with *F. gigantica* show anaemia, reduced PCV and reticulocytosis from week 7 of infection (Sharma *et al.*, 2011).

5.2.2.2 Plasma proteins

Hypoalbuminaemia and hyperglobulinaemia commonly occur in liver fluke infections in all host species. Sheep with subclinical infection showed a decline in plasma albumin from mean values of 32 g/l in controls to 20 g/l, a fall of around 35%. Protein components of plasma are lost with blood but are partially reabsorbed after digestion in the intestine. An expansion of the plasma volume following blood loss also contributes to the fall in protein concentrations (Anderson *et al.*, 1977; Dargie, 1981; Symons, 1989).

The liver is the only site of synthesis of serum albumin, so there is a combined effect of loss through bleeding and reduced replacement through diminished liver function. This is especially true during the biliary stage of the infection where the synthetic capacity of the liver to replace the lost albumin is overwhelmed. In endemic situations fresh infection of the host with metacercariae may further damage the liver, further compromising synthetic capacity. Thus, a progressive loss of plasma albumin occurs in all infected host species but is worse in sheep and less critical in cattle (Anderson *et al.*, 1977). At the extreme, infected sheep on a low-protein diet increased the rate of catabolism of intravascular albumin about threefold and at the same time the plasma half-life of albumin decreased from 500–600 h to below 300 h (Dargie, 1981). The diversion of amino acids to albumin and haemoglobin synthesis compromises the availability of amino acids for protein synthesis in muscle and the body generally, as reflected in loss of body weight and propensity for wool break, and can account for production loss in this species.

Increased immunoglobulin synthesis commences within several weeks of infection and elevated immunoglobulins, including IgM, IgG1, IgG2 and IgE, persist throughout the infection (for examples, see Sykes *et al.*, 1980; Dargie, 1981; Hughes *et al.*, 1981; Jemli *et al.*, 1993). For example, globulin concentrations in sheep doubled from 30 g/l to 60 g/l with subclinical infection (Sykes *et al.*, 1980). Immunoglobulins are produced in response to infection and synthesized by leucocytes at a variety of sites in the body, so this source of plasma proteins is independent of liver pathology.

5.2.2.3 Hepatic enzymes in blood

Parenchymal infection causes death of hepatocytes and this is associated with the release of constituent hepatic enzymes into the blood. Elevated profiles of these enzymes are used to diagnose infections and judge the severity of infection. While elevation occurs for a range of enzymes, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) are the most commonly used clinical markers of parenchymal infection across species. The appearance in the plasma of γ-glutamyl transferase (GGT), an enzyme present in the bile duct epithelium, is an indicator of damage to the bile ducts and is diagnostic for the biliary stage of the disease. Anderson *et al.* (1981) reported that GGT levels in infected calves peaked at a level more than twice that of controls (95 versus 242 IU/l) at 14 weeks of infection. In *F. hepatica*-infected sheep, levels of enzymes fell within 28 days after successful treatment, demonstrating the capacity for liver repair after infection has been eliminated (Kozat and Denizhan, 2010).

5.2.2.4 Leucocyte populations

Parenchymal infection with *F. hepatica* leads to dramatic peripheral blood eosinophilia, which appears soon after infection and peaks late in the parenchymal stage. Eosinophilia persists through the biliary stage and is common across host species. For example, it is marked (approximately 4 times the level in uninfected controls) in riverine buffalo infected with *F. gigantica* (Ganga *et al.,* 2007). Lymphocytes and neutrophils, and occasionally monocytes and basophils, have been observed to increase but the changes are inconsistent.

5.2.2.5 Other blood components

Although the mature flukes occupy the bile ducts and might be expected to interfere with bile production and flow, the occurrence of bilirubinaemia during infection has been reported only rarely (Prache and Galtier, 1990; Ferre *et al.*, 1995).

Levels of high-, low- and very low-density lipoproteins in sheep infected with *F. hepatica* were approximately half those in uninfected controls (Kozat and Denizhan, 2010). These changes are thought to reflect a compromised synthetic capacity for lipoproteins by infected livers. Interestingly, the levels had not returned to the control levels 56 days after flukicidal treatment, which suggests that this synthetic deficiency persists. In sheep infected with *F. hepatica*, glucose levels are lower than in controls (Phiri *et al.*, 2007; Kozat and Denizhat, 2010) with a coincident increased concentration of β-hydroxybutyrate (Phiri *et al.*, 2007).

Recent research across a range of host species infected with *F. hepatica* and those infected with *F. gigantica* has revealed that endocrine responses are detectable in peripheral blood. For example, in *F. gigantica*-infected riverine buffalo, T_3 and T_4 serum concentrations were significantly reduced from week 14 and serum cortisol was also elevated about twofold compared with controls, with a peak reading at 10 weeks of infection (Ganga *et al.*, 2007). Rams chronically infected with *F. gigantica* have reduced serum testosterone (Iliyasu *et al.*, 2014).

Ascorbic acid in the plasma declines steadily throughout infection in sheep (Gameel, 1982a; Kouider and Kolb, 1994) and may indicate oxidative stress in the tissues. Oxidative stress is a common feature of fasciolosis in several species. While the liver is the main focus for this pathology, reactive oxygen species (ROS) and enzymes involved in free-radical generation and radical scavenging spill into serum as markers of oxidative stress.

5.3 Pathophysiology and Metabolic Aspects

5.3.1 Introduction

The liver plays a central role in the physiology of the body, being responsible for a large proportion of the body's amino acid metabolism, for carbohydrate and lipid balance, urea synthesis, detoxification, ketogenesis, albumin and glutathione synthesis as well as aspects of homeostasis. It is not surprising, therefore, that many systemic changes are induced by liver fluke infections, which ultimately cause reduced productivity in livestock. While the changes recorded below vary with stage and intensity of infection, most have been recorded even for subclinical infections involving relatively few flukes (e.g. two or more mature flukes for rats, around 20 mature flukes for sheep).

Because fasciolosis causes disruption of multiple liver functions, the pathophysiology may appear in other body systems indirectly. Some impacts include gastrointestinal effects such as on food intake and ascites (ventral oedema) due to protein loss. Endocrine disruption may be linked to stress or nutrient deficiency. Other impacts are a result of a polarized immune system, which may increase susceptibility to other diseases. New technologies such as transcription analysis, metabolomics and proteomics have been used to describe molecular events in *Fasciola*-infected animals, including in liver and other tissues. The nature of these technologies means that they can provide quantitative data that are unbiased.

In natural infections the impact on productivity is variable, due to host, parasite and environmental factors. For example, adult sheep with 250–350 mature flukes progressively lose weight, starting a few weeks after infection. In young sheep 40–60 adult flukes will reduce the weight gain and wool growth starting within a few weeks of infection but the effect is most prominent during the biliary stage (Hawkins

and Morris, 1978). Studies of weight gain and productivity in cattle with subclinical infections (up to 200 mature flukes) have shown variable outcomes (Charlier *et al.*, 2014).

5.3.1.1 Weight gain, food intake and nitrogen balance

In chronic fasciolosis, reduced weight gain has two apparent causes: sheep with fluke burdens < 200 have reduced feed efficiency, while in sheep with higher fluke burdens anorexia is an additional, consistent and important feature (for reviews, see Dargie, 1987; Symons, 1989). Following infection of buffalo calves with 1000 *F. gigantica* metacercariae, anorexia commences during the parenchymal stage (6 weeks pi) and continues into the biliary phase of infection (Mehra *et al.*, 1999). What causes this reduced appetite is not known. Interestingly, proteomic analysis (Rioux *et al.*, 2008) on plasma of infected mice described increased concentrations of apolipoprotein (Apo A-IV) compared with controls. This lipoprotein is associated with appetite in rats but a mechanism in anorexia associated with fasciolosis has not been explored.

Nitrogen is a limiting element in terms of building and retaining muscle mass and is central to reduced feed efficiency. In heavy infections poor-quality diets and anorexia limit the intake of nitrogen, compounding the nitrogen depletion that has occurred through loss of blood and urinary urea. The most obvious impact of low nitrogen availability is hypoalbuminaemia. Together with the reduced capacity for the liver to produce amino acids and the lack of nitrogen, this may elicit scavenging of nitrogen through muscle catabolism and lead to wasting and, in time, emaciation. In contrast, nitrogen does not appear to be limiting in subclinical multiple-dose infections of sheep, where no differences in nitrogen balance were observed compared with pair-fed controls and there were no changes in plasma urea (Sykes *et al.*, 1980).

5.3.2 Organ function

5.3.2.1 Hepatic function

Liver tissue has remarkable functional redundancy and, unlike most other organs in mammals, is able to regenerate functional tissue after physical or chemical injury. It has been estimated that 60–70% of liver tissue can be lost before significant dysfunction becomes apparent (Jubb and Kennedy, 1970), though this probably depends on what aspect of liver function is examined.

5.3.2.2 Bile flow and composition

It might be expected that the flow and composition of bile would be altered during liver fluke residence in the ducts, but reports show no consistent pattern. In cattle the rate of excretion of iron in the bile increased markedly after week 8 of infection, presumably as a consequence of haemorrhage into the bile. A study by Isseroff *et al.* (1972) showed increases of amino acid levels of the bile in cattle, rabbits and rats during the biliary stage of infection. While much of this could be due to amino acids derived from blood, possible excretion of proline by the flukes could contribute to elevated proline in the bile.

5.3.2.3 Endocrine and reproductive effects

Research reports across a range of host species and in animals infected with *F. hepatica* or *F. gigantica* have revealed effects on endocrine responses. For example, reduced T_3 and T_4 plasma concentrations in *F. gigantica*-infected riverine buffalo were associated with thyroid pathology. In the same animals, serum cortisol was elevated about twofold compared with controls, with a peak reading at 10 weeks of infection. Like the thyroid observations, this was associated with adrenal pathology, presumably in response to stress (Ganga *et al.*, 2007).

Several workers describe declines in reproductive hormones and compromised reproductive function. For example, lower testosterone in infected rams compared with controls has been associated with reduced semen motility and infertility (Iliyasu *et al.*, 2014). Ewes that were oestrus-synchronized and joined during the parenchymal stage of an *F. gigantica* infection had a lower rate of oestrus than controls (66% versus 100%). It was proposed that early embryonic death had occurred in these sheep (Chiezey *et al.*, 2013). Mechanisms for these effects on reproduction have not been investigated but appear to have a systemic pathogenesis.

5.3.3 Effects on metabolism

5.3.3.1 Redox status

Reactive oxygen species (ROS) increase in liver and serum during infection, with a concomitant decline in catalase and glutathione transferase (GST) in cattle (Silva *et al.*, 2017). The imbalance between enhanced generation of ROS due to the catalytic action of fluke secretions on hepatocytes and reduced scavenging enzymes leads to persistence of ROS. This has been shown in cattle and rats (Bottari *et al.*, 2015; Silva *et al.*, 2017).

Kamel *et al.* (2015) investigated oxidative stress in 20 human cases of fasciolosis compared with controls. Increased lipid peroxidation and decreased antioxidant enzymes (e.g. superoxide dismutase and glutathione peroxidase) in erythrocytes were associated with chronic disease, confirming that the patients were in oxidative stress.

5.3.3.2 Mitochondrial bioenergetic metabolism

Van den Bossche *et al.* (1980, 1983) and Rule *et al.* (1989) reported that in mitochondria prepared from infected rat livers, electron transport was not coupled to ATP synthesis. Such effects persisted up to 21 weeks pi with a fluke burden of greater than two. It is likely that uncoupling limits availability of ATP to maintain cellular functions in the liver *in vivo*. Possible mechanisms of uncoupling are through mitochondrial membrane derangements associated with high levels of ('free') fatty acids and a decline in the phospholipid composition in mitochondrial membranes (Lenton *et al.*, 1995). In contrast, the attenuation of tissue respiration in infected sheep was attributed to direct damage to the electron transport chain (Lenton *et al.*, 1996).

5.3.3.3 Carbohydrate metabolism

Two important metabolic functions of the liver are the regulation of the concentration of blood glucose, which derives from hepatic conversion of three-carbon precursors, and secondly, the conversion of two-carbon units into ketone bodies such as acetoacetate and β-hydroxybutyrate, which are energy substrates for tissues, including the brain. Blood glucose concentrations in infected animals are either in the normal range (Rowlands and Clampitt, 1979) or lowered. For example, glucose levels in serum in sheep during the biliary phase were about 4.3 and 5.3 mM in infected sheep and controls, respectively (Ferre *et al.*, 1994). Other reports describe significant reductions in glucose (Phiri *et al.*, 2007) and Kozat and Denzihan (2010) reported that glucose levels in infected sheep were 62% of control levels.

The glycogen content in livers of rats is significantly lower in infected animals than in controls in pair-fed comparisons (Gameel, 1982b). These data are supported by rat liver perfusion experiments (Hanisch *et al.*, 1991) where both hormone-dependent release of glucose from livers (20–60% of controls) and gluconeogenesis (50% of controls) were severely attenuated throughout infection for up to 21 weeks. In contrast, a summary of biochemical data (Kokova and Mayboroda, 2019) indicated that glycogen levels are elevated in infected livers. The report of lower glucose levels associated with higher levels of β-hydroxybutyrate (Phiri *et al.*, 2007) in blood of infected sheep supports the notion of metabolic impairment in the liver and activation of β-hydroxybutyrate synthesis to meet energy needs.

5.3.3.4 Protein metabolism

Classical techniques of analysis of microsomal and cytosolic protein metabolism and catabolism in rats in the early biliary stage (Biro-Sauveur *et al.*, 1994, 1995) provide equivocal evidence for changes in protein synthesis and turnover. Similarly, in sheep infected with 1000 metacercariae, the relative rate of albumin synthesis declined during the parenchymal stage but increased during the biliary stage in comparison with pair-fed controls (Dargie, 1981). These reports probably reflect the capacity of the liver to respond at the different phases of infection.

While such traditional methods have been used to study metabolism, modern '-omics' approaches that study transcriptomes, proteomes and metabolomes offer global views of transcripts, proteins and biochemicals, respectively (see Chapter 11, this volume). They have been used to compare changes in host responses during infection in a range of tissues and samples. Statistically different levels of agents between infected and uninfected animals are generally reported as 'fold' changes termed up- or downregulation that

achieve significant differences (Alvarez Rojas *et al.*, 2015; Fu *et al.*, 2017; Garcia-Campos *et al.*, 2019) (see Chapter 11 of this volume). The high number of changes across different tissues show profound effects of parasite-induced damage and host responses at the tissue level. However, a vast array of proteins are synthesized during fasciolosis. This is evidenced by proteomic and transcriptomic studies where thousands of proteins and transcripts have been detected and hundreds of those are up- or downregulated. Some of these relate to immune or inflammatory responses (Cwiklinski and Dalton, 2018) (see sections 5.4 and 5.5, below). Others indicate changes in metabolic pathways and tissue repair. For example, reports of increased levels of amino acids in various tissues (spleen and liver) and fluids (plasma) (Kokova and Mayboroda, 2019) suggest high rates of amino acid flux and point to significantly higher protein catabolism and synthesis.

Using proteomic analysis, Ruiz-Campillo *et al.* (2017) examined peritoneal fluid samples in early infections of sheep (18 days pi) identifying 176 peritoneal proteins and also confirmed the cell locations of some by immunohistochemistry. Using transcript analysis Rojas-Caraballo *et al.* (2015) detected 128 significantly upregulated genes during early-stage infection (7 days pi) and 308 at late stage (21 days pi) in livers of infected mice. Their important findings were confirmed with real-time polymerase chain reaction. Many upregulated genes coded for representatives of signalling pathways that control biochemical pathways. In fasciolosis some proteins were elevated as a result in hepatocellular damage and response, including fibrin and fibrinogen, which are substrates of *Fasciola* proteases (Mebius *et al.*, 2018), and a range of serum markers of disease such as the liver enzymes that indicate hepatocyte damage. The signalling protein serpin, which has an association with pathways for hepatic fibrosis required for liver repair, is also upregulated (Rojas-Caraballo *et al.*, 2015). Proteins associated with the extracellular matrix of liver (collagen VI, fibronectin, fibrocystin with fold changes of about 5.8) are also represented. Periostin and vascular cell adhesion protein 1 (fold change of 3), which are linked to inflammatory disorders and wound repair, were upregulated and localized to inflammatory cells, probably dendritic cells, in the liver parenchyma (Ruiz-Campillo *et al.*, 2017). Coagulation pathways (Rojas-Caraballo *et al.*, 2015) and haemoglobin-related genes (Alvarez Rojas *et al.*, 2015) are upregulated as required to replace erythrocyte components lost through haemorrhage. Proteomics studies, verified by Western blot, describe upregulated transferrin in serum from sheep at week 9 of infection. Transferrin is associated with anaemia and iron deficiency and is likely to be a response to haemorrhagic anaemia, although its role is complex (Rioux *et al.*, 2008).

5.3.3.5 *Lipid metabolism*

Although no studies have directly addressed the synthesis and turnover of lipids and phospholipids in fluke-infected livers, the concentrations of both the phospholipid and total lipid components of hepatic homogenates decline dramatically in infected rats commencing, respectively, in the second and fourth week pi (Maffei Facino *et al.*, 1990, 1993; Lenton *et al.*, 1995). Consistent with this is a decline in phospholipids and an increase in their degradation products or precursors in both the mitochondrial and microsomal fractions. Increases in the concentration of malondialdehyde in homogenates and in concentrations of conjugated dienes in microsomal preparations from rat liver (Maffei Facino *et al.*, 1990, 1993) are evidence for peroxidative damage to hepatic lipids. The consequences of decreased phospholipid content may be profound and explain many of the functional changes, such as respiratory chain aberrations, increased permeability of hepatocyte membranes and reduced cytochrome P450-associated activities (see below). Metabolomic studies describe increased phosphocholine in liver (Kokova and Mayboroda, 2019) which may result from cell damage or reflect the role of phosphocholine in tissue repair and cell proliferation. Upregulation of apolipoprotein A-IV found in proteomic studies in infected mice (Rioux *et al.*, 2008) also suggests an elevation of lipid transport molecules that may be linked to tissue repair.

5.3.3.6 *Steroid metabolism*

The liver is the principal site of steroid catabolism by the mixed-function oxidase system and so liver insults may be expected to impact steroids. The rate of clearance of exogenous testosterone

was reduced in post-pubertal rams infected with mature *F. hepatica* compared with controls (Fleming and Fetterer, 1986). Such impaired *in vivo* steroid hormone metabolism has been implicated as a possible factor in the failure of fluke-infected livestock to maintain pregnancy (Biro-Sauveur *et al.*, 1994). Rams infected with *F. gigantica* also have reduced serum testosterone, which is coincident with declines in semen volume, sperm motility and sperm concentration linked to infertility (Iliyasu *et al.*, 2014).

5.4 Pathogenesis

5.4.1 Hepatic pathogenesis

Liver damage in fasciolosis is a complex and multifactorial process associated with traumatic action of the parasite during migration and penetration of bile ducts (Dawes and Hughes, 1964), impact by parasite excretory products on hepatic cells (Wesołowska *et al.*, 2012) and immune response leading to an inflammatory reaction directed both at the parasite and at the damaged host cells and tissues (Pérez *et al.*, 2002; Mendes *et al.*, 2010a). There is evidence that several pathological processes proceed simultaneously. For example, within the liver parenchyma migrating juveniles cause haemorrhagic and necrotic tracts, which attract an immune-induced inflammatory reaction, involving migrating larvae but also necrotic cells of the liver (Zafra *et al.*, 2013a). However, lesions and immune responses can be found in the necrotic tracts and also in areas of the liver where parasites are absent, suggesting that excretory/secretory (ES) products released by the parasite can become trapped in the tissue and attract immune cell infiltration (Molina-Hernandez *et al.*, 2015).

Adult flukes within the bile ducts also exert a combined mechanical and chemical action. While feeding, mainly on blood but also hepatic parenchyma adjacent to bile ducts, flukes cause traumatic damage and focal rupture of bile ducts, allowing some parasite eggs to reach the liver parenchyma, causing severe eosinophilic and granulomatous inflammatory responses (Zafra *et al.*, 2013b). However, some duct lesions precede fluke entry into the bile ducts (Dawes, 1963a), induced by ES products released by the parasite during migration. In support of this, bile duct enlargement can be chemically induced (Lopez *et al.*, 1993) and it has been suggested (Isseroff *et al.*, 1977b) that the amino acid proline, essential for collagen synthesis by fibroblasts and released in large quantities by the parasite, can contribute to this pathology (Modavi and Isseroff, 1984).

Liver damage is broadly correlated with the infective dose, with high infective doses causing more severe lesions as well as more acute and fatal disease. However, different studies carried out in sheep (Pérez *et al.*, 2002) and goats (Martinez-Moreno *et al.*, 1999) have shown that trickle infections, simulating natural exposure (Clery *et al.*, 1996), produce more severe hepatic lesions than a single infective dose of an equivalent number of metacercariae. These findings suggest that, while the mechanical and enzymatic tissue-penetrating action of the parasite may be the initial cause of hepatic damage, it is the ensuing immune or wound-healing response as well as simultaneous infection with different stages of fluke and responses to them that play a major role in the hepatic pathogenesis of fasciolosis (Molina-Hernandez *et al.*, 2015).

5.4.2 Mechanical damage

The lesions within the haemorrhagic migratory tracts caused by juvenile flukes arise from abrasion by the parasite tegument and the action of the suckers as the parasite traverses the liver parenchyma. The spines of the tegument that help to maintain the position of the fluke within the tissues and the mechanical interaction between hepatic cells and the parasite tegument are thought to cause sufficient trauma to lead to cell destruction (Zafra *et al.*, 2013a). The adult causes mechanical damage while blood feeding within the bile duct, leading to erosion of the epithelium and puncturing of small blood vessels. In infections of mice (Dawes, 1963b), cattle (Dow *et al.*, 1967) and sheep (Sinclair, 1967), desquamation and ulceration were observed in bile duct regions adjacent to the spiny bodies and in some cases indentation of spines in the tissue was observed.

The oral sucker is the route by which liver flukes gain most of their nutrition. It appears to cause considerable damage to liver tissue and

macerated hepatic cells have been observed inside the sucker and pharynx (Dawes and Hughes, 1964). Even in young flukes the oral sucker evaginates during migration and feeding activities and the muscular pharynx assists in this process. From the sucker, food in the form of cell debris enters the caeca, where it is digested and what is not absorbed is regurgitated. Observation of postures of fixed flukes within excavated tracks in the parenchyma also suggest that the oral suckers are the major organ involved in tissue disruption (Sukhdeo *et al.*, 1988).

The ventral sucker is a holdfast organ which the fluke uses to maintain position while feeding. Dawes (1963b) observed broken cells in the fluke's ventral sucker in mice and assumed that the fluke contributed to the damage. On the other hand, Sukhdeo *et al.* (1988) described papillae in bile ducts of rabbits and concluded that they were the sites of 'permanent' attachment which allowed the fluke to graze on adjacent ulcerated regions of the epithelium.

5.4.3 Fluke excretory/secretory (ES) products and other secretions

Fasciola, in common with other flukes, has a blind-ending gut, so faeces, as well as a number of secreted components, are shed via the mouth in a tidal fashion: food in, waste out by the same route. Other components may be excreted through the median pore situated at the posterior of the body. The parasite tegument can be considered a secretory organ. It is the primary site of interaction between host and parasite; it is a key element in the survival of adult flukes; and it is metabolically active, being continuously sloughed off and replaced (Jefferies *et al.*, 2001). The tegument is also involved in an array of vital functions, including the synthesis and secretion of substances, absorption of nutrients, osmoregulation and protection against host enzymes. It consists of a syncytial layer surrounding the surface of the parasite and is contained by a plasma membrane covered with a thick glycocalyx.

It has long been held that ES proteins play a dual role in pathogenesis of fasciolosis. They are necessary for fluke migration, assisted by proteolytic tissue damage, and for the evasion or modulation of immune responses (Dawes, 1963a; Howell, 1966). They may also have direct toxic effects on cells and can induce apoptosis in the liver. Several proteases have been recovered from fluke homogenates, regurgitant or culture fluids, and the major proteins in ES products of adult and immature stages of *F. hepatica* were biochemically identified as cysteine proteinases (Dalton and Heffernan, 1989) and cathepsin-like proteinases (Smith *et al.*, 1993). In particular, cathepsin B is the major proteinase family produced by juvenile flukes (Chapman and Mitchell, 1982; Wijffels *et al.*, 1994) and cathepsin L proteinases predominate in adult *F. hepatica* gut contents and ES products (Carmona *et al.*, 1992; Dowd *et al.*, 1994). ES products have been studied using modern proteomic techniques (Jefferies *et al.*, 2001) (see Chapter 11, this volume). This has helped elucidate their identities (Cwiklinski and Dalton, 2018) and the pivotal roles of these protein families in pathogenesis, immune modulation and immunopathology of *Fasciola* infection. Various ES products may be acting in concert or sequentially as pathogenic agents and so their individual effects are difficult to appreciate. While 29 proteins are shared between the NEJ 24 h, immature flukes at 21 days pi (Robinson *et* al., 2009) and adult parasites, a distinct protein profile is secreted by each stage indicative of the different environments each stage occupies in the host (Cwiklinski *et al.,* 2018, 2021) (see Chapter 11, this volume). The major classes of proteins that predominate the secretome of juveniles and of adults (Ryan *et al.,* 2020) along with their functions and influence on disease pathogenesis are summarized in Table 5.2.

The essential functions of proteases and other ES molecules such as penetration and migration through host tissues (McGonigle *et al.*, 2008) and catabolism of host proteins (Robinson *et al.*, 2011) are supported by several lines of evidence. *In vitro,* cathepsins are capable of degrading extracellular matrix and basement membrane components, which is evidence of a role in host tissue invasion *in vivo* (Berasain *et al.*, 1997). For example, i*n vitro* models have shown that those proteases efficiently cleave interstitial matrix proteins such as fibronectin, laminin and native collagen (Berasain *et al.*, 1997). RNA interference knockdown of both cathepsin L and cathepsin B transcripts in NEJ parasites blocked their ability to penetrate and traverse the intestinal wall of a rodent host, suggesting that both cathepsins degrade the intestinal tissue to enable

Table 5.2. A list of excretory/secretory molecules, their actions and how they impact pathways, signalling or expression of immune effectors and intermediaries.

Molecule	Actions	Role in pathogenesis
Cathepsins	Prevent expression of TLR3, precluding macrophage activation	Modulation of (innate) immune response
	Suppression of Th1-Th17 response	Tissue destruction
	Proteolysis of cells	
	Degradation of interstitial matrix proteins	
Fatty acid binding protein	Reduction of pro-inflammatory cytokines in LPS-induced models of sepsis	Modulation of inflammatory response
Cathelicidin-like helminth defence molecule	Inhibits lysosomal acidification and prevents macrophage antigen presentation	Modulation of (innate) immune response
	Inhibits formation of the NLRP3 inflammasome and thus release of IL-1β	Modulation of inflammatory response
	Reduces inflammation in models of multiple sclerosis, type 1 diabetes, and allergic asthma	
Mucin	Increases $CD11b^{+}MHCII^{+}$ macrophages during LPS stimulation	Modulation of immune response
	Increases TLR4 expression in DCs	Modulation of inflammatory response
TGF-like molecule	Inhibits SMAD2/3 signalling and induces a regulatory phenotype in bovine macrophages	Modulation of immune response
Kunitz-type molecule	Decreased inflammatory cytokine secretions in DCs	Modulation of inflammatory response
Glutathione S-transferases	Suppress NF-κB pathway stimulation in macrophages	Modulation of inflammatory response
	Antagonize actions of ROS	
Thioredoxin peroxidase /	Induces Ym-1 expression and arginase activity in murine macrophages	Modulation of immune (innate) response
Peroxiredoxin	Antagonizes actions of ROS	Modulation of inflammatory response
	Induction of AAMF phenotype	

AAMF, alternative activated macrophages; DC, dendritic cells; IL, interleukin; LPS, lipopolysaccharide; MHC, major histocompatibility complex; ROS, reactive oxygen species; TLR, Toll-like receptor.

the rapid penetration of the host intestine (McGonigle *et al.*, 2008). Cathepsin L clades are upregulated in the secretome of fluke stages that migrate in liver tissue, suggesting that particular proteases have specialized roles. Other proteases associated with adults activate cathepsin L (e.g. asparaginyl endopeptidase), tissue degradation and the initiation of blood feeding (prolyl-carboxypeptidase and saposin). The reported degradation of fibrinogen could contribute to a role in the feeding and digestion processes providing peptides and amino acids for fluke nutrition (Dowd *et al.*, 1995).

ES products have also been linked to direct toxic damage of host cells. Histological studies in goats (Martinez-Moreno *et al.*, 1999) and sheep (Pérez *et al.*, 2002) described necrotic lesions not directly related to sites of parasite migration, but to

parasite ES products and inflammatory infiltrate. Examples include degeneration of hepatocytes by hypertrophy of the endoplasmic reticulum, an action that appears to be linked to a reduction in cytochrome P450 activity (Maffei Facino *et al.*, 1993). *In vitro* studies demonstrate that a range of molecules from flukes interfere with function and viability of host cells: in rat hepatocytes, Gajewska *et al.* (2006) described decreased metabolic activity and an alteration of protein synthesis when treated with liver fluke ES products; Wesołowska *et al.* (2012) reported reduced survival rate, increased enzyme leakage and altered synthetic capacity after treatment with parasite somatic proteins. Dose-dependent cytotoxicity caused by ES on human hepatocytes has also been observed (Bąska *et al.*, 2013).

A further proposed role for ES products is the induction of apoptosis in different cell populations such as eosinophils, lymphocytes and macrophages (Serradell *et al.*, 2007; Guasconi *et al.*, 2012; Escamilla *et al.*, 2016a). Apoptosis has been considered a means of causing tissue damage and as an immune evasion mechanism (Escamilla *et al.*, 2016a), since it regulates the innate and adaptive immune responses of the host.

ES products are also associated with increases in the generation of free radicals, ROS and nitric oxide (NO), which are intended as effector molecules to kill the parasite. Piedrafita *et al.* (2007) described an antibody-dependent cell-mediated killing of NEJ involving NO production in the resistance of Indonesian Thin-Tail (ITT) sheep to *F. gigantica*; and transcriptome studies in mice revealed production of ROS as one the most significant pathways associated with immune-protection (Rojas-Caraballo *et al.*, 2017). Free radicals are also associated with oxidative stress and hepatic damage in sheep (Saleh, 2008), rats (Bottari *et al.*, 2015) and cattle (Da Silva *et al.*, 2017).

Many trematode parasites produce and store short non-coding RNA fragments called miRNA in excretory vesicles which are excreted and can be recovered from host plasma. *Fasciola* produces a suite of characterized miRNA, while several miRNAs are shared across metazoans, yet others are *F. hepatica*- and *F. gigantica*-specific (Fromm *et al.*, 2017; Ovchinnikov *et al.*, 2020; Ricafrente *et al.*, 2020) (see Chapter 11, this volume). The roles of miRNAs are unknown, but may include immune regulation, tissue growth and modulation of cancer cells.

5.4.4 Inflammatory responses

A cellular inflammatory reaction is mounted by the host as a result of the mechanical and chemical liver damage caused by the migrating juvenile flukes (Dawes, 1963b; Ross *et al.*, 1966; Dow *et al.*, 1968). Fresh tracts fill with cell debris, erythrocytes, lymphocytes, neutrophils, eosinophils and macrophages and damage to the hepatic cells surrounding the tracts is evident. Older tracts may undergo severe necrosis, which is likely due to the effects of parasite ES products and the effects of an inflammatory infiltrate of macrophages. Fibroblasts also accumulate, forming fibrotic granulation or scar tissue. In heavy or prolonged infections, fibrosis of the liver becomes severe. Fibrosis is more marked in cattle than other hosts and may act to restrict the movement of the flukes.

The exact role of the inflammatory response in the pathogenesis of the infection remains unclear. Some data suggest a contribution in limiting host damage caused by the flukes but there is also evidence that inflammation exacerbates the pathological process. Experiments in sheep (Sinclair, 1968, 1970) showed that treatment of infected sheep with the anti-inflammatory corticosteroid, dexamethasone, allowed a more rapid development of flukes and led to increased physical liver damage. A similar situation occurs in mice and rats. Infected athymic (nude) mice almost invariably die during the parenchymal stage with extensive liver damage (Eriksen, 1980). In rats, flukes recovered from athymic hosts were larger than those from heterozygous littermates (Doy and Hughes, 1982) and gross pathology was less severe (Hanisch *et al.*, 1992). For rats, as with sheep, treatment with anti-inflammatory agents led to increased fluke burdens (Baeza *et al.*, 1994).

More recent studies show that the extensive inflammatory infiltrate that occurs as a response to migrating flukes can also damage hepatic parenchyma. This is either by forming granulomas or by a diffuse infiltration of inflammatory cells that occupy and disrupt wide areas of parenchyma, destroying the normal lobular architecture and

compromising liver function (Martinez-Moreno *et al.*, 1999; Pérez *et al.*, 2002). An inflammatory infiltrate is also present in the severely necrotic tracts vacated by migrating flukes. As these cells are not in close contact with flukes it is assumed they are not involved in parasite destruction (Zafra *et al.*, 2013a).

Because the parasite develops, migrates and induces a range of responses over time, the pathophysiology is dynamic and earlier responses overlap in time with newer responses. The peritoneal migration of juvenile flukes induces a marked inflammatory reaction in the peritoneal cavity associated with the infiltration of leucocyte populations, mainly eosinophils, in the peritoneal fluid (Zafra *et al.*, 2013a; Escamilla *et al.*, 2017; Pérez-Caballero *et al.*, 2018a). Because increases in eosinophils and total peritoneal leucocytes commenced from 9 days pi and were particularly marked at 18 days pi (Escamilla *et al.*, 2017), it is possible that these changes in peritoneal leucocyte populations are also related to hepatic penetration of the liver surface.

The sequence of events in the parenchyma appears to be as follows. In necrotic migrating tracts there is an initial infiltration of eosinophils, neutrophils, macrophages (Mendes *et al.*, 2010b), mast cells (Vukman *et al.*, 2013a,b) and CD4 and CD8 T lymphocytes (Pérez *et al.*, 2002), leading to granuloma formation. In the later stages of the infection the inflammatory response pivots to a chronic proliferative reaction. This is associated with fibrosis, a complex process characterized by inflammatory cell infiltrates and excessive deposition of extracellular matrices. Macrophages play a central role in fibrogenesis via production of fibrogenic factors; the most effective factor is transforming growth factor-β (TGF-β). In sheep, the portal fibrosis has been related to the increase of TGF-β gene expression and the augmented infiltration of FoxP3+ T cells in the hepatic lesions (Pacheco *et al.*, 2018). In cattle, CD68- and CD163-positive macrophages are the main cells involved (Golbar *et al.*, 2013).

There is an intimate connection between the inflammatory response and the immune response in *F. hepatica* infection. The innate immune response determines the cell populations involved in the inflammatory response and, least partially, the activation and function of some specific inflammatory cells (Flynn *et al.*, 2010). Eosinophils are a key cell population in the response, including appearing in the circulatory system and in tissues such as the peritoneum and liver during the initial response to migrating juveniles in sheep (Chauvin *et al.*, 1995), bovines (Bossaert *et al.*, 2000), goats (Pérez *et al.*, 2002) and experimental animals (Tliba *et al.*, 2000). Macrophages are another crucial cell population in both innate and inflammatory responses and their involvement and function determine protective or deleterious effects of the reaction. *F. hepatica* can induce alternatively activated macrophages (AAMF) both *in vitro* and *in vivo* and these are generally characterized as having high arginase activity and low inducible nitrogen oxide synthase (iNOS) levels (Flynn *et al.*, 2010; Ruiz-Campillo *et al.*, 2018). AAMF are involved in priming Th2 cell differentiation and they may act as suppressor-type cells given their high interleukin-10 (IL-10) expression (Flynn and Mulcahy, 2008a). There is a strong correlation between the presence of these cells, with suppressor capacity, and susceptibility during secondary infection (Flynn *et al.*, 2007a). A role for AAMF-derived IL-10 during fibrosis and parasite attrition has been proposed (Haçarız *et al.*, 2009).

Interactions between innate inflammatory cells and migrating NEJs are noteworthy. Peritoneal macrophages and granulocytes of infected sheep showed reduced ability to produce ROS and NO (Pérez-Caballero *et al.*, 2018b). Similar effects occur with peritoneal eosinophils and macrophages of infected rats (Jedlina *et al.*, 2011). These findings support the hypothesis that free oxygen radicals can precipitate hepatic lesions such as necrosis in fasciolosis, particularly during the migratory stage of infection. Useful indicators of oxidative stress that occurs in infected animals are the concentration of reduced glutathione (GSH) in cells and elevated ROS in serum. A study by Maffei Facino *et al.* (1993) in rats reported that the concentration of hepatic GSH declines, but other studies do not show this (Galtier *et al.*, 1991a). A decline in cytosolic hepatic GSH has also been reported in infected lambs (Galtier *et al.*, 1986). Further, in rats the concentrations of products of lipid peroxidation, malondialdehyde and conjugated dienes are elevated. Administration of exogenous GSH to infected rats restored hepatic glutathione levels almost to normal and also normalized the levels of malondialdehyde (Maffei Facino *et al.*, 1993).

Apoptosis of inflammatory cells is another immunopathological aspect of liver fluke infection. It has been reported in *in vitro* studies for rat eosinophils and macrophages (Serradell *et al.*, 2007; Guasconi *et al.*, 2012). *In vivo* studies describe apoptosis in eosinophils from hepatic inflammatory infiltrates both during acute and chronic stages of the infection (Escamilla *et al.*, 2016a) and in peritoneal macrophages, eosinophils and lymphocytes during migratory stage of the disease (Escamilla *et al.*, 2017). Increased proapoptotic gene expression has also been reported in peripheral blood mononuclear cells of *F. hepatica*-infected sheep and cattle (Fu *et al.*, 2017; Garcia-Campos *et al.*, 2019). The increase in the ROS production, particularly hydrogen peroxide, induces mitochondrial injury and also leads to apoptosis of leucocytes by the intrinsic pathway (Serradell *et al.*, 2009). The high percentage of apoptotic eosinophils, macrophages and lymphocytes found during early stages of infection in liver inflammatory infiltrates and in peritoneal leucocytes suggests that apoptosis of effector and/or immune cells may play a role in immune evasion of the parasite during the peritoneal and hepatic stages of the infection (Escamilla *et al.*, 2016a, 2017).

5.5 *Fasciola* and its Host

5.5.1 Immune response

The immune response to *Fasciola* infection in ruminants is complex but has several hallmarks. The first is that there is strong evidence from immunological and molecular studies that the immune response is polarized towards a Th2 response with a suppression of Th1 components. This is reflected in systemic and local responses. Secondly, *F. hepatica* induces potent immune suppression or modulation to an extent that animals are susceptible to further infection with liver flukes (and other pathogens).

The developing Th2-driven immune response and suppressed Th1 response appear to promote an immune regulatory environment that both secures parasite survival and prevents excessive immune-mediated damage to the liver (particularly by containing the damage within the parasite tracts and laying down fibrotic tissue). However, this immune response may not be sufficient to prevent host death resulting from organ failure caused by high-level or repeated infections, especially in sheep (Molina-Hernandez *et al.*, 2015). Infection with *F. gigantica* repeats that general pattern (Molina and Skerratt, 2005; Ingale *et al.*, 2008; Kumar *et al.*, 2013), with a Th2-type immune response evident in the hepatic lymph nodes, spleens and sera of the hosts during chronic infection (Sheng *et al.*, 2019). As an exception, in the Indonesian Thin-tailed sheep, a protective immune response has been described (Hansen *et al.*, 1999), mediated by a mechanism of antibody-dependent cell-mediated cytotoxicity involving superoxide-mediated killing during the peritoneal migration of the juveniles (Piedrafita *et al.*, 2007).

In cattle, during the acute stages of infection, a mixed immune response with elevated IL-10, TGF-β, IL-4 and IFN-γ is observed. As infection progresses, Th2/Treg immune responses become more dominant and, during the latter chronic stages, Treg cells release TGF-β and IL-10 cytokines that inhibit inflammatory Th1/Th2 cytokines (Flynn and Mulcahy, 2008b). This immune profile is similar in infected sheep but as infection progresses the Th2 response is amplified in terms of cytokine production (Pacheco *et al.*, 2017) and enhanced gene expression of Th2 but not Th1 cytokines (Alvarez Rojas *et al.*, 2015). However, even when a systemic Th2 immune response dominates, different cytokines are expressed in different tissues; in sheep, IL-5 can be detected in the hepatic lymph nodes while IL-10 is primarily observed in the spleen (Haçarız *et al.*, 2009; Pleasance *et al.*, 2011). By contrast, in goats, IFN-γ and high levels of IL-4 can be detected in both the hepatic lymph node and liver (Mendes *et al.*, 2010a). The chronic diseases in ruminants (cattle, sheep, goats and buffalo), typified by the Th2/Treg responses and suppressed Th1 responses, have a distinctive serological picture in the isotype of circulating antibodies, with high titres of IgG1 and virtually no IgG2 secreted in cattle (Mulcahy *et al.*, 1998), sheep (Hoyle and Taylor, 2003) and goats (Buffoni *et al.*, 2010).

While Th2 and regulatory T-cell cytokines play a role in downregulating host protective Th1 responses during infection with *F. hepatica*, the effect is partly related to the modulatory effect of the parasite on various cells of the innate

immune response. For example, dendritic cell (DC) populations CD11+c are increased during infection in experimental mouse models, displaying an immature phenotype with lower expression of co-stimulatory markers (CD40, CD80 and CD86) and increased expression of CC chemokine receptor 5 (CCR5), resulting in lower responsiveness to Toll-like receptor activation (Dalton *et al.*, 2013). These cells express enhanced levels of intracellular IL-10 and, *ex vivo,* suppress the secretion of antigen-specific IL-17 and IFN-γ from CD4+ T cells independent of IL-10 and TGF-β (Walsh *et al.*, 2009). Another striking feature of the infection is the induction of AAMF with a regulatory/M2 phenotype in both ruminants and rodents (Donnelly *et al.*, 2005; Haçarız *et al.*, 2011). This occurs early in the infection and those M2 macrophages are hyporesponsive to TLR ligands, suggesting a reduced capacity to induce the differentiation of Th1 response and, conversely, an increased ability to promote the polarization towards Th2 cells (Donnelly *et al.*, 2008).

The local immune response in the liver shows a marked infiltration of CD4+ T cells, B cells and IgG+ plasma cells, with less participation of other immune cells (CD8+, gd+ T cells), eosinophils and macrophages (Zafra *et al.*, 2013b). During the biliary stage CD4+ T lymphocytes, B cells (CD79α and IgM+ cells) occupy the peripheral areas of bile ducts. In some portal areas B cells are organized into lymphoid follicles (Pérez *et al.*, 1998, 2005). In chronic hepatic lesions CD8+ T cells were more abundant, particularly in secondarily infected sheep (Meeusen *et al.*, 1995).

The description above is an oversimplification of the immunological picture that occurs during natural *F. hepatica* infection where existing and newly acquired infection occur simultaneously and the parenchymal and biliary immune and inflammatory responses overlap. More studies are needed to determine if the response to one parasitic stage affects other stages of the parasite.

5.5.2 Immune evasion

The existence of immune evasion mechanisms in *F. hepatica* infection has long been suggested and major advances in the understanding of these processes have occurred in recent years (summarized below). Evidence comes from *in vitro* studies on the role of ES products on host cells (Carmona *et al.*, 1993; Jefferies *et al.*, 1996) and in experimental studies in rats (Tliba *et al.*, 2000), sheep (Meeusen *et al.*, 1995) and goats (Martinez-Moreno *et al.*, 1999). The parasite appears to have developed many strategies to physically avoid the immune response (Berasain *et al.*, 2000), to resist effector molecules (Piedrafita *et al.*, 2004) or to suppress (Flynn and Mulcahy, 2008b), subvert (Hamilton *et al.*, 2009) or modulate the immune response of the host (Robinson *et al.*, 2010). Evasion has been observed at all stages of the parasitic life cycle. Together, these factors can help to explain how this parasite can continue to infect and establish even in currently infected and previously infected hosts. Further, because individual flukes can survive for many years and as these chronic infections do not typically kill their hosts, these flukes can produce eggs and continue the life cycle. While parasite biology is not discussed here, evasion provides the capacity to survive for many years and sustain the life cycle through egg production.

During the migration through the intestinal wall and the peritoneum, no host response is observed (Table 5.1) (Boray, 1969; Zafra *et al.*, 2013a,b), suggesting either that these NEJs induce little mechanical damage or that they modulate the immune response by downregulating inflammatory infiltrates and so evade peritoneal immune attack.

The migratory behaviour of juvenile flukes within the liver may itself be an immune evasion tactic. It has been suggested that evasion of Th2 responses that are suited to protecting against parasites at mucosal surfaces is one advantage provided by tissue migration in the life cycles of parasitic helminths (Mulcahy *et al.*, 2005). It has also been described that rapid migration of juvenile flukes in hepatic parenchyma is an immune evasion strategy, as parasites are rarely found in the tracts themselves but have moved on, leaving behind a trail of destruction and being unaffected by effector cells of the infiltrate (Tliba *et al.*, 2000; Zafra *et al.*, 2013b). The differences in the susceptibility of ITT sheep to *F. hepatica* and *F. gigantica* are related not only differences in the innate and acquired immune response but also to the higher migration and

growth rate of *F. hepatica* versus *F. gigantica* (Pleasance *et al.*, 2011).

The prolonged survival of liver flukes in bile ducts has also been attributed to an evasion mechanism, partly by location in a 'secure' environment, scarcely affected by the immune mechanisms of the host, and partly by the release of ES products and the metabolic activity of the parasite tegument. The tegument is the primary site of interaction between host and parasite and is a key element in the survival of adult flukes. It is a metabolically active layer that is continuously sloughed off and replaced during infection, suggesting that this is a method of avoiding the attachment of immune effector cells (Dalton and Joyce, 1987).

F. hepatica flukes modulate the host response from the early stages of infection. It is clear that their capacity for limiting Th1 and Th17 responses and proinflammatory cytokine production (Walsh *et al.*, 2009; Vukman *et al.*, 2013a; Pacheco *et al.*, 2017), inducing alternative activation of macrophages (Donnelly *et al.*, 2005; Flynn *et al.*, 2007a; Ruiz-Campillo *et al.*, 2018; Celias *et al.*, 2019) and promoting proliferation of FoxP3+ regulatory lymphocytes play important roles. Early in the migratory phase (9 days pi), a significant increase of T-reg (Foxp3+) lymphocytes was found in both goats and sheep (Escamilla *et al.*, 2016b; Pacheco *et al.*, 2018) and the regulatory role of those cells may facilitate parasite survival when it is more vulnerable to the immune effector mechanisms. These mechanisms facilitate parasite survival but also limit liver damage by the host response and thus facilitate healing and fibrosis, and even protect from T cell immune-mediated tissue damage such as encephalitis (Walsh *et al.*, 2009; Quinn *et al.*, 2019). In this way the flukes may preserve their predilection site and promote the survival of their host.

Much of the immunomodulatory activity of the parasites is mediated by their ES products and extracellular vesicles (Dalton *et al.*, 2013). The involvement of ES products, such as cysteine proteinases, in immune evasion is supported by experiments demonstrating cleavage of immunoglobulins and the impairment of antibody-mediated eosinophil attachment to newly excysted juveniles (Carmona *et al.*, 1993; Berasain *et al.*, 2000). Moreover, they are believed to immunomodulate T-cell responses during *F. hepatica* infection by suppressing interferon-γ (IFN-γ) production (O'Neill *et al.*, 2000) and degrading surface CD4 molecules in T cells (Prowse *et al.*, 2002). When used as targets for immunoprophylaxis, significant levels of protection, reduction of viability and fertility both in *F. hepatica* and *F. gigantica* have been obtained (reviewed by Toet *et al.*, 2014). As reflected in Table 5.2, the immunomodulatory effects of ES products have been widely studied in recent years (Cwiklinski and Dalton, 2018) and there is clear evidence, from *in vivo* and *in vitro* studies, that secreted molecules from both NEJs and adults interact with host innate immune effector cells (Ryan *et al.*, 2020) and induce potent immune suppression or modulation to an extent that animals are susceptible to further infection with liver flukes (and other pathogens).

F. hepatica ES molecules can induce apoptosis as a means of causing immunoregulation of innate and adaptive immune responses of the host. Serradell *et al.* (2007) observed ES-induced early apoptosis of rat peritoneal eosinophils and that this phenomenon was time- and concentration-dependent. Activation of protein tyrosine kinases (TyrK) and caspases was necessary to mediate the apoptotic process. Studies in rats demonstrate the apoptosis of eosinophils by a caspase-dependent mechanism, causing mitochondrial-membrane depolarization and release of cytochrome c (Serradell *et al.*, 2009).

5.5.3 Impact of concurrent infection

The immunomodulatory mechanisms induced in *F. hepatica* infection and the resulting Th2-biased response appear to have consequences for the host homeostasis and particularly its ability to control concurrent bacterial infections, mainly by intracellular pathogens. Early evidence for this came from observations that animals co-infected with *F. hepatica* and *Salmonella* had higher fatality rates with smaller doses of bacteria, excreted larger numbers of bacteria for longer and showed a greater extent of tissue infection than animals that were not infected with liver fluke (Aitken *et al.*, 1984). On the other hand, when *Salmonella dublin* was given orally, susceptibility to salmonellosis was not increased (Hall *et al.*, 1981). An epidemiological study on Dutch dairy farms found that liver fluke infection

was significantly associated with *S. dublin* and identified fasciolosis as one of the main risk factors (Vaessen *et al.*, 1998).

In mice, co-infection with *Bordetella pertussis* and *F. hepatica* produced a delayed clearance of the bacteria due to suppression of protective Th1 responses (Brady *et al.*, 1999; O'Neill *et al.*, 2000). The immune polarization was shown to be IL-4 dependent, as *F. hepatica* infection did not suppress IFN-γ production by *B. pertussis*-specific T cells in IL-4 knockout mice. Both the ES products and purified cathepsin L proteinases were shown to suppress the *B. pertussis*-specific IFN-γ production by a mechanism mediated, at least in part, by IL-4 (O'Neill *et al.*, 2001).

Due to the immunosuppression of Th1/proinflammatory innate and adaptive responses, the interactions between *F. hepatica* and other pathogens normally controlled by Th1 are especially relevant. This is the case of *Mycobacterium bovis*, the causative agent of bovine tuberculosis (bTB). Flynn *et al.* (2007b) showed that cattle co-infected with the avirulent *M. bovis* strain, Bacille Calmette–Guérin (BCG), had a significantly reduced diagnostic response to the antigen. The main diagnostic methods in bTB are skin test and *in vitro* IFN-γ assay, both of which detect Th1 cell-mediated immune responses against *M. bovis* antigens. Suppression of this diagnostic response has been demonstrated in calves co-infected with *F. hepatica* under experimental conditions (Flynn *et al.*, 2007b, 2009; Claridge *et al.*, 2012). For cattle in the field, a comparison of the spatial distribution of the prevalence of *F. hepatica* infection and incidence of bTB in over 3000 dairy herds in England and Wales showed almost no overlap and logistic regression analysis found that the presence of *F. hepatica* was significantly negatively associated with diagnosis of bTB (Claridge *et al.*, 2012). The evidence from the 13 studies included in the review supports the hypothesis that liver fluke-infected animals are likely to have a reduced response to both the tuberculin skin test and the *in vitro* IFN-γ assay and fewer bacteria recovered/cultured from their lesions (Howell *et al.*, 2019). The study by Claridge *et al.* (2012) using cattle with induced infections also concluded that test accuracy is compromised. On the other hand, in a study of 1494 dairy herds in Northern Ireland where cattle were naturally exposed to both infections, Byrne *et al.* (2019) concluded that there was a lack of support for associations between the pathogens at the herd level.

In a further example, the interaction between *F. hepatica* and *Toxoplasma gondii*, a Th1-inducing protozoan pathogen, has been studied (Miller *et al.*, 2009) and, regardless of whether *F. hepatica* infection preceded or followed *T. gondii* infection, there was little impact on the production of the Th1 cytokines or on the development of classically activated macrophages. By contrast, the production of helminth-activated Th2 cytokines was suppressed by infection with *T. gondii*. The clinical symptoms of toxoplasmosis and the survival rate of infected mice were not significantly altered by the helminth. In this case, the potent Th1 immune responses to *T. gondii* appeared to be capable of suppressing the responses to helminth infection.

5.5.4 Effects on pharmacokinetics of drugs

One of the critical functions of the liver is the detoxification of xenobiotics and endogenous compounds prior to excretion via the bile or urine. Infection with liver flukes affects both the metabolism of xenobiotics and the pharmacokinetics of many drugs. These changes could influence the efficacy, the toxicity and/or the clearance of xenobiotics such as therapeutic drugs or environmental toxins. Given the structural, physiological and biochemical effects of *Fasciola* infection, impacts on drug behaviour are not surprising.

Metabolism of lipophilic xenobiotics generally occurs in two sequential stages, termed Phases I and II. In Phase I, lipophilic compounds are converted, usually by oxidation reactions catalysed by the mixed-function oxidase system. The major actors are the P450 family of haemoproteins and the regulatory processes that control their expression. Several P450 isotypes show a range of specificities to deal with the diversity of xenobiotics and endogenous biochemicals. Several P450 isoforms are expressed constitutively in the liver (e.g. CYP1A2 and CYP2A1), whereas others are induced selectively by exposure to different xenobiotics. Transcriptome analysis has revealed that downregulation of several CYP-family P450s, including CYP2,

CYP3 and CYP4 members, occurs during fasciolosis (Rojas-Caraballo *et al.*, 2015).

In Phase II the oxidized products are conjugated by UDP–glucuronosyl transferases (UDPGT) and GSTs, to produce water-soluble products that are passed in urine. GSH, for example, is both a substrate for GST and an intracellular reducing agent capable of binding directly to endogenous or exogenous electrophilic compounds such as peroxides or free radicals. It is synthesized by the liver and released into the blood to supply other tissues. A decline in GSH concentrations has been reported in infected livers (Maffei Facino *et al.*, 1990, 1993), including in sheep (Galtier *et al.*, 1986).

Changes in drug metabolism may lead to clinical outcomes such as reduced efficacy (due to lowered drug levels) or increased toxicity and prolonged residence time (due to higher levels) possibly requiring longer withholding periods prior to human consumption of animal products. Many studies have been carried out on drug pharmacokinetics and detoxification in *F. hepatica*-infected hosts (for a review, see Behm and Sangster, 1998). However, reports of clinical failures or toxicity of pharmaceutics appear to be sparse. It is likely that the changes in drug levels either have little clinical impact or are unnoticed in practice. Two examples below record observations on two drugs used to treat fasciolosis.

Albendazole (ABZ), a nematocide and fasciolocide of the benzimidazole class, is administered orally and metabolized by S-oxidation. The first step is conversion to the active metabolite ABZ sulfoxide, which is probably a P450-independent step and unaffected during fasciolosis. The metabolism of the sulfoxide to the inactive excretion product, ABZ sulfone, is P450-dependent and in infected sheep the rate of this P450 step is reduced by 47% and its elimination by 87%. This leads to an increase in the mean residence time and area under the time/concentration curve for ABZ sulfone (Galtier *et al.*, 1991b). In another example, nitroreductase activity of the anthelmintic nitroxynil is reduced in infected cattle, indicating a potentially reduced capacity of the animals to detoxify this compound. The maximum tolerated dose of nitroxynil is only threefold higher than the therapeutic dose, so there is increased potential for toxicity in infected animals (Maffei Facino *et al.*, 1985).

5.6 Conclusions

Fasciola spp. are highly successful disease agents and fasciolosis is indeed a complex disease. Central to this are the biology of the fluke itself, including its migratory life cycle, the severe pathology it causes, hosts potentially carrying several life-cycle stages at the same time, its ability to infect multiple host species and its widespread global distribution. An added twist is its ability to infect humans. Our current understanding of the disease processes, based on a mix of historical observation and classic biochemical and immunological techniques, has been greatly enhanced due to the application of modern -omics technologies. This work has also opened up avenues to investigate the mammalian immune system, including immune system polarization and immune evasion. Despite the great challenge of working on a large pathogen that must generally be studied while inside its host, knowledge has expanded in recent years. What remains is to harness our knowledge to successfully control this parasite.

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6 Epidemiology and Control

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6.1 Introduction

Epidemiology is the study of disease in populations and the factors that determine its occurrence. In addition, it includes investigation and assessment of other health-related events in livestock, such as productivity. The study of the epidemiology of fasciolosis in livestock encompasses the factors that affect the prevalence and intensity of infection with the parasite and how these impact on animals, in terms of both clinical disease and the economic effects of productivity losses. The epidemiology of the disease depends on factors such as the infection pressure in the environment and the susceptibility of the host species (or individual) through innate or acquired resistance. The infection pressure in turn depends on factors that affect the free-living and intermediate stages, such as temperature and moisture. Furthermore, the availability of large numbers of susceptible definitive and intermediate hosts will increase the parasite's ability to reproduce itself and result in a high fluke abundance. Figure 6.1 illustrates the interactions of these epidemiological factors that increase the likelihood of fasciolosis. To be effective, control measures depend upon a sound understanding of the epidemiology of the disease so that intervention strategies can be designed to produce the greatest possible benefit in terms of minimizing disease or loss of productivity in animals at risk of infection.

6.2 Parasite, Host and Intermediate Host Species

Pathogenic species of *Fasciola* and conditions suitable for the development of all stages of the life cycle need to be present for disease outbreaks to occur. The two most important species are *Fasciola hepatica* and *F. gigantica*, which both cause disease in domestic animals and humans. *F. hepatica* has a cosmopolitan distribution whereas the distribution of *F. gigantica* is more limited, being restricted to tropical Africa, the Middle East, eastern Europe and southern and eastern Asia. There have been repeated reports of hybrids (or 'intermediate forms') of *F. gigantica* and *F. hepatica* in regions where both species are present (Calvani and Šlapeta, 2021) (see Chapter 13, this volume) and this is of concern, as it is thought that they could acquire advantageous traits such as increased pathogenicity and host range. A third species, *F. jacksoni*, is found in elephants, in which it may cause significant pathogenic effects, but there is genetic evidence to suggest that this parasite should be placed in the genus *Fascioloides* (Rajapakse *et al*., 2019).

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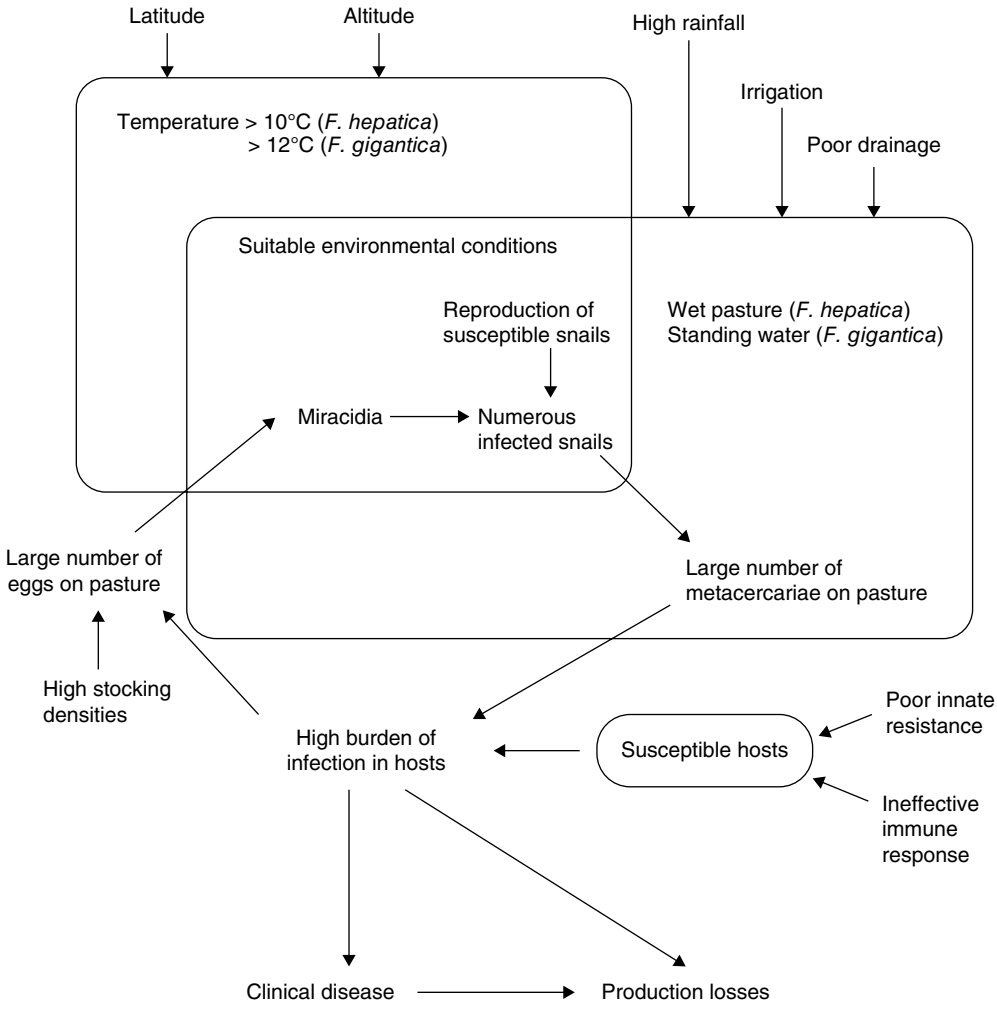

Fig. 6.1. Interactions of epidemiological factors that influence the likelihood of disease due to fasciolosis in livestock.

6.2.1 Definitive hosts

Domestic ruminants such as cattle, sheep and buffalo are the most important species of farm livestock affected by *Fasciola* spp. Although goats, horses, pigs, deer and many other species of herbivore can also be infected (Howell and Williams, 2020), the parasite is of less economic importance on a global scale in these hosts. Humans are also a suitable host and in some areas of the world human fasciolosis is an important cause of ill health (Torgerson *et al.*, 2015) (see Chapter 14, this volume).

6.2.2 Intermediate host species

Liver fluke is absent in areas where conditions are unsuitable for the development of suitable intermediate-host snails. Suitable snails belong to the phylum Mollusca and class Gastropoda and the species of interest fall into the subclass Euthyneura or Pulmonata, depending on the system of classification (Wright, 1971).

Lymnaea spp. snails involved in the transmission of *F. hepatica* are mud-living and amphibious, living in an environmental niche that is subject to flooding and desiccation (Kendall

and Ollerenshaw, 1963; Over, 1982). They are more likely to be found in habitats that are intermittently wet (flush habitats) than in permanently wet sites and in water that is generally slightly acid (Ollerenshaw, 1971; Villegas, 1984) and moving at 15–20 cm/s (Boray, 1964). Distribution is not uniform, because, within each habitat, the snails may be concentrated in small, very wet areas such as ditches and seepages (Kendall and Parfitt, 1975). Snails can travel large distances by drifting in water (Ollerenshaw, 1971). In Australia, large permanent water areas harbouring a few snails are thought to be important in recolonization of temporary water courses (Kendall, 1949; Boray, 1964). Thirty per cent of snails survive a 12-month artificial drought by aestivation (Soulsby, 1982) and even newly hatched snails can survive 2 months of aestivation (Kendall, 1949). Once water returns, snails can breed very rapidly. More detailed information about suitable snail intermediate hosts is provided in Chapter 2 of this volume.

6.3 The Effects of Climate and Environment on *Fasciola* spp.

Parasite distribution in the environment is extremely variable. Despite this variability, for *Fasciola* spp. to complete their life cycle the environment must provide a consistent set of suitable conditions of moisture and temperature for the development of the larval stages and the development of the intermediate host itself. The majority of work on the development of flukes outside the definitive host has been carried out on *F. hepatica*, with a smaller amount of information on *F. gigantica*.

6.3.1 Effect of temperature

6.3.1.1 On fluke eggs

The minimum critical temperature for development of *F. hepatica* eggs is about 9.5°C (Rowcliffe and Ollerenshaw, 1960). Development is inhibited above 30°C. The number of degree-days for complete development varies, with estimates of the development of the miracidium in the egg ranging from 60 days at 12°C to 10 days at 30°C (Rowcliffe and Ollerenshaw, 1960; Gettinby and Byrom, 1991). In the Kenyan highlands, where mean temperatures vary between 10°C and 22°C, the development time of *F. gigantica* eggs has been reported to be from 52 to 109 days, while at a constant temperature of 26°C they take 12–17 days to develop (Dinnik and Dinnik, 1959, 1963; Hussein *et al*., 2010). At 29°C it has been reported that the eggs became fully embryonated and hatched in 11–12 days (Phalee *et al*., 2015).

6.3.1.2 On snail development

A minimum temperature of 10°C is necessary for development of *Galba truncatula* (formerly *Lymnaea truncatula*) and maximum growth occurs at 18–27°C (Kendall, 1953). *G. truncatula* can grow to sexual maturity in 3–4 weeks, depending on the amount of food available and the number of parasites in the snail (Kendall, 1953; Kendall and Ollerenshaw, 1963). *Lymnaea viatrix* reaches maturity in 24–27 days (Lara *et al*., 1988). Snail growth is slow at temperatures below 10°C but increases rapidly up to 25°C and then falls again at higher temperatures (Claxton *et al*., 1999). The number of eggs produced is related to the food supply available and *L. viatrix* may produce up to 5000 eggs in its lifetime of at least 200–305 days (Lara *et al*., 1988). *G. truncatula* may live for 12–14 months (Over, 1982). Under laboratory conditions, *Lymnaea viridis* has been shown to grow rapidly, reaching 12 mm in 37 days and producing eggs at 18–24 days old (Lee *et al*., 1994).

6.3.1.3 On parasite development within the snail

At 15°C, *F. hepatica* cercariae will complete development in the snail in 80 days (Gettinby and Byrom, 1991). A minimum temperature of 10°C is required for shedding but above this minimum the temperature appears not to be as critical (Kendall and McCullough, 1951). Above 20°C mortality of the cercariae increases. In the *G. truncatula–F. hepatica* model, the release of cercariae was believed to be optimum at a constant temperature of 20°C. However, exposing snails to a fluctuating temperature regime between 12°C and 20 °C, mimicking natural

conditions, leads to an increase in the number of snails shedding cercariae and greater cercarial production (Rondelaud *et al.*, 2013). Once infected, the snail appears to remain infected for life (Kendall, 1965). Mean temperatures above 23°C are unsuitable for the transmission of *F. hepatica* (Malone *et al.*, 1998)

The minimum temperature for the development of *F. gigantica* in *L. auricularia/natalensis* is greater than that required for the intermediate stages of *F. hepatica*. Development to cercariae does not occur below 12°C; it takes 73 days at 15°C and 25 days at 30°C. Emergence of the cercariae takes 129 days at 15°C and 43 days at 30°C (Al-Habbib and Al-Zako, 1981). Dinnik and Dinnik (1963) reported that the development of *F. gigantica* in *L. auricularia/natalensis* took 33 days at a constant 26°C maintained experimentally but required between 69 and 197 days under the prevailing environmental conditions in Kenya.

6.3.1.4 On metacercarial survival

At 12–14°C, up to 100% of metacercariae can survive for 6 months while only 5% survive for 10 months. Metacercarial survival is reduced in hot conditions. Boray and Enigk (1964) found that cysts remained viable for only 3 days at 20°C and a relative humidity of 75–80%, whereas they survived 122 days at 10°C and 90% relative humidity. Cysts have been shown to survive and remain infective for 70 days when alternately frozen to −5°C and warmed to +10°C for 12 h each day, whereas freezing at −20°C may render the metacercariae non-infective (Boray and Enigk, 1964). For prolonged survival, the relative humidity needs to be above 70%, with cysts surviving on moist hay for 8 months and on silage for 57 days.

6.3.1.5 Implications for the epidemiology of the infection

In countries where there are only two months of the year when the mean temperature is greater than 10°C, parasites will have to overwinter in the snail to complete their development. Since snails rarely live longer than 1 year it can become difficult for the disease to establish (Gettinby and Byrom, 1991). Fasciolosis is therefore absent in Iceland and northern Scandinavia, despite the availability of suitable intermediate hosts. In temperate climates, where there is year-round rainfall, temperature is still the restricting factor. When there is a mean day and night temperature above 10°C for approximately 6 months of the year, the parasite is often endemic. A cycle of summer/winter snail infection has been described for north-western European climates such as Britain, Holland and Denmark (Ollerenshaw and Rowlands, 1959; Ollerenshaw, 1970; Shaka and Nansen, 1979). Most development of the larval stages takes place during the spring and summer months and ceases during the winter. The 'summer' infection of snails results from the hatching of overwintering eggs or eggs passed in the spring, with metacercariae appearing on pasture from August to October. A smaller 'winter' infection of snails is due to the infection of snails in the autumn: larval development ceases during the winter months and commences again in spring, resulting in pasture contamination with metacercariae in May and June.

Disease outbreaks usually relate to the peak time of pasture contamination with metacercaraie. In climates with moderate winter temperatures parasites can overwinter as eggs, pasture metacercariae or developmental stages in snails, development resuming when the temperature reaches 10°C in the spring. Although Gaasenbeek *et al.* (1992) reported successful overwintering as metacercariae or as developmental stages in snails in the Netherlands, numerous studies have shown that the most important overwintering stage is the egg in similar climates. Several studies in northern Europe support the view that overwintering eggs develop at the same time as eggs that have been shed the following spring (Hope-Cawdery, 1975; Shaka and Nansen, 1979; Gaasenbeek *et al.*, 1992). The dominance of the summer infection of snails has also been described in Ireland (Ross, 1967; Ross and Todd, 1968), Scotland (Armour *et al.*, 1970) and Sweden (Novobilský *et al.*, 2014). Livestock are infected from late summer onwards as a result of this early summer infection of snails. Thus, late autumn/winter represents a period of peak fluke infection (Munita *et al.*, 2016; Byrne *et al.*, 2018). More chronic disease in both sheep and cattle is seen in winter and early spring due to the ingestion of lower numbers of metacercariae. Occasionally disease

is seen in summer from the winter infection of snails. The pattern of transmission is similar in north-western USA. In Idaho, peak transmission to sentinel steers occurred between August and November, indicating the summer infection of snails predominating in this region (Hoover *et al*., 1984); there is little evidence of overwintering metacercariae or larval stages within snails, possibly due to low winter temperatures.

In regions further south, such as central western France, the increasing summer temperature and longer season allow an extra summer cycle (Leimbacher, 1978). In warmer conditions still, stages other than the egg play a much more important role in the overwintering of the parasite. In Australia, because there is often no winter housing of stock, contamination of pasture is continuous. Even in southern areas of Australia conditions may favour the development of eggs and larval stages for 9 months of the year. In these circumstances significant overwintering takes place in the form of infected snails, which can result in heavy infection of sheep as early as mid-spring (Boray, 1969).

F. hepatica is the only *Fasciola* species endemic to both the north or south American continents. In tropical and subtropical areas transmission is driven by seasonal rainfall similarly to *F. gigantica* in tropical Africa and Asia (see below). For example, the major annual transmission occurs in cattle in the wet season (July–February) in Colombia (Valencia-López *et al*., 2012). Likewise, there is a marked increase in cattle infection in Costa Rica associated with the rainy season (Ernesto Alpízar *et al*., 2013).

F. gigantica, having higher temperature requirements for development of intermediate stages, is largely confined to the tropics and warm temperate regions. In some endemic areas, such as eastern Europe and the southern part of the former USSR, seasonal development is likely to follow a similar pattern to *F. hepatica*. Both *F. hepatica* and *F. gigantica* are endemic in many tropical countries; however, *F. hepatica* often predominates at higher altitudes, partly because of the lower temperature requirements of the developmental stages.

In the tropics where *F. gigantica* predominates, seasonal rainfall patterns will drive transmission. A common pattern seen is a higher prevalence in livestock during or immediately after the rainy season. In India, *F. gigantica* infection of *L. auriculus* snails correlated positively with temperature, rainfall and humidity, which occurs between May and September. This in turn leads to peak prevalences of infection in cattle and buffalo between October and February (Yadav *et al*., 2007). In Pakistan, highest levels of infection are seen immediately after the rainy season (i.e. September–October) (Qureshi *et al*., 2012). In Zimbabwe, higher levels of infection in cattle have been recorded during the wet season (Pfukenyi *et al*., 2006). In Nigeria, highest prevalences of *F. gigantica* in livestock have been found during and directly after the rainy season (Schillhorn van Veen *et al*., 1980; Gboeloh, 2012). In Zambia, animals are most intensely infected just after the rainy season and lowest in the cool dry season (Phiri *et al*., 2005). It has been reported that animals grazing close to the shores of Lake Chad have a much higher risk of infection with *F. gigantica* (Jean-Richard *et al*., 2014), presumably because of the more suitable habitat for the intermediate host in such areas.

6.3.2 Effect of moisture

6.3.2.1 On the parasite

Fasciola spp. require moisture for transmission, proliferation and survival; miracidia need wet surfaces to find snail hosts; snails need moisture to develop; cercariae do not normally emerge from snails unless there has been recent rainfall; and metacercariae need humidity to survive. Where there is seasonal rain, transmission will be limited to the wet season, unless land is irrigated or there are permanent water courses. Ideal conditions, when precipitation exceeds potential evapotranspiration resulting in increased water in the environment, favour both the intermediate host and intermediate stages. However, a good example where *Fasciola* is highly endemic despite a desert climate is in Egypt in the Nile valley and especially in the Nile Delta. For example, buffalo in the Nile Delta have been reported to have a prevalence of between 9% and 20% of infection with *F. hepatica* (El-Tahawy *et al*., 2017). Even in Libya, which is an extremely arid country with no permanent rivers, *Fasciola*-infected snails have been found in desert oases (El-Serety *et al*., 2008).

Fluke eggs will not develop while in the faecal mass. Moisture is necessary to break up the mass and even the presence of soil may reduce the rate of development of the egg (Rowcliffe and Ollerenshaw, 1960). Eggs in moist faeces can survive for at least 10 weeks in the summer and 6 months in the winter in the UK. However, if the faecal material dries out, there is rapid mortality of the eggs (Ollerenshaw, 1971). In Spain 55% of overwintering eggs arrested in wet faeces survived compared with 20% arrested in water (Luzón-Peña *et al.*, 1992). *F. gigantica* eggs have a greater mortality in dung exposed to sunlight compared with that located in the shade (Suhardono *et al.*, 2006b).

Free-swimming cercariae, released from snails, encyst by secreting a cyst wall and losing their tails. Encystment usually takes place on a surface (such as vegetation) but some cysts may develop air-filled lacunae in the outer cyst wall and float freely (Esclaire *et al.*, 1989). Between 6.8% and 10% of shed parasites will form such free-floating metacercariae (Boray, 1969; Esclaire *et al.*, 1989). Once encysted, the metacercariae are thought to be infectious to the definitive host; 2-day-old cysts are known to be able to excyst within the host (Ollerenshaw, 1971). The longevity of metacercariae, however, depends on moisture in, and temperature of, the environment (see above).

6.3.2.2 On the snail

G. truncatula is a mud snail which, if the environment dries out, may undergo a prolonged state of aestivation, during which transmission of the parasite is suspended. When the rains return, there can be rapid recolonization of the environment. *Lymnaea bulimoides*, an important intermediate host in the southern USA (Zukowski *et al.*, 1991), also undergoes aestivation during summer drought (Malone *et al.*, 1984). In many areas, such as in Australia and Spain, the prevalence of fasciolosis is higher than might be expected from the regional climate as a result of increased moisture from irrigation (Meek and Morris, 1979; Uriarte *et al.*, 1985). In such circumstances, summer warmth or winter freezing could affect the viability and development of the parasite and the snail intermediate host.

Although *G. truncatula* can survive periodic droughts through aestivation, other species of lymnaeid snails have different requirements for moisture. *Lymnaea tomentosa* and *L. columella* live in habitats that are permanently wet (Mitchell, 1995). The important snail hosts of *F. gigantica* differ from those of *F. hepatica* in that they are aquatic. There is little evidence that they can aestivate (Soulsby, 1982), thus the continuous presence of free water is required for their development.

6.3.2.3 Soil type

Snails require clay soils that accumulate a great amount of moisture, and that contain minerals such as calcium (vertisols) needed to form shells (Deplazes *et al.*, 2012, 2016). Some studies have found that soil characteristics may be predictive factors for fasciolosis (McCann *et al.*, 2010; Selemetas *et al.*, 2015): regions that were positive for the disease had deep soils with poor drainage. Soil with very fine sand has been reported as a positive predictor of *F. hepatica* herd prevalence in the UK. This may be due to soil consisting of very fine particles having poorer drainage (McCann *et al.*, 2010). Intermediate hosts have also been reported to be more abundant on loamy soils (Charlier *et al.*, 2011). Acid ferralsol soils of < 5.5 pH are unsuitable habitats for snail intermediate host species (Malone *et al.*, 1998).

6.3.2.4 Implications for the epidemiology of the infection

In Mediterranean climates, such as Spain, moisture can be the limiting factor for overwintering eggs (Luzón-Peña *et al.*, 1992) and, as in northern Europe, these overwintering eggs affect the intensity, but not the timing, of the spring contamination with miracidia. The dry winters of the semi-arid region of the Iberian Peninsula restrict contamination to areas adjacent to ponds, streams and irrigated water courses. Luzón-Peña *et al.* (1995) reported that excessive summer temperatures have a lethal effect on snails and metacercariae in parts of Spain. Similarly, in parts of the southern USA, excessive summer heat and/or drought prevent transmission (Boyce and Courtney, 1990; Zukowski *et al.*, 1991). In Florida, fluke transmission occurs almost exclusively between December and June, with the peak months being

February, March and April (Boyce and Courtney, 1990). Despite there being adequate moisture in the summer, a break in the cycle often occurs in April or May when high temperatures and low rainfall cause desiccation of snail habitats. When the summer rains begin a month or two later, excessively high temperatures seem to prevent the snails reappearing. In other southern states, such as Louisiana and Texas, transmission occurs between February and July (Craig and Bell, 1978; Malone *et al*., 1984). Here, replenishment of soil moisture begins in autumn and by February the snails are shedding large numbers of cercariae. Provided rainfall is sufficient, this shedding continues and livestock become infected. During the first sustained drought of the summer, however, snails aestivate, metacercariae rapidly die and transmission ceases.

Although *L. auricularia*/*natalensis* is associated with tropical environments that have adequate temperatures for the development of *F. gigantica* all year, a growth cycle has been shown to occur in Malawi, where snails were found to reach peak abundance around March/April each year (Tembely *et al*., 1995). Both the total fluke counts and the faecal egg counts are highest at the beginning of the wet season in west Africa (reviewed by Schillhorn van Veen, 1980). This is when snail populations are rising. Large numbers of infected snails are present at the end of the wet season and beginning of the dry season, when most ruminant hosts are infected. Acute fasciolosis is then seen towards the end of the dry season. Similarly, there is a cycle of development of snails and infection in cattle and buffalo in India related to the rainy season (Chaudhri *et al*., 1993).

6.4 Effects of Climate Change

The dependence on the presence of moisture and suitable temperatures for the transmission of *Fasciola* means that the epidemiology of fasciolosis may change as a result of climate change. In the UK this has been modelled by Fox *et al*. (2011) and suggests that the increased temperature will lead to increasing risk for fasciolosis, with serious epidemics in areas of the UK such as the west of Scotland and in parts of Wales where there is ample rainfall. In contrast, the east of England is predicted to have little or no disease by 2040 because of decreased rainfall and resulting soil moisture deficits, which impact adversely on the intermediate stages of the parasite. Rising temperatures may also allow winter transmission in certain areas in south-west England and Wales. Consequently, there is the possibility of fasciolosis changing from a seasonal to a year-round threat.

Models that include much of Europe have suggested that the current climate is highly suitable for *Fasciola* across the European Union, with the exception of some parts of the Mediterranean region. Caminade *et al*. (2015) simulated climatic suitability for fasciolosis and observed that it significantly increased during the 2000s in central and north-western Europe. This was consistent with an observed increased in ruminant infections. The simulation showed that recent trends are likely to continue in the future with the estimated pattern of climate change for northern Europe, possibly extending the season suitable for development of the parasite in the environment by up to 4 months. For southern Europe, the simulated burden of disease may be lower, but the projected climate change will increase the risk during the winter months since the simulated changes in temperature and moisture support the development of the free-living and intra-molluscan stages between November and March.

In New Zealand, the significance of *F. hepatica* in farming systems is probably underestimated and this risk is predicted to increase with global warming due to climate change (Haydock *et al*., 2016). Outbreaks of acute fasciolosis in sheep are now being linked to climate change in Italy (Bosco *et al*., 2015) which was hypothesized to be due to increased temperatures, increased rainfall and increased number of rainy days compared with previous years.

6.5 Management Factors Affecting Transmission

Mowing of pastures, proportion of grazed grass in the diet of livestock and length of grazing season are significant predictors for the spatial distribution of *F. hepatica*, in addition to climatic and environmental factors such as rainfall and temperature (Bennema *et al*., 2011). The major

cause of loss of infectivity of pasture appears to be death of the metacercariae, except where conditions of rapid pasture growth cause dilution of the infection (Ollerenshaw, 1971).

The viability of *F. hepatica* metacercariae in silage appears to be poor, potentially as a consequence of lactic acid production and proteolytic bacteria proliferation during the initial fermentation stages. Likewise, *F. gigantica* metacercariae viability in fermented wastelage, consisting of buffalo faeces, molasses and rice straw, appears to be poor. Studies have demonstrated that survival of metacercariae in hay is somewhat variable and is most prolonged under cool conditions. Poorly stored old hay originating from contaminated pastures poses a potential risk of fasciolosis (reviewed by John *et al.*, 2019).

In Cambodia, infection of cattle with *F. gigantica* coincided with the rice harvest, which suggested that feeding of fresh rice stalks and stubble after the rice was harvested was the main source of infection. Furthermore, transmission ceased when cattle were moved away from low-lying land to above the flood height and a change of diet to dry crop residues (Suon *et al.*, 2006).

F. gigantica metacercariae can survive up to 4 months on rice straw when it is stored above 60% relative humidity. Local farming practices can also promote parasite transmission. When untreated livestock manure is used as fertilizer, there is the possibility that fluke eggs may be spread on to otherwise uncontaminated pastures. Furthermore, waterlogged rice paddy fields provide an ideal habitat for the snail intermediate host. *F. gigantica* metacercariae typically encyst on herbage at the water level; hence, the lower portion of the rice stalk is often highly contaminated (Suhardono *et al.*, 2006a). Buffalo fed on a diet of rice straw with the lower 40 cm of each blade removed do not succumb to infection. However, livestock fed the highly contaminated lower regions of rice straw present with gross pathological signs consistent with acute infection (Mahato and Harrison, 2005).

6.6 Other Effects on Transmission

6.6.1 Source of infection

Metacercariae derived from cattle were found to be less infective than those from sheep, although the metacercariae of bovine origin eventually developed into flukes that produced more eggs than those of ovine origin (Dixon, 1964). Similarly, *F. gigantica* from sheep were shown to infect a smaller proportion of *L. auricularia*/*natalensis* snails than those from buffalo (30% and 82%, respectively), while metacercariae developing from infections derived from sheep had a higher mortality than those of buffalo origin (Al-Kubaisee and Altaif, 1989).

6.6.2 Competing infections

The presence of other species of parasites in the snail, such as the pulmonary fluke of frogs, *Haplometra cylindracea*, may reduce development of *Fasciola* spp. (Whitelaw and Fawcett, 1982). In recent years, the rumen fluke *Calicophoron daubneyi*, which also uses the mud snail *G. truncatula* as an intermediate host, has increasingly been detected in domestic ruminants. It is speculated that the presence of this trematode in the intermediate host population could potentially lead to displacement of *F. hepatica*, as one infection usually dominates another (Rondelaud *et al.*, 2007; Jones *et al.*, 2017; Beesley *et al.*, 2018; Munita *et al.*, 2019). Furthermore, when treating the definitive hosts against *F. hepatica*, this can result in an advantage for *C. daubneyi* as this parasite is not covered by the usual treatments against liver fluke (Mage *et al.*, 2002). One implication is that high levels of relatively less pathogenic rumen flukes may suppress the more pathogenic *F. hepatica* and thus limit livestock losses where both parasites are co-endemic. However, in contrast, in Ireland a positive association between infection with liver fluke and rumen fluke has been described (Naranjo-Lucena *et al.*, 2018).

6.6.3 Co-morbidities

Co-infection of animals with other pathogens may have an effect on the severity of disease or susceptibility to infection or may compromise diagnosis. In recent years, it has been hypothesized that infection of cattle with *Fasciola* makes such animals less sensitive to diagnostic tests for bovine tuberculosis. This would have important

implications for *Fasciola*-endemic areas such as the UK and Ireland that also have substantial problems with bovine tuberculosis. However, most studies showed a small or non-significant effect, so the clinical and practical importance of the observed effect is likely to be modest, although it could be more significant in particular groups of animals, such as dairy cattle (Howell *et al*., 2019). A study in Cameroon has suggested an association between infection with *F. gigantica* and bovine tuberculosis that is breed dependent (Kelly *et al*., 2018).

In the Netherlands, bovine fasciolosis has been shown to be highly associated with *Salmonella dublin* infection (Vaessen *et al*., 1998). An association between bovine fasciolosis and shedding of *E. coli* O157 has also been suggested (Howell *et al*., 2018). There is some evidence that there are interactions between infection of cattle with *F. hepatica* and the zoonotic cestode *Echinococcus granulosus* (Hidalgo *et al*., 2020). However, it is not yet clear how this could have an effect on the epidemiology of diseases caused by either parasite.

There may be interactions between trematode infections resulting from a common intermediate host rather than a direct effect on the definitive host (see above).

6.7 Resistance to Fasciolosis in Livestock

Understanding the resistance of livestock to infection with *Fasciola* or to the pathogenic effects of the parasite is important in developing strategies for the control of fasciolosis. These include using or developing resistant breeds or individuals or developing future vaccination strategies. Infection with *Fasciola* may result in a degree of acquired resistance, which varies depending on the host species. In addition, some species show a degree of innate resistance: horses are less susceptible than ruminants (Nansen *et al*., 1975); pigs are only significantly susceptible when under 8 weeks old (Nansen *et al*., 1972).

6.7.1 Resistance in sheep

Numerous studies in sheep have demonstrated that naive animals, sensitized with infections of *F. hepatica*, generate no significant protection to challenge infections (Sinclair, 1971; Sandeman and Howell, 1981; Boyce *et al*., 1987), though innate resistance between breeds of sheep does vary. The best example of resistance is found in Indonesian Thin-tailed sheep to *F. gigantica*, although they do not appear to have such resistance to *F. hepatica* (Roberts *et al*., 1997; Pleasance *et al*., 2011; Cameron *et al*., 2017). Other examples include St Croix sheep, which develop less than half the parasite burden compared with Barbados Blackbelly sheep (Boyce *et al*., 1987). Similarly, Gruner *et al*. (1992) demonstrated that Romanov sheep have greater resistance than Merinos. In Ethiopia there is experimental evidence to suggest that the Horro breed of sheep is more resistant to fasciolosis caused by *F. hepatica* than are Arsi or Menz breeds (Eguale *et al*., 2009).

There has also been a number of studies documenting variation between individuals within breeds (Meek and Morris, 1979; Sandeman and Howell, 1981; Boyce *et al*., 1987). Khallaayoune *et al*. (1991) reported a particularly marked variation in the Timahdit breed of sheep in Morocco: in a flock in which the prevalence of infection was only 50%, 10% of untreated lambs died of fasciolosis. This may indicate different degrees of resistance in individual animals and the prospect for selective breeding.

6.7.2 Resistance in cattle

Although susceptible to infection, cattle have been assumed to be relatively resistant to infection compared with sheep with a tendency to develop chronic infections. Evidence implies that there is little acquired concomitant immunity to *F. hepatica*. Studies by Clery *et al*. (1996) suggested that cattle with a chronic natural infection remain as susceptible to experimental infection as fluke-naive animals. Recent studies have confirmed the earlier work and suggest that chronic infection of cattle is via modulation to a non-protective Th2 response (Graham-Brown, 2016). In a study in Canada, cows with a mean age of 5 years had a prevalence of up to 68% (depending on the season) with an observed pattern of egg passage closely resembling those of single, primary infections (Bouvry and Rau, 1986). Recent studies in Uruguay with

a large dataset of 31,000 cattle also demonstrated that prevalence increased with age (da Costa *et al*., 2019). Consequently, if cattle are exposed to *Fasciola* infection, immunity to reinfection may not develop. Therefore, it must be assumed that adult and older cattle are likely to be infected with this parasite in endemic areas. This is in sharp contrast to many nematode infections such as ostertagiosis or dictycaulosis where good protective immune responses evolve following exposure. Older cattle cannot be ignored either from the point of view of clinical and subclinical disease or as a potential source of pasture contamination. There is some recent evidence that differences in genetics may result in a variation in the tolerance of cattle to *F. hepatica* infection (Hayward *et al*., 2021a).

Bos indicus cattle appear to be more resistant than *B. taurus* to infection with *F. gigantica* (Bitakaramire, 1973; Castelino and Preston, 1979). There is evidence that there is variation between breeds of *B. indicus* and there is also some evidence to suggest that buffalo are more resistant than cattle to infection with *F. gigantica* (Molina *et al*., 2005). Castelino and Preston (1979) and Mochankana and Robertson (2018) reported an increase in the percentage of cattle infected with *F. gigantica* with age. It has also been suggested that chronic infection with *F. gigantica* is modulated by a non-protective Th2 type of immune response (Sheng *et al*., 2019).

6.8 Economic Effects of Fasciolosis in Livestock

The effects of clinical fasciolosis can range from sudden death, when there is a massive challenge infection, to chronic underperformance. Subclinical infections, which often go unnoticed, produce marked economic effects.

6.8.1 Effects on live-weight gain and wool production

Ovine fasciolosis can result in significant blood loss representing a loss of metabolizable energy. This, together with impaired appetite and impaired nitrogen retention, can have an adverse effect on weight gain (reviewed by Hope Cawdery, 1984). Sinclair (1962) reported a 70% reduction in weight gain in sheep with a mean burden of 200 flukes. Sykes *et al*. (1980) demonstrated a reduction in feed intake, and a depression of live-weight gain in groups of infected sheep despite the absence of clinical signs of disease. It has also been reported that sheep with the lowest condition scores have the highest prevalence of fasciolosis (Alemu and Chala, 2019). Hawkins and Morris (1978) developed models relating live-weight changes in sheep with fluke burden. Weekly growth rates of wool and live-weight gain decreased with increasing fluke burdens. Burdens of 346 flukes or greater resulted in weight loss and lamb mortality; lower burdens of 46 flukes resulted in 13.6% decrease in wool production and a 5.1% decrease in weight gain. Reductions of 40% in wool production, due to fasciolosis, have been recorded by other workers (Roseby, 1970; Edwards *et al*., 1976).

In cattle there is both more data and more recent data. Early work demonstrated that modest fluke infections can result in significant reductions in performance. Infections as low as 54 flukes per animal have been shown to reduce weight gain by 8–9% even though this degree of infection results in no clinical signs of disease. Recovery in performance occurs about 26 weeks after experimental infection. Larger fluke burdens, such as occur after experimental infections with 1000 metacercariae, can reduce weight gains by 28% in previously uninfected animals (Ross, 1970; Hope Cawdery *et al*., 1977). Significant effects on performance in beef cattle have been reported in animals that have been infected by natural challenges. In Belgium, Genicot *et al*. (1991) reported an 18% increase in weight gain in flukicide-treated double-muscled Belgian Blue cattle compared with untreated controls. Most importantly, the increased profit resulting from the improved productivity was 4.2 times the cost of treatment. A negative association between *F. hepatica* exposure and milk yield across 606 dairy farms has been reported, with a 15% decrease in yield associated with exposure to flukes (Howell *et al*., 2015).

The use of large datasets confirmed the effects of liver fluke on the performance of beef cattle. Data from over 300,000 cattle slaughtered at a large abattoir in Scotland between 2005 and 2010 demonstrated that fluke-infected cattle had, on average, 0.63 kg lower carcass

weight, lower confirmation scores and a lower price (£1.50 per carcass) compared with non-infected animals (Sanchez-Vazquez and Lewis, 2013). A second study investigated nearly 170,000 cattle slaughtered in 2013–2014, also in Scotland. This demonstrated that animals with liver fluke damage had on average 10 days greater slaughter age compared with animals with no evidence of fasciolosis (Mazeri *et al*., 2017).

A recent meta-analysis has analysed the results of multiple studies on the influence of liver fluke on production in cattle and sheep (Hayward *et al*., 2021b). There were significant negative effects of fluke infection on daily weight gain, live weight and carcass weight (9%, 6% and 0.6% reductions in performance, respectively), but not total weight gain or milk production. In general, experimental infections had a larger effect than seen in observational studies, younger animals were more likely to have a reduced weight gain and the effects on live-weight gain increased across the course of the experiment.

It is clear that fasciolosis can result in substantial economic effects, considering the production effects described above and the widespread prevalence of infection in agricultural animals. Consequently, a number of estimates for the economic effects of fasciolosis have been made on a national or global scale. In Turkey, national estimates for the financial cost in cattle were approximately US$42.8 million in 2010 (Sariözkan and Yalçin, 2011). Likewise in Switzerland bovine fasciolosis was estimated to cost a total of €52 million per year (Schweizer *et al*., 2005a). In Germany, €42 million losses per annum in dairy farms has been attributed to *F. hepatica* infection (Fanke *et al*., 2017). Across 16 European countries and Tunisia and Israel, the economic costs of fasciolosis have been estimated at €634 million per annum (Charlier *et al*., 2020). It has been estimated that in southern and eastern Asia between 51 and 118 million cattle or buffalo are infected with *F. gigantica*, resulting in annual economic losses of AU$4 billion and AU$11 billion per annum. This represents between 11% and 26% of large ruminants infected (over 452 million large ruminants) (Copeman and Copland, 2008). The global cost of fasciolosis has also been previously estimated as several billion US dollars (for example, see Boray, 1985).

All this evidence suggests that the economic impact of fasciolosis in livestock is substantial. However, despite the published reports, the true costs of the diseases have a large degree of uncertainty. One important reason is that a single disease-centred economic approach can lead to an overestimate of disease losses. Most studies on the economic impacts of livestock diseases have a single disease focus with an advocacy role. They produce a large figure for monetary losses which is used as an argument for mobilizing the necessary resources to control the disease. Disease losses can be difficult to attribute when co-morbidities occur (which is well known in human health economics). In the early stages of the Global Burden of Disease (GBD) study into human mortality and morbidity, for some age/gender categories, the sum of the deaths ascribed to individual diseases substantially outnumbered actual reported deaths (Murray and Lopez, 1994). A new initiative, the Global Burden of Animal Diseases (Rushton *et al*., 2018) will address this issue by first estimating a health loss envelope – the difference between optimal output and actual observed output. Within this envelope, all disease losses will be placed, including the losses due to fasciolosis.

6.9 Control Options for Fasciolosis

The previously widespread strategic use of fasciolicides, in which all potential liver fluke carriers are treated at certain times of the year, varying from one to four times a year, is described in various studies (e.g. Parr and Gray, 2000). Nevertheless, and despite many years of research to control fasciolosis, this disease is currently not losing any of its importance worldwide. On the contrary, the incidence seems to be increasing globally (Fairweather, 2011). This is related to various factors, including climate change (as discussed above), that could play an important role (Gauly *et al*., 2013). This could even result in fasciolosis being an all-year-around problem in temperate regions under certain circumstances (Fox *et al*., 2011). Also, the intensification of land use by artificial irrigation channels (Alba *et al*., 2021) and environmental protection measures can lead to an increase in

suitable intermediate-snail habitats (Malan *et al.*, 2009). Environmental protection restricts drainage of wet pastures, the closure of open ditches and of course the use of molluscicides (Fairweather, 2011).

Another challenge is the increase in resistance to flukicides, above all triclabendazole but also albendazole (Fairweather, 2011; Carmona and Tort, 2017) (see Chapter 7, this volume). Further problems are the withdrawal periods for all drugs, which are a major challenge, especially in organic farming due to the extension (doubling) of the withdrawal period (Takeuchi-Storm *et al.*, 2019), as well as the limited availability of certain preparations in some countries (Keyyu *et al.*, 2009; Marques *et al.*, 2020).

The aim of up-to-date control must therefore be to avoid infection, if possible, by taking into account the epidemiological situation on the farms (Jones *et al.*, 2018) to decrease the infection pressure on the definitive hosts (Munita *et al.*, 2019), so that affected animals will not be completely free from the infection but nevertheless remain as healthy and economical as possible. To achieve this goal, the epidemiological situation on farms must be known in order to design an adapted control strategy which, in addition to treatments, includes pasture and feeding management (Knubben-Schweizer and Torgerson, 2015; Jones *et al.*, 2018). The correct implementation of adapted control recommendations has been shown to reduce the prevalence in affected herds (Knubben-Schweizer *et al.*, 2010). To reach this goal, farmers, veterinarians and consultants need to be sensitized to this parasitosis and trained in the possibilities of control options that go beyond strategic treatment (Schweizer *et al.*, 2005b; Fairweather, 2011; Munita *et al.*, 2016).

6.9.1 Control based on intermediate-host habitats

For a farm-specific control, on the one hand the affected group of animals and on the other hand the infectious grasslands must be identified (Fig. 6.2). If, for example, only the young stock become infected on a farm, but not the dairy cows, the control strategy will differ from a control strategy in an epidemiological situation where dairy cows get infected (Knubben-Schweizer and Torgerson, 2015; Takeuchi-Storm *et al.*, 2018). Usually, farmers ask for control recommendations if bulk-tank milk ELISA is positive. For a control strategy based on the epidemiological situation on the farm, as a next step the diagnosis must be confirmed using laboratory diagnostics: at least five animals from the group of young stock and at least five animals from the group of dairy cows should be examined for infection (Knubben-Schweizer *et al.*, 2010). Suitable methods are both serology and coproscopy (faecal egg counts) (FEC) as well as coproantigen ELISAs (Takeuchi-Storm *et al.*, 2018) (see Chapter 10, this volume).

The following should be considered when choosing the method for the initial assessment of a herd with suspected fasciolosis.

- *Coproscopy and coproantigen ELISA*. The lifespan of the parasite in cattle can be up to 26 months (Ross, 1968). If young stock become infected on young-stock pastures (e.g. alpine pastures), these animals still carry parasites when they return to their home farm. Without treatment, the infection persists until at least the end of the first lactation. In the case of dairy cows, coproscopy or coproantigen ELISA is therefore only recommended as the diagnostic procedure of choice from the second lactation onwards when the herd problem is first clarified, since an infection in the dairy-cow pastures can only be inferred from this point in time. More recent studies show a possibly even longer lifespan, which confirms that caution is required when interpreting the results of coproscopy or coproantigen ELISA to assess the epidemiological situation on a farm (Takeuchi-Storm *et al.*, 2018).
- *Serology*. In contrast to coproscopy, serology records an infection during the prepatent period, but antibodies persist for 6–9 months (in individual cases up to 18 months) (Hutchinson and Macarthur, 2003; Takeuchi-Storm *et al.*, 2018). Antibodies can therefore be easily detectable up to the second lactation after a natural infection was acquired in the second grazing season of young stock. Therefore, to clarify a herd problem with the aim of localizing the source of infection

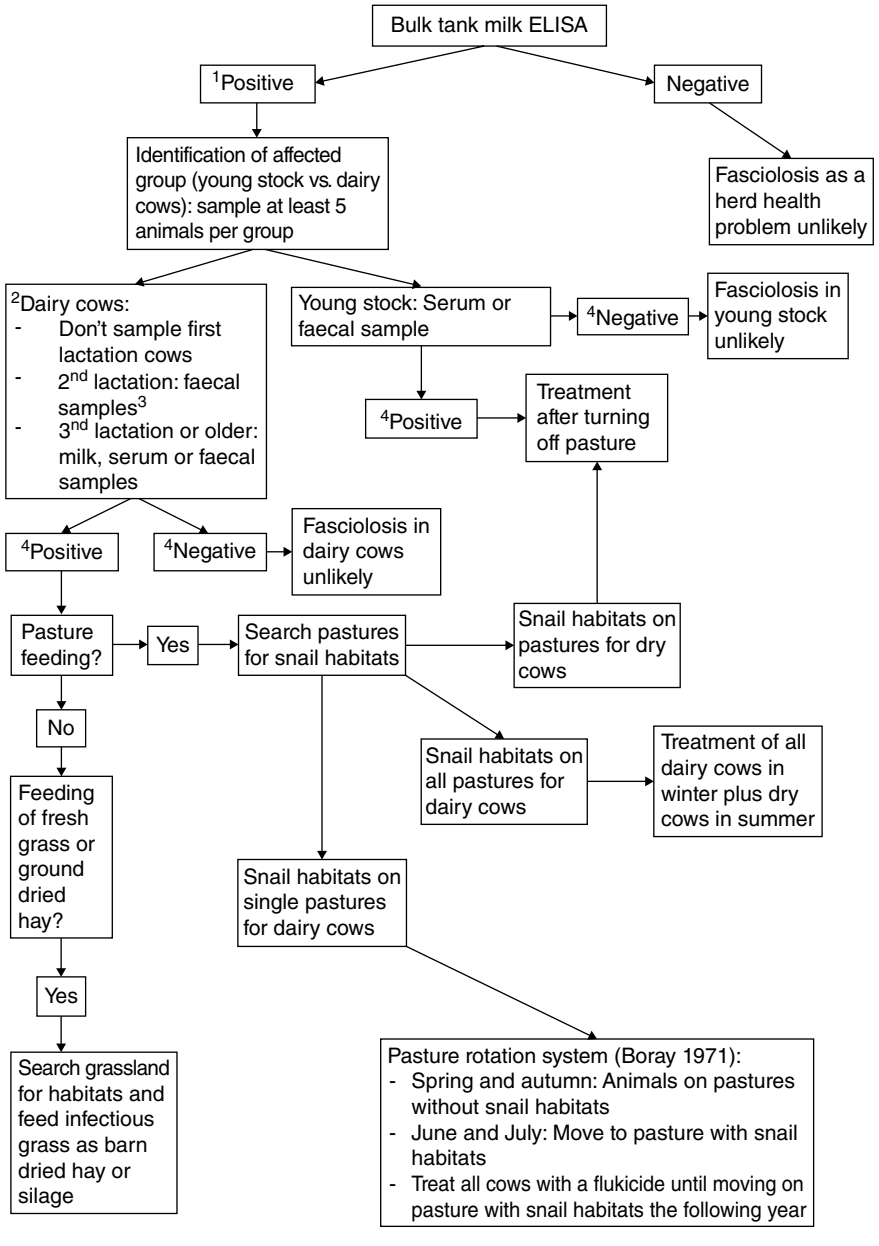

Fig. 6.2. Follow-up of herd health problem 'bovine fasciolosis' (Knubben-Schweizer and Torgerson, 2015). [1]Positive if at least 20% of the lactating cows shed antibodies (Duscher *et al.*, 2011). [2]Coproscopy (faecal egg counts): only animals, that are in the herd for at least one year. Serum and milk samples (ELISA): only animals that are in the herd for at least 2 years. [3]By examining 2×10 g of faeces, sensitivity can be increased from 69% to 86.1% (Rapsch *et al*., 2006). [4]Positive = at least one out of five samples positive; negative = all samples negative.

(young-stock pastures versus dairy-cow pastures), serology is only recommended from the third lactation onward.

- In addition, only animals that have been on the farm for at least 1 year should be examined. Animals bought in during the previous year should not be used for diagnosis in the herd. In the case of newly purchased animals, an examination should be carried out immediately after the purchase in order to avoid the spread of infections in the herd through the introduction of the parasite.

After confirming the diagnosis and identification of the affected animal group, the pastures should be examined for the presence of intermediate-snail habitats (Knubben-Schweizer *et al.*, 2010).

Evidence of permanent snail habitats is of crucial importance, namely small water sources that do not dry up, or that dry out only for a short time in the year. Temporary habitats such as slowly drying puddles along shady forest edges are to be classified as low risk provided that there is no permanent habitat in sight.

Typical permanent habitats for the intermediate host, *G. truncatula*, which predominantly occurs in Europe, are spring water, marshes, ditches, swampy banks of slow-moving streams and occasionally drinking troughs (Fig. 6.3) (Schweizer *et al.*, 2007; Takeuchi-Storm *et al.*, 2018). These permanent habitats usually offer the intermediate host suitable living conditions all year round (Heppleston, 1972) but can vary in their extent due to influence by environmental factors (Takeuchi-Storm *et al.*, 2018). The basic occurrence of such habitats in temperate zones mainly depends on factors such as groundwater, vegetation and water permeability of the soil and less on precipitation (Rapsch *et al.*, 2008; Kuerpick *et al.*, 2013). This also applies to the occurrence of the intermediate host, with extreme temperatures additionally having an influence on the snail population (Rößler, 2016). Rainfall does have an impact on the occurrence of temporary habitats (Fig. 6.3) (Heppleston, 1972); and their number and size, and thus the risk of infection, depend on the prevailing weather conditions, especially precipitation (Heppleston, 1972; Munita *et al.*, 2016). Floods can also lead to the appearance of temporary habitats.

As rainfall especially alters the size of permanent habitats and the number and size of

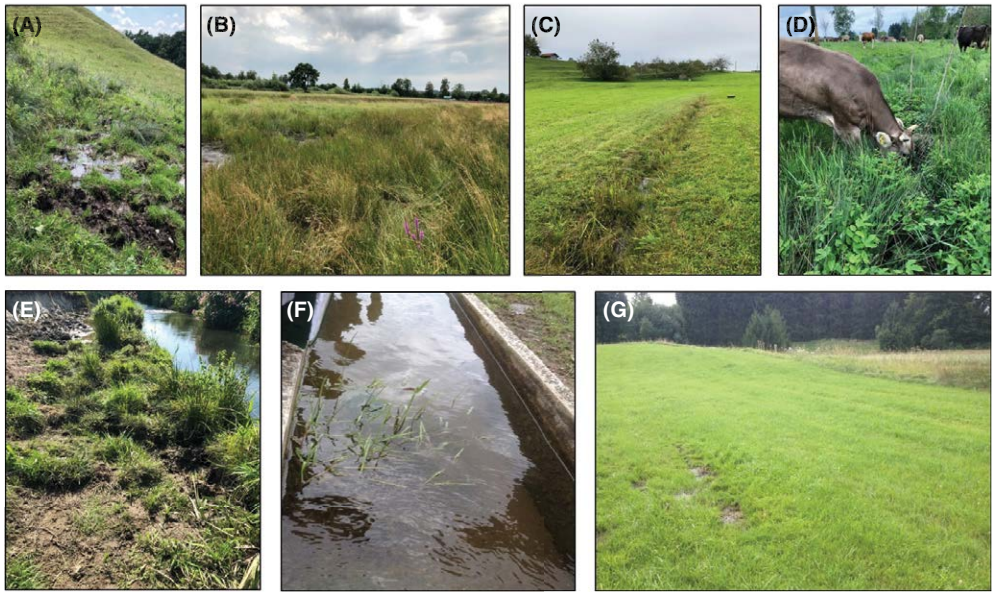

Fig. 6.3. **(A)** Spring water, **(B)** marsh, **(C, D)** drainage ditches, **(E)** swampy banks of a slow-moving stream and **(F)** drinking troughs are all suitable permanent habitats for *G. truncatula*. **(G)** Tractor tracks make a temporary habitat for *G. truncatula* next to a marsh (in the background).

temporary habitats, fencing off habitats (which is a control method often used in practice) is probably of limited use as fences in a distance of a few metres will not prohibit the snails from moving to nearby temporary habitats, especially in 'wet' years (Knubben-Schweizer and Torgerson, 2015). Figure 6.3 shows a tractor track in which *G. truncatula* was found. Fences can help keep definitive hosts away from habitats such as ditches (Fig. 6.3).

As mentioned above, chemical molluscicides (e.g. copper vitriol) (Mehl, 1932) are no longer a control option, due to environmental protection. Alternatively, calcium cyanamide is in use, with no known effect either on the snail or on the infection pressure (Bossaert *et al*., 1999). Plant-based molluscicides are subjects of current research (Isah *et al*., 2020).

Since the occurrence of permanent habitats is dependent on local conditions as well as farm management factors such as drainage and drinking troughs, grasslands with snail habitats can occur even in areas with a relatively low fasciolosis risk, and vice versa (Charlier *et al*., 2011). The identification of habitats at farm level is therefore an important prerequisite for farm-specific control based on the epidemiological situation on the farm (Knubben-Schweizer and Torgerson, 2015). Identifying habitats needs some expertise and is time-consuming (Beesley *et al*., 2018; Jones *et al*., 2018; Takeuchi-Storm *et al*., 2018) and there are various efforts to facilitate habitat identification, especially for inexperienced people. Rondelaud *et al*. (2011) identified plants as indicators, with sharp-flowered rush (*Juncus acutiflorus*) being often associated with the occurrence of *G. truncatula*. Research is also being carried out into methods for the detection of environmental DNA of intermediate host snails and free-living parasite stages from water and plant samples (Jones *et al*., 2018; Davis *et al*., 2020).

Classification of pastures

Fasciolosis is considered to be a herd problem on a farm if the infection has been diagnosed in at least one animal and if a habitat for the intermediate host has been found on at least one pasture. To develop an operationally adapted control strategy, the pastures are classified according to the type of use (young stock, dry cows, or lactating cows) and according to the risk of infection (pastures with snail habitats or pastures without snail habitats). Based on this classification, the farm can be assigned to one of the following control groups.

1. Young stock are affected and snail habitats are found on pastures for young stock.

The following control strategy is applied: after turning off pasture, the animals are ideally treated with triclabendazole in order to capture all parasite stages in the liver.

2. Dairy cows are affected and snail habitats occur on all dairy-cow pastures on the farm.

In this case too, the control strategy is by treatment. It is important to treat all cows in winter to prevent egg excretion in spring when the animals are turned out on pasture. Whether this happens at the beginning or at the end of the stable period depends largely on the active ingredient used. With this strategy, egg excretion on pasture is greatly reduced and, as a result, the parasite cycle is delayed. The success of the winter treatment has been demonstrated in sheep, among others (Taylor *et al*., 1994). Additional treatment of dry cows during the grazing period is recommended, as this can have a positive effect on the health and profitability of the dairy cow during the start phase of lactation (Charlier *et al*., 2012).

Dairy cows are affected and snail habitats are found on single dairy-cow pastures on the farm.

This epidemiological situation is frequently found in Switzerland (Knubben-Schweizer *et al*., 2010). In this case the pasture rotation system according to Boray (1971) is recommended. The most important principle is to prevent egg excretion on infectious pastures. This can be achieved by treating all animals with an effective drug prior to being moved to an infectious pasture. This is followed by a pasture rotation scheme: the cows should be moved to pastures without snail habitats in spring. In June and July, the animals can graze on pastures contaminated with metacercaria, but must be moved to pastures without snail habitats before the end of the prepatency period (8 weeks) so that excreted eggs cannot develop further. In the period between grazing from the infectious pastures and renewed grazing in the following year, all

animals must again be treated with an effective drug. With this method, infection pressure is assumed to decrease and might (especially in combination with regular diagnostics) even lead to a reduced use of medication.

In areas with year-round grazing, the following procedure is recommended by Boray (1971). From February to the end of May the animals should graze on snail-free lots. During this time, the animals should be treated and may be moved to pastures with snail habitats in June and July. Afterwards, the animals are brought back to snail-free pastures from August to the end of November, treated and allowed to graze on pastures with snail habitats again in December and January.

If dry cows are grazed separately from lactating cows on the farm, their pastures should also be examined. If snail habitats can be detected there, the procedure corresponds to that of the young animals (treatment after turning off pasture).

3. If snail habitats are present on hay fields, then grass from infectious hay fields should not be fed as grass or ground-dried hay (as metacercariae can survive for 4–6 months) but should be conserved as barn-dried hay or silage (Enigk and Hildebrandt, 1964; Enigk *et al.*, 1964).

4. Purchased animals must be examined before being moved to the infectious pastures to prevent the parasitosis from being reintroduced.

Various substances are available for treatment (see Chapter 7, this volume). Triclabendazole is the only active ingredient with good effectiveness against the adult and juvenile stages. This is therefore the drug of choice when treating during the grazing season. If treatment is carried out during the period in which the animals are kept indoors, substances that are primarily effective against the adult stages can be used. In this case, however, it should be taken into account either that the treatment is carried out twice, or that the time interval between the end of grazing and treatment is long enough to ensure that the active ingredient used is optimally effective (Munita *et al.*, 2016).

6.9.2 Strategic treatment

Strategic treatment without taking into account the epidemiological situation on a farm is the traditional way to control this parasitosis. It is also the only method of control if all dairy-cow pastures on a farm have snail habitats or if pasture rotation according to Boray (1971) is not possible for management reasons. Usually, all dairy cows are treated with a flukicide during the stall period. Munita *et al.* (2016) recommended using triclabendazole for this until the intensity of the infection has decreased significantly. From then on, other substances can be used alternately. In year-round treatments, four treatments with albendazole resulted in a significant reduction in the proportion of animals that excreted *F. gigantica* eggs compared with untreated animals (Keyyu *et al.*, 2009).

The disadvantage of both winter treatment and year-round treatment is the milk withdrawal time for all approved preparations. Therefore, the treatment of dry cows is recommended as an alternative. The use of closantel at the beginning of the dry period can under certain conditions have a positive effect on the milk quality (Köstenberger et al., 2017) or milk yield (Charlier et al., 2012) of the subsequent lactation.

6.9.3 Vaccination

Vaccination of livestock against fasciolosis would be a valuable tool to prevent disease and economic losses. Presently there are no commercially available vaccines, although work on the development of vaccines has shown considerable promise. Progress and strategies for new vaccine development is covered in detail in Chapter 12 of this volume.

6.10 Risk Maps, Models and Forecasting Systems

As the occurrence of fasciolosis is linked to environmental conditions, attempts have been made for many years to use meteorological data and soil data either to create maps from which the regional risk of the existence or occurrence of intermediate host habitats can be inferred (e.g. Tum *et al.*, 2004; Rapsch *et al.*, 2008) or to develop forecasting systems that predict the risk of infection for the coming grazing season based on past weather data (e.g. Ollerenshaw and Rowlands, 1959; Malone *et al.*, 1987).

The first forecasting model was created in the 1950s for Anglesey, a UK island off the coast of Wales. The influencing factors here were temperature and humidity (Ollerenshaw and Rowlands, 1959). This model was extended to the rest of England and Wales in the 1960s (Ollerenshaw, 1966) and later verified and further developed in Louisiana, USA. A model based on the Thornthwaite water budget was created, which serves as a measured value for soil and surface water availability and has the great advantage that the potential evapotranspiration can be calculated with sufficient accuracy from the few data that are available (Malone *et al.*, 1987). Another model from Northern Ireland, published in 1990, used a computer-based information system to obtain more accurate data on prevalence by linking a meteorological dataset to a slaughterhouse dataset. The authors found that the prevalence of the previous year had a major influence on the current prevalence (McIlroy *et al.*, 1990).

Beginning in the 1990s, the use of new technologies such as geographical information systems (GIS) significantly facilitated the development of spatial risk models for fasciolosis. Suitable software was able to incorporate climate and environmental data in equal measure to correlate them with epidemiological data on disease prevalence and in this way ultimately determine risk factors for the occurrence of a disease. Mapping the risk factors in the GIS then made it possible to predict the spread of a disease in areas for which no prevalence data were available and helped to explain emerging distribution patterns (Clements and Pfeiffer, 2009; McCann *et al.*, 2010). The advantage of GIS is that digital data, for example on soil types and hydrology, as well as on climatic conditions and prevalence (Malone's Red River Basin Model) (Malone *et al.*, 1992), are equally integrated into the program, displayed there in different layers, and correlations can be revealed (Malone *et al.*, 1998). Such combined datasets can be visualized graphically in the form of maps. The grid depends on the resolution of the data used.

Zukowski *et al.* (1991) were the first to attempt to predict the fasciolosis risk on an individual-farm level on the basis of a GIS model. Their GIS model of the habitats of *L. bulimoides*, which is considered an intermediate host for *F. hepatica* in south-west Louisiana, was based primarily on soil hydrological aspects. With the aim of site-specific habitat modelling for marshland that was used as pastureland, the authors revised it in 1993 by incorporating newly acquired knowledge about preferred snail habitats and other factors such as livestock. When compared with habitats known from earlier studies, their approach showed very good agreement (Zukowski *et al.*, 1993).

Malone *et al.* (1992) were also aware that previous models, which had placed the transmission in the context of annual climate fluctuations, were only suitable for regions, but not for use at farm level, as site-specific peculiarities such as soil hydrology, soil types, slopes and thus the the presence of habitats, but also management factors, were not included, or only to a limited extent. Based on the results of Zukowski *et al.* (1991), Malone *et al.* (1992) developed a comparable GIS model for the Red River Basin of Louisiana to take this knowledge into account. A review by Malone and Zukowski (1992) summarized that a suitable forecast system must combine the following information: climatic conditions such as rain and temperatures, taking into account fluctuations depending on the climatic zone and year; indicators of soil hydrology to check the existence of habitats; and finally models of population dynamics to develop and review control measures.

A few years later, with Malone's participation, two projects were launched in Ethiopia (Yilma and Malone, 1998) and East Africa (Malone *et al.*, 1998), both of which saw opportunities for the use of agroclimatic databases. In 1998, Yilma and Malone reverted to the prediction system developed in 1987 at Louisiana State University (Malone *et al.*, 1987) to be able to make GIS-based prognoses for the risk of infection with *F. hepatica* and *F. gigantica* in Ethiopia. The mathematical combination of potential evapotranspiration, growth degree-days and precipitation amounts resulted in a forecast index. A fasciolosis risk was only to be expected from index values above 600. Furthermore, correlations between the known distribution of fasciolosis, the forecast index and data on climate (precipitation, potential evapotranspiration, mean temperature), vegetation and terrain (slope, soil moisture, mean altitude above sea level) were tested. Regionally specific transmission patterns were discovered depending on the geographical location and the altitude above sea

level. For four different agroclimatic zones of Ethiopia a monthly forecast model resulted from this, from which the authors derived recommendations for strategic *Fasciola* control in each of the four zones. It has been successfully validated using prevalence data from the literature (Yilma and Malone, 1998).

In the same year a risk model was created for East Africa, also for *F. hepatica* and *F. gigantica*, using the model developed for Louisiana in 1987. Monthly precipitation data, temperature, potential evapotranspiration, Normalized Difference Vegetation Index (NDVI), vegetation period, altitude and irrigation status were included as variables in the risk model. The resulting forecast index, in combination with the GIS model, enabled forecasts that were consistent with prevalences from monitoring and literature data. There were differences in the distribution and frequency of occurrence of *Fasciola* spp. depending on the region (humid versus dry-hot). According to the authors, to be able to make location-specific predictions more precise data, for example daily instead of monthly climate data, would be necessary (Malone *et al*., 1998).

Without the aid of GIS, Bossaert *et al*. (1999a) developed a climate-dependent model based on the approach of Hope Cawdery *et al*. (1978). They looked at the individual steps in the cycle of the liver fluke, including the intermediate host population, to predict the time it would take before the development of the free-living parasite stages would be completed. They compared their model with serological and coprological data and concluded that precipitation within Belgium did not play a major role in the risk of fasciolosis. Rather, according to their findings, the temperature with a cut-off value of 10°C for snail activity and development of free-living parasite stages is the primary limiting factor (Bossaert *et al*., 1999b).

Tum *et al*. (2004) published a GIS model for *F. gigantica* in Cambodia. Flood, river proximity, land use, slope, altitude and the buffalo and cow density were used as factors. What is striking in comparison with the previous models is that neither temperature nor precipitation were used. This is because these are not important determinants in Cambodia. In contrast to snowmelt in temperate regions, precipitation hardly affects the annual flooding of the rivers and the temperatures change only a little throughout the year and are, therefore, favourable for the snail population all year round. In a subsequent validation of the model using faecal samples, overall good agreement was found, suggesting that the model is suitable for identifying high-risk areas with subsequent initiation of targeted control programs (Tum *et al*., 2007).

Four model classes were evaluated for Victoria, Australia, using an earlier dataset from a slaughterhouse survey. Areas of high prevalence were identified and these were explained based on prevailing environmental variables. Precipitation, irrigation, temperature-adjusted precipitation and NDVI were used as variables. It was found that irrigation had the greatest impact (Durr *et al*., 2005).

In southern Brazil, the prevalence of *F. hepatica* was also determined on the basis of liver examinations at slaughterhouses. The climatic and altitude conditions of the affected areas were then linked to the prevalence using a GIS (Dutra *et al*., 2010).

Since predictions on the risk of fasciolosis in the UK were still based on the Ollerenshaw index, as already mentioned, McCann *et al*. (2010) aimed to create a GIS-based fasciolosis model on a much more precise scale of postcode areas. Based on tank milk ELISA results, positive cattle herds were geo-referenced and associated with covariates in various multiple regression models. The latter included maximum, average and minimum daily temperatures and precipitation amounts from all five years before the sampling time, summarized in differently granulated datasets, information on the texture of the topsoil, soil pH and content of various chemical elements, the height above sea level, the gradient and the land quality. The model that most precisely explained the prevalence data showed that the amount of precipitation from the five years before the sampling was a better predictor for fasciolosis than the precipitation in the year of the sampling. In addition, the deviation of the maximum temperature in the year of sampling from the pattern of the previous years was a significant variable. In summary, the authors suggested that, in areas with year-round precipitation, the temperature should primarily be viewed as a limiting factor.

An extensive study was carried out by Cruz-Mendoza *et al*. (2011) in Mexico: sheep, goats and cattle were coprologically tested for fasciolosis and were repeatedly subjected to treatment with anthelmintics and fasciolicide.

Monthly averages, from data collected on a daily basis, of rainfall and soil temperature were correlated with the information on hosts and intermediate hosts. Snails and cercariae release were observed for 42 months in a hyperendemic region. Peaks in the snail population could be observed parallel to or immediately after periods of high precipitation. Four months later, infection peaks were recorded in the ruminant population. The effects of the use of fasciolicides can be read from the reduced number of cercaria about 5 months after therapy.

Forecast models have also been developed for Belgium in which the relationship between management, climate and environmental factors was examined and their influence on the spatial distribution of *F. hepatica* infections. For this purpose, three different models were developed and then compared with the prevalence data determined using ELISA from tank milk samples. The best model contained both climate and environmental as well as management variables and consisted of the following determinants: annual precipitation; mowing of pastures; proportion of cut grass in forage; and length of the grazing season. This combined model described the spatial distribution of *F. hepatica* infections better than models that contained only climate and environmental variables or only management factors. This study shows that it is important to include management factors in the risk calculation (Bennema *et al*., 2011).

In 2011 and 2014 two additional studies were presented on the spatial distribution of fasciolosis and its risk factors in Belgium (Charlier *et al*., 2011, 2014). Fine-scale risk models were necessary because farms with and without fasciolosis could coexist in high-risk and low-risk areas. Based on the results of Zukowski *et al*. (1993), 39 Belgian farms were investigated serologically using tank milk ELISA, and associated pastures were searched for habitats and intermediate hosts. Habitats were geo-referenced and linked in a GIS with data on the management and known risk of the areas. As the occurrence of intermediate host snails had a strong influence on exposure to *F. hepatica*, even stronger than the number of potential habitats themselves, the type of cattle watering, the drainage of pastures and the time of turnout on pasture, risk factors for the colonization of potential snail habitats were determined. Larger habitats were more likely to harbour *G. truncatula* or *Radix* spp. and loamy soils favoured the occurrence of snails.

To create a further basis for a fine-scale risk map, Charlier *et al*. (2014) investigated four farms that reacted positively to *F. hepatica* in the tank milk ELISA over the entire pasture season. Habitats were identified and classified. To describe the population dynamics of *G. truncatula*, a monthly inspection of potential habitats was carried out, including the search for snails, differentiation between old and young snails and temperature and pH measurements of water and soil on site. The occurrence of so-called indicator plants, the growth of which is likely to be positively correlated with the occurrence of snails, was also taken into account. Attention was also paid to management factors such as fencing or mowing the habitats. Temperature and precipitation values from a weather station 18 km away were subjected to validation on the basis of values measured on site. To understand the course of the infection in the herds, blood and faeces were examined before, after and during the winter after the grazing period. The results demonstrated a close correlation between air and soil temperature. The occurrence of *G. truncatula* also proved to be positively correlated with the type of habitat, measured amounts of precipitation, the soil and water pH value and the presence of *Ranunculus* spp. in the habitat. There was a negative correlation for the factors 'mowing' and 'water temperature'. No correlations could be found between the snail frequency and the herd prevalence, which was attributed to the fact that some animals had been treated and, in addition to *G. truncatula*, other snails that had not been examined could also be considered as intermediate hosts. Nevertheless, the habitats should be viewed as the bottleneck of liver fluke transmission; in the future they could be more easily recorded using aerial images from drones, for example, and incorporated into a GIS for model generation together with the above-mentioned influencing factors.

Since the detection of potential intermediate-host habitats by means of field surveys or photographs is associated with a great deal of effort, De Roeck *et al*. (2014) suggested the use of a high-resolution satellite-based remote sensing system. Using the data from the study by Charlier *et al*. (2011), a method for the semi-automated detection of potential habitats such as ponds, ditches and wetlands was successfully developed.

These data were also used to evaluate the methodology. This enabled the detection of bodies of water with an area of more than one square metre with an accuracy of 87% and was able to identify even wetlands. Data collected in this way could be incorporated into future GIS models.

Kuerpick *et al.* (2013) collected data on the seroprevalence of fasciolosis in German dairy herds using tank milk samples. In 2012, these were integrated into a GIS at the postcode area level together with presumed risk variables. The latter included the following factors: livestock and farm density; monthly averages of temperature, precipitation and humidity in the year of sampling; type of land use; and proportion of the area of standing water in the respective postcode area. No influence of precipitation on the seroprevalence could be found. A positive correlation was found, among other things, between the prevalence data and the proportion of grazed areas and the bodies of water per postcode area. This can be explained by the biology of the intermediate host and grazing as the main source of infection for cattle. However, the model indicated that other factors that were not taken into account could also play a role.

A model developed in Iran assumes a strong connection between climatic conditions and fasciolosis outbreaks. Linking the Ollerenshaw index and GIS-associated data resulted in a climate-based risk map for Iran, which identified four risk classes according to various indices. Overall, it was judged that only 3% of the country's area bears a high risk of fasciolosis due to heavy rainfall in summer and autumn (Halimi *et al.*, 2015).

A large study in Ireland of over 4000 dairy farms indicated that 82% had been exposed to *F. hepatica* infection (Selemetas and deWaal, 2015; Selemetas *et al.*, 2015). A total of 108 variables, including averaged climatic data for the period 1981–2010 and contemporary meteorological data for the year 2012, such as soil, subsoil, land cover and habitat maps, were investigated for a possible role as predictor of fasciolosis. A total of 74 high-risk clusters and 130 low-risk clusters of fasciolosis were identified. Several climatic variables (monthly and seasonal mean rainfall and temperatures, total 'wetdays' and 'raindays') and environmental datasets (soil types, enhanced vegetation index and normalized difference vegetation index) were used to investigate dissimilarities in the exposure to liver fluke between clusters. Rainfall, total wetdays and raindays, mean temperature and soil type were the significant classes of climatic and environmental variables explaining the differences between significant clusters. The risk model presented 100% sensitivity and 91% specificity and an accuracy of 95% correctly classified cases. A risk map of exposure to liver fluke was constructed with higher probability of exposure in western and north-western regions. The model obtained by means of the random forest method recognized primarily precipitation-associated parameters as influential. In contrast to the conclusions of McCann *et al.* (2010), the climatic parameters close to the sampling time had higher influence than the values of previous years. In addition, daily measured values could be more informative than monthly or seasonal averages. Among the other parameters, only the soil type was of moderate relevance with regard to the risk of fasciolosis.

Based on tank milk ELISA results from Belgium, Germany, Ireland, Poland and Sweden, two modelling approaches were pursued in parallel within the GLOWORM project, namely random forests and boosted regression trees. They included 49 co-variables, including surface temperature, vegetation indices and rainfall values. The clustering of data points represented an important intermediate step to do justice to the heterogeneity of the investigated area and the limiting factors that presumably vary depending on the region. Both models revealed temperature and precipitation as the most important factors in predicting exposure risk for fasciolosis. The vegetation did not seem to play a major role in this. For many regions, especially from different climatic zones, the data required for large-scale modelling was still lacking (Ducheyne *et al.*, 2015).

An analysis by Olsen *et al.* (2015) studied the relationships between prevalence data based on the results of official meat inspections and potential risk factors for the occurrence of fasciolosis in Denmark. Details on herd size and composition, animal purchase and data on the characteristics of the environment were taken into account. Hotspots for *F. hepatica* infections could be determined via cluster analysis. The presence of watercourses, wetlands and pastureland was positively associated with the risk of fasciolosis. Cultivated areas and arid areas revealed negative correlations with the risk of

infection. The risk could be increased by purchase and being in neighbourhoods of herds that tested positive. Likewise, non-dairy herds of medium size were characterized by an increased risk of fasciolosis.

For Switzerland, an interactive map with a grid of 100 m × 100 m was designed modelling the monthly risk for the occurrence of *G. truncatula* and the free-living stages of *F. hepatica* (Rapsch *et al*., 2008). The factors used were temperature, precipitation, soil types including groundwater and forest cover. The interactive map was then evaluated by means of a field survey taking different data sources for the occurrence of *G. truncatula* into account. This demonstrated moderate sensitivity, specificity and predictive values of the model (Baggenstos *et al*., 2016). It was assumed that it is difficult to simultaneously model local microclimatic factors and ground condition as well as artificial habitats. This issue has been noted previously; since most habitats are locally limited habitats, the aim should be to create risk maps at farm level, taking management factors also into account (McCann *et al*., 2010; Bennema *et al*., 2011; Charlier *et al*., 2011; De Roeck *et al*., 2014).

In Germany, a risk map for one farm was developed and validated (Schade-Weskott, 2019). The risk map was based on a regression model predicting the occurrence of *G. truncatula* using a generalized linear mixed model which was based on 13 covariates, of which eight of the variables were terrain and soil dependent and seven were temperature variables (Rößler, 2016). For the validation the farmland was divided into grid fields of 100 m × 100 m. The risk per grid field was calculated, allocated to one of three risk categories and subsequently coloured according to that category. The validation of the map was performed by conducting ten field surveys and a risk map was generated for each field survey. Furthermore, a median map was created, providing an overview for the entire time frame of the field surveys (Fig. 6.4). The validation showed, that even though the median map was well suited to depict the actual predominant conditions, the modelled risk of the ten individual maps was generally overestimated. Thus, even though risk maps can be beneficial for the awareness of farmers, veterinarians and consultants for a potential fluke problem on a farm or in a certain region, in order to assess the epidemiological

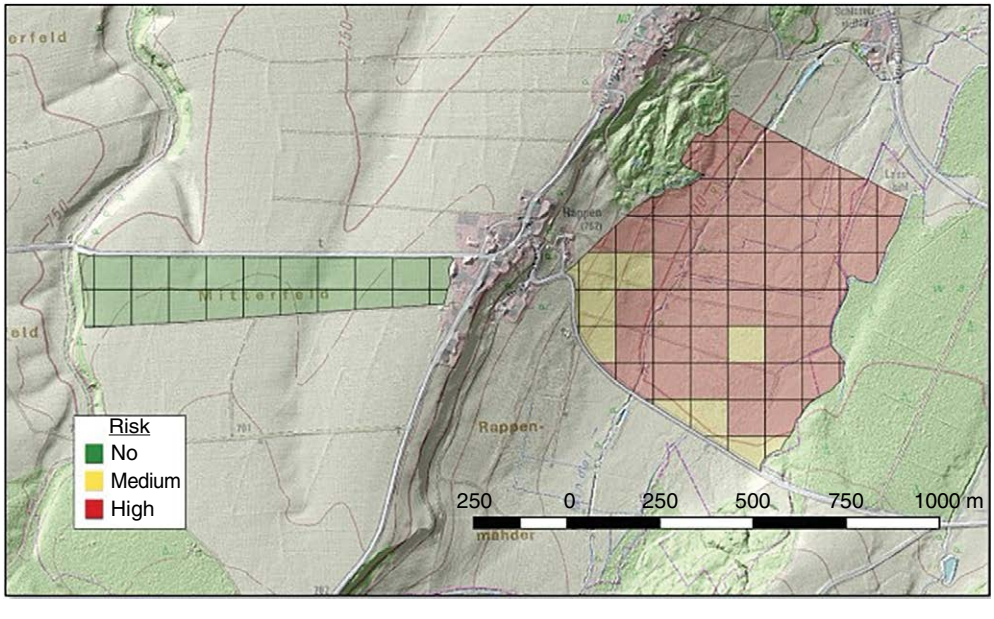

Fig. 6.4. Example of a risk map for the occurrence for *G. truncatula* at farm level. The map shows the median yearly risk arising from the grassland on a 100 m × 100 m grid basis on one dairy farm in Germany with fasciolosis as a herd health problem (Schade-Weskott, 2019).

situation at farm level a farm visit and evaluation of grassland are of central importance.

6.11 Conclusions

As long ago as 1883 it was suggested that fasciolosis was preventable and that control measures should be integrated (Thomas, 1883). Although Thomas's reasoning may have been influenced heavily by the lack of effective fasciolocides, it still has applications today and adequate control should involve a range of the methods described in this chapter. In all cases, however, local knowledge of the disease epidemiology is vital to maximize the efficacy and cost effectiveness of *Fasciola* control.

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7 Flukicidal Drugs: Pharmaco-therapeutics and Drug Resistance

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7.1 Introduction

The purpose of this chapter is to review our current understanding of the action of flukicides against *Fasciola hepatica* and *Fasciola gigantica*, particularly the former given the greater body of work reported in the literature for this species. We illustrate how the drugs work and how they are absorbed, distributed, metabolized and excreted by the host, which in turn dictates exposure of the parasite to the active drug or drug metabolites. To achieve optimal efficacy, it is vital that a given drug can gain access and accumulate within the parasite, hence we report on what we know about the main route of drug entry into *F. hepatica*. Finally, as we face the threat of drug resistance in *Fasciola* spp., we consider how we can determine when a parasite population has become resistant to a given drug and how they have evolved and adapted to overcome its effects, especially to the highly effective drug triclabendazole (TCBZ).

7.2 Pharmaco-therapeutics

The efficiency spectra of drugs used to treat *Fasciola* spp. liver fluke infections at the time of the first edition of this book were summarized in Tables 7.1 and 7.2 in that edition (Fairweather and Boray, 1999a). Since that time, some drugs have fallen out of use, while new formulations (e.g. pour-ons) and new combinations of drugs have come on to the market, including: (i) closantel (CLOS) + albendazole (ABZ) oral drench; (ii) CLOS + Abamectin oral drench; (iii) CLOS + oxfendazole (OXF) oral drench; (iv) CLOS + ABZ + Abamectin + levamisole (LEV) oral drench; and (v) TCBZ + ivermectin (IVM) pour-on. The majority of flukicides focus on the control of *F. hepatica*; consequently, an updated summary of the efficacy of drugs used to treat *F. hepatica* infections in sheep and cattle is presented here in Table 7.1. Figure 7.1 gives a spectrum of efficacy of drugs used in sheep against weekly stages of fluke development.

The developmental stage of a fluke that is killed is probably of greater significance than the precise age of the fluke. Location in the host is important, as flukes will be in very different physiological environments if they are in the liver parenchyma compared with the bile ducts, which will impact on what the flukes are exposed to in terms of the drugs and their metabolites. For instance, following TCBZ treatment in

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Table 7.1. Efficacy of drugs used to treat infections of *Fasciola hepatica* in sheep and cattle.

Anthelmintic	Route of administration	Stage of fluke killed	Minimum age of fluke killed (efficacy ≥ 90%)	Use for sheep	Use for cattle
Nitroxynil	Injection	From late immature	8 weeks (sheep)	√	√
		Adult	10 weeks (cattle)	√	√
Oxyclozanide	Oral	Adult	≥ 12 weeks	√	√
Closantel	Oral	From late immature	6–8 weeks	√	X
Closantel	Injection	Adult	≥ 12 weeks	X	√
Clorsulon	Oral	From late immature	8 weeks	X	√
Clorsulon	Injection	Adult	≥ 12 weeks	X	√
Albendazole	Oral	Adult	≥ 12 weeks	√	√
Triclabendazole	Oral	From early immature	1 week	√	√
Oxyclozanide + levamisole	Oral	Adult	≥ 12 weeks	√	√
Closantel + mebendazole	Oral	From late immature	6–8 weeks	√	X
Closantel + ivermectin	Injection	From late immature	7 weeks	√	√
Closantel + ivermectin	Pour-on	From late immature	7 weeks	X	√
Clorsulon + ivermectin	Injection	Adult	≥ 12 weeks	X	√
Triclabendazole + ivermectin	Oral	From early immature	1 week	√	√
Triclabendazole + moxidectin	Oral	From early immature	1 week	√	X
Triclabendazole + moxidectin	Pour-on	From late immature	6–8 weeks	X	√
Triclabendazole + abamectin	Pour-on	From late immature	6–8 weeks	X	√
Triclabendazole + levamisole	Oral	From early immature	2 weeks	√	√
Triclabendazole + oxfendazole	Oral	From early immature	2 weeks	√	√
Nitroxynil + clorsulon + ivermectin	Injection	From early immature	2 weeks	X	√

√, drug formulation used in host; X, drug formulation not used in host

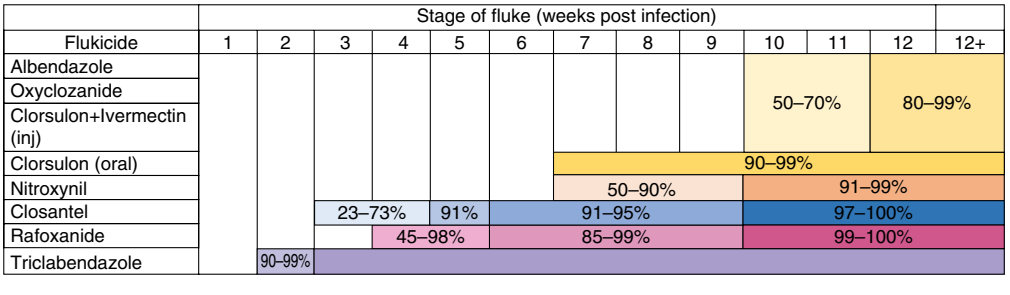

Fig. 7.1. Spectrum of efficacy of drugs at recommended dose rates against drug-susceptible *Fasciola hepatica* in sheep. Efficacy is shown against stages of fluke development in weekly intervals, based on drug doses recommended at licensing of product.

sheep, large differences in TCBZ-sulfoxide (TCBZ.SO) concentration levels have been observed at 24 h post-treatment (pt) among the different tissues/fluids where the fluke can be exposed to the drug, including liver (0.41 µg/g), plasma (10.8 µg/ml) and bile (15.2 µg/ml) (Moreno *et al.*, 2014). Schemes have been put forward to link age of fluke to stage of development (Wood *et al.*, 1995; Behm and Sangster, 1999). For the purposes of drug efficacy and drug resistance we will use the following developmental stage terms.

- Early immature flukes have penetrated the liver capsule and are migrating through the liver parenchyma: ~1–5 weeks post-infection (pi).
- Late immature flukes have completed the liver migration phase and are entering the bile ducts, though are not yet mature: ~6–8 weeks pi.

- Mature adult flukes are resident in the bile ducts and are producing eggs: ~9–14 weeks pi onwards.

Fluke development is inherently variable. For example, flukes develop more quickly in sheep than in cattle and there is variation amongst flukes in the same animal, between host animals and in how the infection is acquired (Robinson *et al.*, 2001). In addition, it has been shown that different fluke isolates develop at different rates (Fairweather, 2011a). In clonal infections, where multiple genetically identical flukes infect the liver, a range of sizes and differences in fecundity amongst clones was observed, likely due to the different environments within the liver (Hodgkinson *et al.*, 2018). To complicate matters further, it is known that label claims for efficacy can vary as a result of differences in formulation of the same drug manufactured by different companies and regulatory schemes operating in different countries (Love, 2017). In other words, the same active drug may be prepared at different dosages with different inactive excipients and in different forms for different routes of administration. It is also known that different generic TCBZ formulations display different pharmacokinetic behaviour, which could affect their flukicidal activity (Ortiz *et al.*, 2014). This argues for better quality control in the drug approval process.

7.2.1 Mode of flukicidal action

The intention here is to focus on developments since publication of the first edition (Fairweather and Boray, 1999a). To date, by far the majority of drug efficacy studies have been performed for *F. hepatica* and unless otherwise stated the details in this chapter refer to that species of liver fluke. A number of ultrastructural studies using comparative scanning and transmission electron microscope (SEM and TEM, respectively) have been carried out on liver flukes exposed to drugs *in vitro* or recovered from drug-treated hosts. Of particular note is the challenge we face in having drugs that will effectively target drug-resistant parasites, particularly populations resistant to TCBZ.

7.2.1.1 Benzimidazoles

TRICLABENDAZOLE. TCBZ is a halogenated benzimidazole (BZ) compound that, unlike the BZ methylcarbamate compounds (i.e. ABZ), holds an excellent efficacy against immature and mature stages of *F. hepatica*. TCBZ is administered orally to cattle (12 mg/kg), sheep (10 mg/kg) and goats (15 mg/kg). There is also a TCBZ formulation available for use in humans (Egaten®, Novartis; 250 mg/tablet, 10 mg/kg in two doses given 12 h apart). TCBZ possesses extremely high efficacy against all intra-mammalian stages of fluke, both adult and juvenile, down to 1-week-old flukes (Boray *et al.*, 1983; Smeal and Hall, 1983; Turner *et al.*, 1984). At elevated dose levels, TCBZ has good activity when given to sheep 1 day pi (Boray *et al.*, 1983). Following its administration, TCBZ is metabolized (by both host and fluke) into a number of metabolites: TCBZ.SO, TCBZ sulfone (TCBZ.SO_2), hydroxy-TCBZ (OH-TCBZ), hydroxy-TCBZ sulfoxide (OH-TCBZ.SO) and hydroxy-TCBZ sulfone (OH-TCBZ.SO_2) (Hennessy *et al.*, 1987; Mottier *et al.*, 2004; Virkel *et al.*, 2006; Mestorino *et al.*, 2008). TCBZ.SO is considered the active form of the drug, but TCBZ.SO_2 has been shown to cause a 41% reduction in fluke burden against 4-week-old fluke infection in sheep (Büscher *et al.*, 1999).

Results of studies carried out *in vivo* on the time-course of TCBZ action have revealed changes to the main tissues that were similar to those observed *in vitro*, strengthening the notion that TCBZ targets microtubules in the fluke (for specific details see Toner *et al.*, 2011; Hanna *et al.*, 2015a and references cited therein). In summary, a severe necrosis of the posterior half of the body was seen at 72 h pt, and by 96 h pt the tegument had been completely stripped off, with most effects seen between 48 h and 72 h pt. Egg formation had ceased at 48 h pt and the testis was most affected. TCBZ targeted spindle formation in all actively dividing cells, triggering a cascade of events that led to apoptosis in the testis, vitelline tissue and ovary. The time-course of drug-induced changes seemed to fit well with the pharmacokinetics of TCBZ in the host and the pattern of movement of flukes from the bile ducts to the gall bladder prior to their ultimate expulsion.

A comparative SEM and TEM study carried out *in vitro* on the effect of TCBZ, TCBZ.SO and

$TCBZ.SO_2$ on adult flukes showed that surface disruption caused by the three compounds was similar, but the disruption caused by parent TCBZ was slightly greater. Internal changes were greatest following $TCBZ.SO_2$ treatment and, while TCBZ.SO was also disruptive, TCBZ was far less so. Combining the results for surface and internal changes, the order of severity of disruption was $TCBZ.SO_2$ > TCBZ.SO > TCBZ (Halferty *et al*., 2009a). As the level of $TCBZ.SO_2$ peaks after that for TCBZ.SO, it may further disrupt flukes already affected by the action of TCBZ.SO. The hydroxy forms of TCBZ.SO and $TCBZ.SO_2$ have also been shown to be capable of disrupting the tegument of *F. hepatica* (I. Fairweather, unpublished observations), so overall drug action may be due to the combined effect of several metabolites.

With respect to the timescale of drug action, two studies were carried out in sheep, one against early immature (4-week-old) flukes and one against mature adult (12-week-old) flukes. Detachment and movement of adult flukes from the bile ducts to the gall bladder occurred between 48 h and 72 h pt; and by 96 h pt very few flukes remained, all of which were dead (Toner *et al*., 2010a). A similar time-line operated with immature flukes (Halferty *et al*., 2008). A separate study has shown that egg production stops 24 h after treatment with TCBZ (Hanna *et al*., 2012). Stunting and suppression of egg production in adult flukes surviving treatment with TCBZ and the delayed development of immature flukes following treatment have been described by Büscher *et al*. (1987).

ALBENDAZOLE. ABZ is the only BZ methylcarbamate compound available to control fasciolosis in domestic animals (McKellar and Scott, 1990). It is recommended for the treatment of mature *F. hepatica* (> 14 weeks old) at the oral dose of 7.5 mg/kg (sheep) or 10 mg/kg (cattle). Dosing differences between sheep and cattle are related to the higher systemic exposure observed for the ABZ active metabolite, ABZ-sulfoxide (ABZ.SO) in sheep compared with cattle. ABZ is marketed in three forms: as the pro-drug netobimin; as ABZ itself; and as the active sufoxide metabolite (ABZ.SO, or ricobendazole). Incubation in ABZ.SO led to a block of the transport of secretory bodies from the tegumental cells to the tegumental surface membrane, breakdown of the Golgi complexes in the cell bodies and a reduction in tubulin immunostaining within the tegumental system (Buchanan *et al*., 2003; McConville *et al*., 2006). Moreover, it has been shown that liver fluke β–tubulin isotype 2 binds ABZ and so is a potential target of the drug (Chambers *et al*., 2010). ABZ is effective against adult TCBZ-resistant (TCBZ-R) fluke, implying that it may bind to a different site on the β-tubulin molecule than TCBZ, an idea supported by published data (Robinson *et al*., 2004b; Ranjan *et al*., 2017) (see section 7.2.1.5).

ABZ causes stunting of flukes and severe disruption of the reproductive system (Lang *et al*., 1980; Masaba, 1981; Malone *et al*., 1982; Carneiro *et al*., 2019). Flukes recovered 5–6 weeks pt showed gross degeneration of the testes, ovary and vitelline cells, and fewer eggs than normal were observed in the uterus. Moreover, the eggs were abnormal and displayed reduced hatchability for 3 weeks pt (Lang *et al*., 1980). Severe degenerative apoptotic changes in the testis tubules of *F. hepatica* over a 4-day period post-treatment with ABZ was observed, which led to a virtual cessation of sperm production (Carneiro *et al*., 2019). Inhibition of the embryonation of fluke eggs has also been demonstrated by Coles and Briscoe (1978) and by Alvarez *et al*. (2009), a property that has been exploited for the diagnosis of ABZ resistance (see section 7.3.1).

A relatively rapid time-frame for action of ABZ against *F. hepatica* can be inferred from morphological studies, where reductions of 94% and 95% were seen at 48 h pt and 96 h pt, respectively. The number of eggs per gram of faeces also declined by 93% at 48 h pt, dropping further to 99% at 96 h pt (Carneiro *et al*., 2019).

COMPOUND ALPHA. Compound alpha is a TCBZ derivative, with a similar spectrum of activity, capable of killing flukes as young as 3 days old in both sheep and cattle, and is also active against newly excysted metacercariae *in vitro* (Hernández-Campos *et al*., 2002; Ibarra *et al*., 2004; Montenegro *et al*., 2004). The pharmacokinetics of compound alpha are similar to those reported for TCBZ (see above). Morphological studies *in vitro* and *in vivo* reported the impact of compound

alpha on multiple tissues within the fluke consistent with anti-microtubule action and indicate that compound alpha may share the same tubulin target with, and have a similar mode of action to, TCBZ. However, the action of compound alpha may be more complex. It disrupts several structures: the tegument of TCBZ-R *F. hepatica* without affecting tubulin immunostaining the sub-tegumental muscle blocks and the spines, all of which points to an action directed against actin (see McConville *et al.*, 2012 and references therein). This idea is supported by the presence of multinucleate cells in the testis of treated flukes, presumably as a result of inhibition of cytokinesis; a similar phenomenon was observed following incubation with the microfilament inhibitor, cytochalasin B (Stitt and Fairweather, 1991).

Following treatment with compound alpha, a ~40% reduction in numbers of adult flukes was noted at 72 h (McConville *et al.*, 2012). Egg formation stopped within 24 h, but the main changes to the tissues occurred between 48 and 72 h pt. The effect was more rapid with immature (4-week-old) flukes than with adult flukes: at 72 h pt, almost 90% of immature flukes were dead, compared with only 23% of adult flukes. However, about 50% of recovered flukes displayed a discoloration in the midbody region, which coincided with the loss of the tegument.

Compound alpha showed efficacy against adult and immature stages of the Sligo TCBZ-R isolate *in vitro,* but not *in vivo* (sheep), where no reduction in fluke burden was seen at 3 days, 4 weeks and 12 weeks pi. Consequently, the potential of compound alpha to replace TCBZ for the treatment of TCBZ-R fluke infections may be limited unless activity against TCBZ-R flukes can be demonstrated.

7.2.1.2 Salicylanilides

CLOS is a salicylanilide-derived compound, used in cattle and sheep for treating liver fluke ≥ 7–8 weeks old (Mohammed-Ali and Bogan, 1987). CLOS is a lipophilic drug formulated for oral and/or sub-cutaneous (sc) administration. It acts on the fluke by disrupting energy metabolism, since it is an uncoupler of oxidative phosphorylation (Fairweather and Boray, 1999a,b) and induces a rapid spastic paralysis of the fluke at therapeutically relevant concentrations (Skuce and Fairweather, 1990).

Given the impact of CLOS on energy metabolism, it is not surprising that it has an effect on those tissues that have a high energy requirement, namely, the reproductive organs and especially the vitelline cells that provide the protein material for eggshell formation and nutrients for the developing embryo (Hanna *et al.*, 2006). The latter have a very high rate of secretory activity, cell division and turnover to maintain the prodigious level of egg production. CLOS has been shown to cause clastogenic changes, including chromosomal abnormalities and misshapen sperm, to somatic and germ cells of mice following treatment (Donya and Hassan, 2007), an action that may be relevant to its effect on liver fluke.

While CLOS is less effective against immature flukes (Table 7.1), it has been shown that treatment of 5-week-old flukes in cattle leads to a retardation of their development and an extension of the pre-patent period. Surviving flukes were also smaller than normal and released fewer eggs, although the eggs hatched normally (Hanna *et al.*, 2006). Similarly lower egg embryonation rates with a delayed start of egg production were reported from flukes recovered from CLOS-treated hosts (Maes *et al.*, 1988). A low ovicidal activity of CLOS against eggs from untreated flukes has also been observed by Ceballos *et al.* (2017). Solana *et al.* (2016) reported an altered shape for eggs recovered from bile after treatment of an adult infection in sheep and the percentage of embryonated eggs that hatched was significantly reduced. Stunting of flukes following treatment has also been reported for the related salicylanilide, RAFOX, by Presidente and Knapp (1972), Stammers (1975a) and Mohammed-Ali and Bogan (1987).

The time-course of CLOS action appears to be quite rapid; the number of flukes recovered declined sharply at 24 h and 36 h pt (sheep infection: Scarcella *et al.*, 2016). At 2 days pt, only inactive, flaccid and partially fragmented flukes were recovered (sheep infection: Hanna *et al.*, 2015a) and all flukes had been eliminated by 3 days pt (rat study: Skuce and Fairweather, 1990). Severe and widespread disruption of the tegument and gut of the flukes was seen at 36 h

and 48 h pt in each of the studies, along with changes to the reproductive organs.

There is a correlation between the efficacy of salicylanilides such as CLOS and RAFOX and the level of glutathione *S*-transferase (GST) activity in flukes (Miller *et al.*, 1994); this is discussed in greater detail in section 7.6.1. In addition, the interaction of salicylanilides with β–tubulin and with P-glycoprotein (Pgp)-linked drug efflux pumps is discussed in section 7.6.1.

7.2.1.3 Halogenated phenols

Nitroxynil (NITROX) is a trematodicidal compound highly effective against mature stages (from 8 weeks pi) of *F. hepatica* (Boray and Happich, 1968), which also holds nematocidal activity. There has been an assumption that NITROX disrupts energy metabolism in the fluke, as an uncoupler of oxidative phosphorylation. However, there is little direct evidence for this from experiments with *F. hepatica* itself (see Fairweather and Boray, 1999a,b). Perhaps of greater significance is the rapid spastic paralysis of flukes observed at therapeutic drug concentrations (Fairweather *et al.*, 1984).

Early morphological studies carried out *in vivo* (in sheep, rabbits and rats) showed that NITROX affected the reproductive system of *F. hepatica*, with the testes consistently being most sensitive to drug action, with changes evident within 4 h of treatment (Stammers, 1975a,b, 1976). NITROX is known to be capable of inducing clastogenic effects in somatic and germ cells of mice following treatment (Donya and Hassan, 2007). Delayed development of the reproductive system and stunting of the fluke were observed following treatment, with fewer eggs produced by the fluke *in vivo* as a result of the disruption of oogenesis, possibly due to atrophy of the ovary; egg hatchability was also reduced (Stammers, 1975a, 1976). Inhibition of growth and delayed development of immature flukes surviving treatment have also been observed by Lucas (1967).

NITROX caused progressively severe disruption to the surface morphology of the tegument, as exemplified by blebbing, swelling, loss of spines and tegumental sloughing, which was focused at the anterior end and dorsal surface, possibly as the result of the internal changes to the tegumental syncytium and tegumental cells. The gut was more severely affected than the tegument *in vivo*, with disruption of the gut lamellae, swelling of the mitochondria, dilation of the cisternae of the granular endoplasmic reticulum (GER), decreased production of secretory bodies and increased numbers of autophagic bodies. Interestingly, the extent of the fine structural changes induced by NITROX is different across fluke isolates (see McKinstry *et al.*, 2009 and references therein). Ranking isolates according to their susceptibility to NITROX – Cullompton (TCBZ-susceptible, or TCBZ-S) > Sligo (TCBZ-R) > Oberon (TCBZ-R) > Fairhurst (TCBZ-S) – does not correspond to their susceptibility to TCBZ: Cullompton > Fairhurst > Oberon > Sligo (Coles and Stafford, 2001; Walker *et al.*, 2004).

The time-course of drug action appears to be quite rapid, as flukes retrieved 2 days pt were dead, showing signs of autolysis and disintegration (Hanna *et al.*, 2015a). Following treatment of infected rabbits, dead flukes were recovered 24 h pt, but none were necrotic (Stammers, 1975b). In one study in rats, no flukes survived 24 h after treatment (Stammers, 1975b), while dead flukes were recovered at 3 days pt, though changes were evident at 1 day pt (McKinstry *et al.*, 2007).

The possibility of using NITROX in drug combinations against TCBZ-R *F. hepatica* was explored using *in vitro* morphological studies on TCBZ-R adults (Sligo isolate). NITROX + TCBZ.SO at half maximum blood levels *in vivo* (50 μg/ml and 7.5 μg/ml, respectively) caused more severe disruption than the sum of their effects when used alone, indicating potential synergy (McKinstry, 2008, unpublished data). Disappointingly, no efficacy was observed against early immature (4 weeks pi) TCBZ-R (Sligo isolate) flukes *in vivo* in sheep with a combination of NITROX + TCBZ at normal dose rates: 10 mg/kg for each drug (Forbes *et al.*, 2014).

7.2.1.4 Sulfonamides

Clorsulon (CLOR) is available as injectable formulation to be administered by the subcutaneous (sc) route in cattle (2 mg/kg) or by the oral route in sheep and cattle (recommended dose at 7 mg/kg). CLOR is frequently used in association with ivermectin for sc administration to cattle. It

is believed to target two glycolytic enzymes, namely 3-phosphoglycerate kinase and phosphoglyceromutase, although the relevance of this observation is questionable (see Fairweather and Boray, 1999a,b for discussion of this point).

Drug combination studies using TEM and SEM to assess CLOR + TCBZ or TCBZ.SO at half-normal concentrations against TCBZ-S fluke (Cullompton isolate), both *in vitro* and *in vivo*, showed more disruption than each drug on its own (Meaney *et al.*, 2006, 2007). Whether the results indicate true synergy is open to question, as it is difficult to quantify morphological changes. Certainly, they represent an additive effect between two drugs which have different chemical structures and different modes of action. CLOR has also been used in drug combinations to treat TCBZ- and salicylanilide-R fluke infections.

Flukes recovered from CLOR-treated cattle, as observed by Malone *et al.* (1984) and Yazwinski *et al.* (1985), were shorter and narrower than control flukes. Egg production by the flukes was also shown to be markedly reduced and the eggs were abnormal. As for the other drugs discussed above, it has been suggested that CLOR suppresses fluke growth and maturation (Malone *et al.*, 1984; Yazwinski *et al.*, 1985).

The timescale of drug action appears quite rapid, based on rodent studies: flukes were considered to be 'dead' by 25–30 h pt and were expelled by 50–60 h pt (Schulman *et al.*, 1979), or showed little sign of movement after 48 h treatment and, although intact at 72 h pt, they were inactive and severely damaged (Meaney *et al.*, 2003).

7.2.1.5 Phenoxyalkanes

Diamphenethide is highly active against early immature flukes up to 6 weeks of age, even against flukes as young as 1 day old; it becomes less active against older flukes as they develop to maturity. Despite this activity against the pathogenic early immature stages, diamphenethide has fallen out of favour and is no longer used to treat the disease; refer to Fairweather and Boray (1999a,b) for further information on mode of action. A more recent study showed that diamphenethide binds to zone 2 of β-tubulin, the same site to which TCBZ is thought to bind (Ranjan *et al.*, 2017).

7.2.1.6 Artemisinins

The artemisinins, originally isolated from the wormwood plant, *Artemisia*, are a major group of therapeutic compounds, well established as antimalarials and used in the treatment of human schistosomiasis (Utzinger *et al.*, 2007; Chaturvedi *et al.*, 2010). A number of semi-synthetic derivatives, including artemether and artesunate, have been tested against *F. hepatica*, *in vitro* and against fluke infections in rats. They displayed high levels of activity against adult and juvenile flukes, with relatively greater activity against adults (Table 7.2). Further modification of the artemisinin molecule led to the synthesis of an extensive range of fully synthetic 1,2,4-trioxolane and 1,2,4,5-tetraoxane derivatives, including OZ78 and MT04 (Zhao *et al.*, 2010; Wang *et al.*, 2011). It has been shown that they are effective against immature and adult fluke infections in rats (Table 7.3). Of particular note is that artemether and OZ78 showed an extremely high level of efficacy against the TCBZ-R Oberon isolate in a rodent model (Keiser *et al.*, 2007). Artemisinins are known to cause the collapse of the membrane potential of mitochondria, leading to their swelling and inhibition of electron transfer and oxidative phosphorylation. In addition, artemisinins are known to cause the oxidation of membrane proteins, such as ion pumps. For greater discussion of these points, see O'Neill *et al.* (2017 and references therein).

A number of artemisinin derivatives (artemether, artesunate, OZ78 and MT04) have been tested against natural fluke infections in sheep, but provided disappointing results (Tables 7.2 and 7.3). Moreover, toxicity problems have been reported in trials involving artesunate (Keiser *et al.*, 2010; O'Neill *et al.*, 2015a). The efficacy of artemisinins in treating fasciolosis in humans in Vietnam showed that initially (10 days pt) symptom control was better with artesunate than TCBZ but it was less effective by the end of the study (3 months pt) (Hien *et al.*, 2008). Similarly, in a second study, artemether was seen to exert little or no activity against fasciolosis in phase-2 trials in Egypt (Keiser *et al.*, 2011).

Faecal egg count reductions have been observed following treatment of liver fluke infections in sheep with artemether, artesunate,

Table 7.2. *Fasciola hepatica*. Fluke burden and faecal egg count reductions following treatment with the semi-synthetic artemisinin derivatives, artemether and artesunate.

Compound	Host	Dose (mg/kg)	Route of administration	Fluke burden reduction (%)	Faecal egg count reduction (%)	Adult / immature	Reference
Artemether	Rat	100	Oral	30	–	Adult	Keiser *et al*. (2006a)
		200	Oral	100	–	Adult	
		400	Oral	100	–	Adult	
		200	Oral	82	–	Immature	
	Rat	200	Oral	100	–	Adult (R)	Keiser *et al.* (2007)
	Sheep	40	Oral	17.4	39	Adult	Keiser *et al*. (2008)
		80	Oral	39.2	0	Adult	
		40	Intramuscular	0	0	Adult	
		80	Intramuscular	65.3*	24.2	Adult	
		160	Intramuscular	91.3*	64.9*	Adult	
Artesunate	Rat	100	Oral	30	–	Adult	Keiser *et al*. (2006a)
		200	Oral	71	–	Adult	
		400	Oral	100	–	Adult	
		200	Oral	46	–	J	
	Sheep	40	Intravenous	77.4	68.9	Adult	Keiser *et al*. (2010a)
		40	Intramuscular	91.9*	97.6*	Adult	
		60	Intramuscular	87.1*	93.2*	Adult	

R = TCBZ-R isolate; *= significant value.

Table 7.3. *Fasciola hepatica*. Fluke burden and faecal egg count reductions following treatment with the synthetic artemisinin derivatives, OZ78 and MT04.

Compound	Host	Dose (mg/kg)	Route of administration	Fluke burden reduction (%)	Faecal egg count reduction (%)	Adult/ immature	Reference
OZ78	Rat	50	Oral	52.7	–	Adult	Keiser *et al*. (2006b)
		100	Oral	100	–	Adult	
		200	Oral	100	–	Adult	
		400	Oral	100	–	Adult	
		50	Oral	66.7	–	Immature	
		100	Oral	100	–	Immature	
		100	Oral	100	–	Adult (R)	
	Rat	100	Oral	52	–	Adult	Zhao *et al.* (2010); Wang *et al.* (2011)
	Sheep	50	Oral	0	0	Adult	Keiser *et al*. (2010b)
		50	Subcutaneous	0	0	Adult	
	Sheep	100	Intramuscular	0	49	Adult	Meister *et al*. (2013)
MT04	Rat	25	Oral	70.8	–	Adult	Kirchhofer *et al*. (2011)
		50	Oral	100	–	Adult	
		100	Oral	100	–	Adult	
		50	Oral	60.7	–	Immature	
		100	Oral	100	–	Immature	
	Rat	50	Oral	48	–	Adult	Wang *et al*. (2011)
		100	Oral	100	–	Adult	
	Sheep	100	Intramuscular	92*	98.5*	Adult	Meister *et al*. (2013)

R = TCBZ-R isolate; *= significant value.

OZ78 and MT04 (Tables 7.2 and 7.3), although they were not always statistically significant and do not correlate with fluke burden reductions or any drug efficacy (Meister *et al.*, 2013). In a separate study in the rat model, egg production became abnormal within 3 days of treatment, due largely to a decreased supply of vitelline cells (O'Neill *et al.*, 2015b).

For artemether, the peak plasma concentration (t_{max}) in the rat after an oral dose is reached after only 29 min and the elimination half-life ($t_{½}$) is ~2 h (Li *et al.*, 1998). For artesunate, the corresponding figures are 20 min and 42.6 min, respectively. No elimination of flukes was seen 72 h after artemether treatment in one study, while in another study 'dead' flukes were still recovered at 12 days pt. For artesunate, elimination was evident at 96 h pt, but results for this drug are variable, with flukes still being recovered at 12 days pt. Comparing the impact of treatment on different fluke tissues, disruption to the tegumental surface was limited, whereas internal tissues were severely affected. For artemether, the testis was the first tissue to show signs of disruption, at 24 h pt. At 48 h pt, the tegumental system, gut and vitellaria showed evidence of change. Abnormal egg formation was apparent in the uterus at 72 h pt. Changes became progressively more severe with time. For artesunate, the gut and vitellaria were the first tissues to show signs of disruption, at 24 h pt, while changes to the tegumental system and testis were evident at 48 h pt. Changes to all tissues became more severe with time (see Keiser *et al.*, 2010, 2011; O'Neill *et al.*, 2015a,b, 2017 and references therein for further detail).

Artemisinin compounds have demonstrable activity against TCBZ-S and TCBZ-R flukes. Despite the disappointing results in sheep infections obtained to date, it would be worth persevering with the synthesis and testing of new derivatives and improved formulations, especially against infection with immature fluke.

7.2.1.7 Mirazid

Mirazid is marketed in Egypt for use as a fasciolicide; it contains a purified oleoresin extract of myrrh, derived from the plant *Commiphora molmol*. It has been shown to be effective in the treatment of fluke infections in both humans and animals, with efficacy rates of 90% and above (Massoud *et al.*, 2001; Haridy *et al.*, 2003; Abo-Madyan *et al.*, 2004; Soliman *et al.*, 2004). The efficacy studies were carried out on natural infections, following diagnosis based on the detection of eggs in the faeces. To date, no controlled efficacy trials (CET) have been conducted.

Few studies have been carried out to determine the action of Mirazid against *Fasciola* spp. Massoud *et al.* (2012) showed that Mirazid causes disruption of the tegument of *F. gigantica in vitro*. A more recent *in vivo* study (in rodents) has shown that Mirazid causes severe disruption of fluke tissues in a TCBZ-R isolate of *F. hepatica* (Abdelaal *et al.*, 2017a). The disruption evident in the sub-tegumental muscle blocks may be linked to the increasingly sluggish movements exhibited by the flukes at recovery. Myrrh is known to possess (mammalian) smooth muscle-relaxing properties, possibly via a calcium antagonism effect (for references, see Abdelaal *et al.*, 2017a). Therefore, part of the action of Mirazid may involve a neuromuscular action on the somatic musculature.

Mirazid has been shown to suppress egg production by TCBZ-R flukes *in vitro*. The effect was very rapid, becoming evident after only 6 h incubation and before major changes to the ovary, vitelline follicles and Mehlis' gland were observed; by 24 h pt, there were no eggs in the uterus (Abdelaal *et al.*, 2017b). Massoud *et al.* (2001) also observed a decrease in, and cessation of, egg production in patients following Mirazid treatment. The timescale of drug action is difficult to judge because of the variable recovery of flukes from treated rats. Dead flukes were recovered at 7 days pt, but death and elimination could have occurred as early as 2 days pt (see Abdelaal *et al.*, 2017a for further detail). No pharmacokinetic data have been published for Mirazid, so it is difficult to correlate the morphological changes observed with the time-course of drug metabolism and action, and potential speed of elimination.

The ability of Mirazid to act against TCBZ-R flukes shows that the drug has a potential role to play in fluke control and this is worth investigating further, to define its efficacy profile (against different life-cycle stages) and optimum dosing regimes.

7.3 Flukicide Pharmacokinetics

Pharmacokinetics includes the integrated description of the processes of drug absorption, tissue distribution, metabolism and excretion, which altogether determine the exposure of a target parasite to the active drug/drug metabolites. The activity of most modern flukicidal compounds depends on their binding to a target receptor. The pharmacokinetic behaviour determines the drug concentration achieved in the environment surrounding the receptor, named 'biophase', which largely affects the resultant pharmacological effect. For most nematodicidal and cestodicidal compounds, the physicochemical properties such as water/lipid solubility, dissolution rate and ionization determine the capacity of the drug to accumulate into the target parasites and its consequent clinical efficacy. Most flukicidal drugs have a common pharmacokinetic feature characterized by high systemic concentrations and long duration of persistence (Table 7.4) based on their high affinity/binding for plasma proteins. Understanding the pharmacokinetic behaviour and the identification of different factors affecting drug activity is relevant for achieving optimal flukicidal control. The main and specific pharmacokinetic features for the most widely used chemical families of flukicidal compounds are described below.

7.3.1 Benzimidazoles

TRICLABENDAZOLE. Overall, BZ anthelmintics are extensively metabolized in all mammalian species studied (Lanusse and Prichard, 1993). TCBZ is no exception and the parent drug is not detected in plasma after its oral administration to sheep (Hennessy *et al.*, 1987), cattle (Sanyal, 1995) and goats (Kinabo and Bogan, 1988); TCBZ.SO and TCBZ.SO_2 are the only systemically available metabolites measured in TCBZ-treated animals. An efficient first-pass oxidation process, mediated by the flavin monooxygenase (FMO) and cytochrome P-450 (CYP450) enzymatic systems, was found to be involved in the sulfoxidation and sulfonation of TCBZ to form TCBZ.SO and TCBZ.SO_2, respectively. Additionally, the sulforeduction of TCBZ.SO back to form the TCBZ parent compound occurs via the ruminal microflora (Virkel *et al.*, 2006). TCBZ.SO accounted for 43% (sheep) (Ceballos *et al.*, 2010) and 32% (cattle) (Lanusse *et al.*, 2018), respectively, of total analytes recovered in the bloodstream after the intraruminal (ir) administration of TCBZ. The main plasma metabolites TCBZ.SO and TCBZ.SO_2 have a long persistence in sheep and cattle for a period of 144 h pt, thought to be due to their strong binding (> 99%) to plasma proteins (Hennessy *et al.*, 1987). TCBZ binding to bovine serum albumin is higher than that observed for TCBZ.SO (Chambers *et al.*, 2010). In contrast to other flukicidal drugs such as CLOS and NITROX, the high TCBZ plasma protein binding does not limit its tissue distribution and body clearance. A large volume of distribution (Vd = 7.69 l/kg) and a total body clearance (Cl_B = 0.70 l/h × kg) has been reported for TCBZ in sheep (Virkel *et al.*, 2009). The higher systemic exposure of TCBZ metabolites compared with that observed for other BZ compounds offers some advantages to TCBZ in its activity against blood-feeding adult flukes.

Peak plasma concentrations of TCBZ metabolites are attained at approximately 18 h pt (TCBZ.SO) and 36 h pt (TCBZ.SO_2) in treated sheep. Hydroxylation of TCBZ and its two main metabolites also occurs in the liver, but the products are secreted into the bile, mainly in their conjugated forms (Hennessy *et al.*, 1987; Moreno *et al.*, 2014). Low TCBZ concentrations were recovered in bile, with TCBZ.SO, TCBZ.SO_2 and hydroxy-TCBZ (OH-TCBZ) derivatives being the major unconjugated biliary metabolites (Hennessy *et al.*, 1987; Moreno *et al.*, 2014). Approximately 45% of the administered dose is eliminated by bile with only 6.5% excreted in urine, mainly as TCBZ.SO and TCBZ.SO_2 in treated sheep (Hennessy *et al.*, 1987). With regard to compound alpha, which shows similarities to TCBZ, the maximum blood levels of compound alpha -SO and -SO_2 are reached quite quickly, at 14 and 23 h pt, respectively (Ramírez *et al.*, 2009).

The impact of either fasting (Lifschitz *et al.*, 1997; Sánchez *et al.*, 1997, 2000) or modifying feeding management (Hennessy *et al.*, 1995) has been extensively investigated as a tool to enhance drug systemic activity and nematodicidal activity in ruminants. It has been observed that fasting prior to TCBZ treatment in goats resulted in significantly higher systemic exposure of TCBZ metabolites, compared with fed animals

Table 7.4. Comparative dose levels, routes of administration and plasma pharmacokinetic parameters for the main flukicidal drugs used in sheep and cattle: triclabendazole (TCBZ), albendazole (ABZ), closantel (CLOS), nitroxynil (NITROX) and clorsulon (CLOR).

	TCBZ[a]		ABZ[b]		CLOS		NITROX		CLOR	
	Sheep	Cattle	Sheep	Cattle	Sheep	Cattle	Sheep[c]	Cattle[c]	Sheep	Cattle[d]
Dose (mg/kg)	10	12	7.5	10	10	5	10	10	7	2
Admin. route	ir	oral	ir	ir	sc	sc	sc	sc	oral	sc
Cmax (µg/ml)	14.0	12.1	1.92	0.82	119	59.8	104.4	109.6	1.60	1.29
Tmax (d)	0.94	0.79	0.67	0.47	1.62	2.81	4.80	0.41	15.2	6
$AUC_{0\text{-}t}$ (µg.d/ml)	27.3	17.4	1.83	0.43	1570	898.5	1188	1446	80.6	nr
T½el (d)	0.73	0.72	0.25	0.11	15.9	10.6	9.50	12.1	27.9	nr
PDP	1–144 h	1–168 h	1–60 h	0.5–48 h	1.5 h–27 d	1–62 d	2 h–60 d	2 h–60 d	1–96 h	nr
Reference	Ceballos *et al*., 2010	Ortiz *et al*., 2014	Alvarez *et al*., 1999	Sánchez *et al*., 1997	Suárez *et al*., 2011	Cromie *et al*., 2006	Moreno *et al*., 2010	Moreno *et al*., unpublished data	Sundlof and Whitlock, 1992	EMEA, 1995

[a]Plasma pharmacokinetic parameters for the TCBZ sulfoxide metabolite obtained after TCBZ administration. [b]Plasma pharmacokinetic parameters for ABZ sulfoxide metabolite obtained after ABZ administration. [c]Plasma pharmacokinetic parameters for NITROX obtained after its co-administration with ivermectin. [d]AUC estimated from time 0 to infinite.
$AUC_{0\text{-}t}$, area under the plasma concentration vs time curve; Cmax, peak plasma concentration; d, day(s); h, hour(s); ir, intraruminal; nr, not reported; PDP, plasma detection time period; sc, subcutaneous; Tmax, time to the Cmax; T½el, elimination half-life.

(Gokbulut *et al.*, 2010). A similar effect could be expected in sheep and cattle, since similar fasting-induced changes have been described for ABZ (Lifschitz *et al.*, 1997; Sánchez *et al.*, 2000), possibly due to delayed gastrointestinal transit time.

The interaction between co-administered drugs may induce changes in the pharmacokinetic behaviour of either molecule. After intravenous (iv) co-administration of TCBZ and the macrocyclic lactone IVM in sheep, an enhanced TCBZ.SO plasma concentration was reported (Lifschitz *et al.*, 2009). Conversely, methimazole (MTZ)-mediated inhibition of TCBZ oxidative metabolism by sheep liver microsomes has been reported (Virkel *et al.*, 2006). However, when TCBZ was administered by the ir route simultaneously with IVM (subcutaneous, sc) and MTZ (intramuscular) (im) (Ceballos *et al.*, 2010), the plasma concentration profiles of TCBZ.SO and $TCBZ.SO_2$ were similar to those observed after the administration of TCBZ alone. The fact that IVM modifies TCBZ metabolites after its simultaneous iv administration (Lifschitz *et al.*, 2009), but not after IVM (sc administration) and TCBZ (ir administration), implies that the potential drug–drug interaction may be influenced by the route of administration used for each compound.

ALBENDAZOLE. The extremely low water solubility of ABZ limits its formulation to a micronized suspension to be administered by oral/ir routes in ruminants. ABZ particles must necessarily become dissolved in the enteric fluids to facilitate drug absorption through the gastrointestinal mucosa. The pattern of ABZ dissolution determines the rate and extent of its absorption (systemic bioavailability), its maximal plasma concentration, its subsequent distribution to target tissues and its overall disposition kinetics. The low gastrointestinal absorption of BZ methylcarbamates is related to their poor water solubility, which is notably increased by low pH values such as those observed in the abomasum and facilitated by the rumen acting as a drug reservoir by slowing the digesta transit time (Lanusse and Prichard, 1993). Thus, the length of time the administered BZ suspension remains in the abomasal compartment may affect the dissolution rate and the subsequent absorption of ABZ in the gut. The plasma level of ABZ.SO reflects the amount of ABZ dissolved at the gastrointestinal level. First-pass metabolism is relevant to the kinetic behaviour of ABZ/metabolites in ruminants. Intestinal, liver and lung metabolic pathways have been implicated in this phenomenon in most animal species (Virkel *et al.*, 2004). Once absorbed, ABZ is extensively metabolized in the liver microsomal fraction in all the species studied (Gyurik *et al.*, 1981); the FMO (Galtier *et al.*, 1986) and CYP450 enzymatic systems (Souhaili-El Amri *et al.*, 1987) are primarily involved in ABZ biotransformation. The successive ABZ oxidations lead to more polar and less anthelmintically active metabolites. In terms of binding to parasite tubulin, ABZ parent drug is more potent than its sulfoxide metabolite, while the sulfone ($ABZ.SO_2$) is an inactive derivative (Lacey, 1990; Lubega and Prichard, 1991). The sulfoxide and sulfone metabolites dominate the plasma profile in sheep treated with ABZ (Marriner and Bogan, 1980; Hennessy *et al.*, 1989; Lanusse *et al.*, 1995) and are the major analytes recovered in urine, the main route of elimination (Hennessy *et al.*, 1989). Additionally, the metabolic sulforeduction of ABZ.SO to form ABZ has been shown to occur in ruminal and intestinal fluid contents from sheep and cattle (Lanusse *et al.*, 1992). Since ABZ has a greater affinity for parasite tubulin than its sulfoxide metabolite (Lacey, 1990; Lubega and Prichard, 1991), this bacteria-mediated reduction may have significant importance for the efficacy against gastrointestinal parasites. However, ABZ.SO appears to be the main molecule implicated in ABZ activity against *F. hepatica*, which may indicate that this bacterial reduction of ABZ would be less relevant for its flukicidal therapeutic activity.

The extensive tissue distribution of ABZ and its metabolites has been described in sheep (Alvarez *et al.*, 1999) and cattle (Sánchez *et al.*, 1997). However, differential distribution patterns among the parent drug and its main metabolites, based on their differential lipophilicity, may be expected. The distribution rate, which is defined by the apparent volume of distribution (Vd), depends on molecular weight, lipid solubility and plasma protein binding of each drug/metabolite. ABZ shows a binding of less than 50% to plasma protein, a relatively high Vd, and a relatively fast elimination rate in ruminant species. Differences in polarity are reflected in a differential tissue distribution pattern for ABZ and ABZ.SO. A higher Vd has been described for ABZ in sheep (1.47 l/kg) (Alvarez *et al.*, 1999)

compared with that observed for ABZ.SO (1.04 l/kg) (Aksit *et al.*, 2015).

Fasting-induced changes in ABZ absorption have resulted in pronounced enhancement in the systemic availability of ABZ metabolites (Lifschitz *et al.*, 1997; Sánchez *et al.*, 2000). This management practice, which is now a worldwide recommendation for nematode control, could be useful when ABZ is indicated as a part of flukicidal therapy.

7.3.2 Salicylanilides

As with other salicylanilides, CLOS is extensively bound to plasma proteins (> 99%, mainly albumin), which extends the persistence of therapeutic levels in the bloodstream and limits its pattern of tissue distribution (McKellar and Kinabo, 1991). The particular mode of CLOS action requires accumulation in the liver fluke cells. Consequently, its therapeutic effect will depend on its ability to reach high and sustained concentrations within the parasite, and to bind to its specific receptor for sufficient time to cause the therapeutic effect. The strong binding to plasma protein facilitates the oral ingestion of CLOS by haematophagous mature liver flukes, accounting at the same time for its limited metabolism and tissue distribution and extended terminal plasma half-life. In fact, CLOS is poorly metabolized in the host liver. Ninety per cent of the excreted compound corresponds to the unchanged parent drug (Michiels *et al.*, 1987) and a small apparent volume of distribution (< 0.15 l/kg) has been described (Swan, 1999). CLOS formulations can be administered by the oral or sc routes in ruminants, but a lower systemic availability is expected after its oral administration. Thus, an increased dose is required to ensure equivalent anthelmintic efficacy. For example, in sheep an oral dose of 10 mg/kg is recommended, whilst the sc treatment is usually 5 mg/kg. More than 80% of CLOS was shown to be associated with the particulate phase of ruminal fluid (Swan *et al.*, 2000) and this extended association helps to explain the low absorption and relative availability observed after oral administration. On the other hand, this association may also influence the absorption rate according to the level of feed intake; the slower digesta flow rate in sheep on low versus high feed intake resulted in a reduction of the proportion of the dose passing through the abomasum (Hennessy and Ali, 1997), which accounted for an increased absorption and a higher CLOS systemic exposure in those animals (Hennessy and Ali, 1997).

The potential pharmacokinetic interaction between CLOS and IVM (cattle) (Cromie *et al.*, 2006) or moxidectin (sheep) (Suarez *et al.*, 2013) has been investigated. No differences in the CLOS plasma disposition kinetics were observed, indicating that neither the absorption nor the distribution of CLOS were influenced by the presence of the macrocyclic lactone in either host.

CLOS is used as an extra-label or off-licence product in goats, although goats are not merely 'little' sheep and the pharmacokinetic behaviour of CLOS differs between sheep and goats. A faster elimination resulted in an almost threefold lowering of AUC (area under the ROC curve, where ROC = receiver operating characteristic) in goats and could dramatically reduce the sustained action of CLOS in this animal species compared with sheep (Hennessy *et al.*, 1993). Consequently, dose adjustment based on pharmacokinetic and efficacy data must be considered when an anthelmintic drug (including CLOS) is used as an extra-label product in a different host, such as goats.

7.3.3 Halogenated phenols

NITROX suffers a microflora-mediated nitro-reduction in the rumen, which accounts for its low efficacy following oral and ir treatments. Consequently, NITROX is recommended to be administered by parenteral routes at a dose rate of 10 mg/kg in both sheep and cattle. In both species, NITROX is well absorbed after its sc administration and binds strongly to plasma proteins (~98% of the absorbed fraction), mainly to albumin (Alvinerie *et al.*, 1991), and is eliminated mainly by urine. Due to its high affinity for plasma proteins, NITROX has a low tissue distribution and, therefore, the amount of drug reaching the liver parenchyma seems to be insufficient for the treatment of immature flukes at the recommended dose rate. High NITROX concentrations were measured in plasma samples of treated sheep as early as 2 h pt (Moreno

et al., 2010). Plasma concentrations remain high (above 40 μg/ml) for 10 days pt and were quantifiable in the bloodstream for at least 60 days (Moreno *et al.*, 2010). A similar plasma pharmacokinetic behaviour has been observed in cattle (L. Moreno, 2014, unpublished data). NITROX parent compound is the main compound systemically recovered in treated animals, since no major metabolites have been identified. Studies carried out in different animal species demonstrated that NITROX plasma concentrations in the bloodstream are higher than those measured in tissues (EMEA, 1998). Animal body weight seems to affect the NITROX disposition kinetics after its sc administration to sheep. The drug systemic exposure was markedly higher in sheep with 'high body weight' compared with that observed in 'low body weight' animals (Moreno *et al.*, 2010), differences that were reflected in the AUC, where higher values were obtained for the heaviest animals. The impact of this weight-related pharmacokinetic difference on the flukicidal response of the drug remains to be elucidated.

7.3.4 Sulfonamides

Information on CLOR pharmacokinetic disposition in domestic animals is scarce. One study of the plasma disposition kinetics of CLOR in sheep and goats after its administration by the iv and oral routes showed that bioavailability of orally administered CLOR was ~55% in goats and 60% in sheep, but was highly variable in sheep, ranging from 95.6% to 16.3% (Sundlof and Whitlock, 1992). If these differences were observed under controlled experimental conditions, it is expected that this erratic absorption pattern would be much higher under 'field/farm' conditions. Thus, potential differences in flukicidal efficacy could be related to differences in drug absorption among treated animals. CLOR peak plasma concentration was attained at 14–15 h after oral administration in goats and sheep. Absorption from the gastrointestinal tract effectively prolonged the elimination of CLOR, extending its plasma elimination half-life about twofold in both sheep and goats. Following its oral administration, CLOR particles reaching the rumen are adsorbed to digesta solid content, where the slow mixing and long digesta residence time, combined with the large rumen volume, assists absorption by delaying the rate of passage of drug down the gastrointestinal tract (Hennessy, 1993). Thus, the rumen may act as a drug reservoir prolonging the duration of drug absorption and/or outflow down the gastrointestinal tract. It has been demonstrated in rats that CLOR binds predominantly to erythrocytes, binding to red blood cell carbonic anhydrase (Schulman *et al.*, 1979, 1982). Similar to that observed for other flukicidal drugs, the high binding of CLOR to blood components limits its tissue distribution and prolongs its persistence in the systemic circulation. In fact, a low distribution volume of 0.57 l/kg has been described in sheep (Sundlof and Whitlock, 1992). Renal excretion of the parent drug is the main route of CLOR elimination (Sundlof and Whitlock, 1992). A fast CLOR elimination process has been described in goats compared with sheep (Sundlof and Whitlock, 1992), indicating that goats are more effective in their ability to eliminate CLOR than sheep. These differences in drug disposition between sheep and goats may account for the reduced efficacy of CLOR reported in goats after an oral dose of 3.5 mg/kg, with efficacy against mature flukes at 100% in sheep and only 83% in goats (Ostlind *et al.*, 1977; Sundlof *et al.*, 1991).

After the ir administration of CLOR to cattle (10 mg/kg), a peak plasma concentration (3 μg/ml) of the parent drug was attained at ~24 h pt (EMEA, 1995). However, an earlier (6 h pt) peak plasma concentration was reported after its sc administration (EMEA, 1995). For cattle, in contrast to sheep and goats, the major fraction of an oral CLOR dose was excreted in the faeces (approximately 70%) and a minor fraction (about 30%) in urine (EMEA, 1995). CLOR is eliminated mainly as the unchanged parent drug, with a low proportion of acetaldehyde (2.9%) and butyric acid (6.2%) metabolite derivatives.

7.4 Mechanisms of Drug Entry into Adult Liver Flukes

The activity of flukicidal molecules is based on their affinity for a specific receptor, but also depends on the ability of the active drug to reach that receptor within the target parasite. Thus, drug entry and accumulation in target helminths are important factors when considering

how to achieve optimal efficacy. Consequently, it is considered crucial to investigate and understand the main route of drug entry into *F. hepatica* in order to optimize therapeutic strategies for fluke control. There are two potential routes of drug entry into mature *F. hepatica*: oral ingestion from the blood; and/or transtegumental diffusion from the bile. Work developed over the years at the Laboratory of Veterinary Pharmacology, CIVETAN (Centro de Investigación Veterinaria de Tandil), Tandil, Argentina has evaluated the relative importance of these drug entry routes in *F. hepatica in vivo* using TCBZ, ABZ and CLOS.

The routes of TCBZ entry into *F. hepatica* were initially assessed *in vitro*. Equivalent TCBZ.SO concentrations were recovered from mouth-ligated (unable to orally ingest) and non-ligated adult *F. hepatica* (Mottier *et al.*, 2004, 2006), which demonstrated the relevance of drug entry through the external surface. Furthermore, there was a clear tendency towards an enhanced concentration profile within the parasite for the most lipophilic compounds (TCBZ, TCBZ.SO, TCBZ.SO_2) compared with their respective hydroxylated derivatives (OH-TCBZ, OH-TCBZ.SO and OH-TCBZ.SO_2) (Mottier *et al.*, 2004). As drug lipophilicity is the main factor determining drug penetration across the tegument of trematodes (Fetterer and Rew, 1984; Alvarez *et al.*, 2000, 2001), the main route of acquisition of TCBZ/metabolites by mature *F. hepatica* under *in vitro* conditions was demonstrated to be by passive diffusion through its external surface (Mottier *et al.*, 2004, 2006). This was supported by morphological studies, visualized by SEM and TEM, where disruption to the tegument was similar in ligatured and non-ligatured flukes, indicating that restricting the oral uptake of drug does not affect the ability of TCBZ.SO to enter the fluke and exert its effect (Toner *et al.*, 2010b). However, the picture was quite different when TCBZ/metabolite accumulation into liver flukes was evaluated under *in vivo* conditions. Moreno *et al.* (2014) assessed the time-course and pattern of *in vivo* accumulation of TCBZ/metabolites into adult *F. hepatica* specimens recovered post-treatment from infected sheep. TCBZ.SO and TCBZ.SO_2 were the only molecules recovered in the bloodstream, with peak plasma concentrations of 10.8 μg/ml (TCBZ.SO) and 12.6 μg/ml (TCBZ.SO_2). The same metabolites were also the main molecules accumulated within the adult flukes, reaching similar peak concentrations at 24 h pt (Moreno *et al.*, 2014). In the same experiment, TCBZ concentrations were quantified in bile that certainly provides the potential for substantial chemical contact with the liver-dwelling *F. hepatica*. However, low levels of TCBZ (0.14 μg/g at 24 h pt) were measured within the collected flukes. Furthermore, only very low concentrations of OH-TCBZ were measured in the liver fluke, even when its concentrations in bile were relatively high (Moreno *et al.*, 2014). Contrary to what was observed in the *in vitro* trials, these data confirm that oral ingestion is a main route of drug entry into adult liver flukes exposed to TCBZ/metabolites *in vivo*. The presence of low concentrations of TCBZ within the adult fluke may be related to some degree of trans-tegumental diffusion from bile or by direct oral ingestion from portal blood.

The pattern of *in vivo* ABZ/metabolites accumulation into *F. hepatica* recovered from ABZ-treated sheep has been investigated (Alvarez *et al.*, 2000). High concentrations of ABZ were measured in liver tissue. However, ABZ sequestered in the liver tissue is not available to mature flukes located in the bile duct. ABZ.SO was the main analyte recovered in *F. hepatica* and in bile samples collected from ABZ-treated sheep (Alvarez *et al.*, 2000), and only low ABZ concentrations were measured in both the fluke tissue and bile. As described for TCBZ, the pattern of ABZ.SO metabolite accumulation *in vivo* in the liver fluke appears to be related to its high systemic drug exposure in the host, with oral ingestion being the main route of access into the parasite.

It has been shown, in *ex vivo* studies involving mature *F. hepatica* incubated in buffer, that TCBZ/metabolites (Mottier *et al.*, 2004) and ABZ/metabolites (Alvarez *et al.*, 2001; Mottier *et al.*, 2006) can accumulate in the parasite by a trans-tegumental diffusion process. However, the physicochemical characteristics of the fluids surrounding the parasite can limit this process (Alvarez *et al.*, 2004). The partitioning of the active drug/metabolites between an aqueous fluid (buffer or incubation medium in the *ex vivo* assays) and the lipoid tissue of the parasite may facilitate the accumulation of the drug within the parasite. This drug partitioning phenomenon may be different *in vivo* for sites of parasite location such as the biliary tract, where the

bile-induced micelle formation may affect the diffusion of the active drug/metabolite into the target parasite. The quantities of ABZ, TCBZ (Alvarez *et al.*, 2004) and CLOS (Ceballos *et al.*, 2017) recovered from *F. hepatica* incubated *ex vivo* in bile were significantly lower than those obtained after incubation in buffer as incubation medium. Altogether, these findings indicate that the physicochemical characteristics of the tissues and fluids surrounding the parasite may be relevant to the process of drug diffusion into the parasite. For instance, a given drug concentration is not equivalent when in the gastrointestinal fluid content, in a mucosal tissue or in the bile. This phenomenon may also explain many therapeutic failures observed in parasite control in both human and veterinary medicine which, in some cases, have contributed to exposure of target parasites to sub-therapeutic drug concentrations and to the development of drug resistance.

CLOS is an extremely lipophilic molecule, extensively bound (> 99%) to plasma proteins, mainly albumin (Mohammed-Ali and Bogan, 1987). As a consequence of this high plasma protein binding and its low metabolism, an extended therapeutic level of CLOS has been observed in treated animals. The *in vivo* pattern of CLOS accumulation in adult *F. hepatica* has been investigated after oral or sc administration to sheep (Ceballos *et al.*, 2017). CLOS parent drug was recovered in plasma, bile and liver in specimens of *F. hepatica* recovered from animals sacrificed at 12, 24 and 36 h pt. Plasma concentration increased after oral administration of CLOS from 36 µg/ml (12 h pt) to 38.8 µg/ml (24 h pt) and 57 µg/ml (36 h pt). A similar trend was observed in adult liver flukes recovered from treated animals (Ceballos *et al.*, 2017). The highest concentrations of CLOS were measured in plasma and *F. hepatica*, after either oral or sc administration. Although low concentrations were measured in liver tissue, the pattern of CLOS accumulation in *F. hepatica* matched its high plasma systemic availability. In fact, a significant positive correlation ($P < 0.05$) between individual plasma and *F. hepatica* concentrations of CLOS was observed after its oral and sc administration, which can only be explained by the oral ingestion of CLOS due to the haematophagous behaviour of *F. hepatica*. The presence of low CLOS concentrations in bile may contribute to some degree of complementary drug accumulation by a trans-tegumental diffusion process.

Studies on CLOR-treated fluke and identification of morphological changes by SEM and TEM by the group at The Queen's University of Belfast showed severe changes to the fluke surface: swelling, blebbing, sloughing of the apical membrane, loss of spines and complete removal of the tegument. This was particularly evident in older (44 weeks pi) rather than younger (8 weeks pi) flukes (Meaney *et al.*, 2003). The changes in the gut were more severe than those in the tegument and occurred more quickly, pointing to oral uptake as the predominant route of drug entry into the fluke. Further observations of ligatured versus non-ligatured fluke (Meaney *et al.*, 2004) revealed that the greatest degree of disruption was observed in flukes given access to both routes of drug uptake, whilst the least disruption was evident in ligatured flukes, supporting the concept that oral ingestion of CLOR is the principal means of entry (Meaney *et al.*, 2004). With respect to the route of entry for other drugs, for Mirazid it was shown that the tegument was more consistently and severely affected than the gut, which suggests that trans-tegumental uptake is the predominant route, rather than oral uptake (Abdelaal *et al.*, 2017a). The fact that activation of artemisinin compounds depends on interaction with iron-containing compounds, such as haemoglobin in the blood, suggests that artemisinins are taken up orally by the fluke, rather than by trans-tegumental uptake. A similar mechanism has been proposed for schistosomes (Xiao *et al.*, 2003). In support of this idea, the gut of *F. hepatica* was consistently more seriously affected by artemisinin treatment than the tegument and surface disruption to the fluke was less severe than internal changes (O'Neill *et al.*, 2015a).

Altogether, the accumulated data clearly indicate that oral ingestion is the main route of drug entry into liver fluke exposed to flukicidal drugs *in vivo*. Consequently, the systemically available flukicidal drug concentrations are relevant for their trematodicidal effect. The systemic concentrations may notably differ according to the drug formulations used. Several generic formulations of flukicidal drugs have been introduced into the veterinary pharmaceutical market in different regions of the world, following expiry of the original patent of the first approved (pioneer) formulation. However, differences in excipient composition or quality, and quality of the active ingredient, for example, may lead to

differences in absorption, plasma drug exposure and, eventually, in the clinical efficacy of those preparations. In fact, differences in plasma availability of TCBZ.SO (Ortiz *et al.*, 2014) and ABZ.SO (Suárez *et al.*, 2011) were observed among different formulations orally administered to cattle and sheep, respectively. Differences in formulation quality may substantially affect drug dissolution and consequent gastrointestinal absorption, which in turn could affect drug effectiveness. Further work is needed to ensure the bioequivalence of flukicidal formulations before their introduction into the market. This is a critical issue, considering the worldwide spread of resistance-related therapeutic failures in liver fluke control.

As mentioned before, most of the available flukicidal drugs have a common pharmacokinetic property characterized by high systemic concentrations (high systemic exposure) and long persistence, which can be explained mainly by their high binding to plasma proteins. However, there are important differences between different chemical groupings within the available flukicidal drugs. Table 7.4 shows the main pharmacokinetic parameters observed for common flukicidal drugs after their administration to sheep and cattle. Haematophagous mature liver flukes are exposed to markedly different systemic concentrations according to the pharmacokinetic features of each of the flukicidal drugs. The integrated understanding of the relationship between parasite exposure to active drug concentrations and the mechanism of drug action plays a critical role. Significantly lower TCBZ and ABZ plasma concentrations are needed to kill adult *F. hepatica* compared with CLOS, NITROX and, to a lesser degree, CLOR. This indicates that mature liver flukes are particularly susceptible to BZ action, which may be related to the disruption of transport processes in the tegument and the progressively severe disruption to the tegumental surface described above in section 7.2.1.1.

7.5 Flukicide Resistance

Two decades after stating in the first edition (1999) of this volume that 'Drug resistance in *F. hepatica* is not yet a major problem ...', it is clear that flukicide resistance is a significant and growing concern (Kelley *et al.*, 2016; McMahon *et al.*, 2016; Fairweather *et al.*, 2020). Over this time-frame, there has been extensive study of drug-resistant *F. hepatica* as they emerge in the field and more detailed characterization of drug-susceptible and drug-resistant parasites in the laboratory.

7.5.1 Detecting anthelmintic resistance in *Fasciola* spp.

Definitive evidence of anthelmintic resistance in *Fasciola* spp. involves showing a causal link between the phenotype (lack of susceptibility to a drug) and the genotype (detecting the gene(s) responsible for conferring the drug resistance). However, understanding the genetic mechanism(s) responsible for flukicide resistance is still a work in progress (see section 7.6). The ability to detect resistant parasite populations must rely on robust, well validated methods and clear definitions of resistance (Fairweather *et al.*, 2020). Crucial to diagnosing flukicide resistance is the ability to distinguish between drug efficacy and drug resistance, which means access to high-quality efficacy reference data for a given drug (see Tables 7.1 and 7.8; see also Tables 7.1 and 7.2 in *Fasciolosis* (1st edn, 1999).

Over the past few decades there have been a number of advances in the tools used to diagnose drug resistance in *F. hepatica*; however, they all suffer from a lack of standardization, leaving questions as to their accuracy and comparability. Best practice is to apply a number of tests to define resistance, particularly in the absence of a gold standard. A number of tests are available, including the controlled efficacy test (CET), faecal egg counts (FECs) and FEC reduction tests (FECRTs), serological and coproantigen methods and egg hatch tests (EHTs). For comprehensive reviews of flukicide resistance reports published in the past few years, refer to Kelley *et al.* (2016) and more recently Fairweather *et al.* (2020). Here, we aim to complement these reviews by highlighting how each test has been used to detect drug resistance, with a particular emphasis on the relative merits of the different diagnostic approaches. In addition, we identify key considerations for their standardization.

7.5.1.1 Controlled efficacy test (CET)

The CET compares parasite populations in treated and untreated animals infected with *Fasciola* spp. and was used in the earliest studies reporting resistance to RAFOX and CLOS (Boray, 1989; Boray and De Bono, 1989; Swan, 1999). It was also the diagnostic of choice for the initial report of TCBZ resistance in *F. hepatica* (Overend and Bowen, 1995) and it is still considered the gold standard method for detecting drug resistance. Wood *et al.* (1995) provided a useful framework for CET with *Fasciola* spp., but there has been no standardization of the process per se. For example, how should we produce metacercariae representative of the parasite population being tested? What are the recommended numbers of animals within each treatment group to allow for variation in infection rates? What percentage efficacy/reduction reference or 'cut-off' values should be used for determining resistance for each flukicide? In the absence of such guidelines for liver fluke, the World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines developed for nematodes are often employed (Wood *et al.*, 1995; Coles *et al.*, 2006).

Thirteen studies have applied the CET to demonstrate flukicide resistance in *F. hepatica* isolates originating from eight countries (Table 7.5); as yet, no CETs have been conducted for *F. gigantica*. The majority of CETs have been carried out with sheep, with only one study in cattle (Walker *et al.*, 2004). A common question raised about drug resistance in *Fasciola* spp. is its stage specificity, especially for TCBZ given its efficacy against fluke from a few days of age in sheep (dose 12.5 mg/kg; Boray *et al.*, 1983). The CET remains one of the few means of robustly assessing the efficacy of drugs on different parasite stages; for example, TCBZ resistance has been evaluated by CET at 2, 3, 4, 6, 8, 10, 12, 15 and 16 weeks pi (Table 7.5).

Wood *et al.* (1995) stated that a single CET was considered sufficient to confirm resistance and provided some guidance on experimental design: 5–6 animals per treatment group; an inoculum of 60–80 and 150 metacercariae in sheep and cattle, respectively; necropsy 2–3 weeks pt for mature infections; and the need for a statistically significant difference in worm burdens between groups. They also defined the formula for expressing the percentage efficacy:

$$\text{Efficacy (\%)} = \frac{\begin{array}{c}(\text{Mean of parasites}\\ \text{in control group}) -\\ (\text{Mean of parasites}\\ \text{in treated group})\end{array}}{\begin{array}{c}(\text{Mean of parasites}\\ \text{in control group})\end{array}} \times 100$$

Whilst this formula has been widely adopted for defining efficacy, in reality there is a great deal of variation in how CETs have been conducted. The majority of studies had a control (untreated) group and used a minimum of three sheep per treatment group, with a dose rate of 10 mg/kg and 7.5 mg/kg for TCBZ and ABZ, respectively (Table 7.5). However, as few as three sheep have been used per study, a range of metacercarial doses of between 44 and > 500 per animal have been administered and mean recovery rates (number of adult parasites recovered in untreated controls as a proportion of the infective dose) ranged from 2.5% to 55%. One of the most important considerations in any CET trial is the source of parasite material used for experimental infection (Fairweather, 2011a). The majority of metacercariae used for infection were derived from experimentally infected snails, with the exception of Overend and Bowen (1995) who shed metacercariae from wild infected snails collected from pasture grazed by sheep harbouring TCBZ-R *F. hepatica* (21% on FECRT). High levels of genetic variation are known to exist in *F. hepatica* populations naturally infecting sheep and cattle (for example, see Walker *et al.*, 2007; Walker *et al.*, 2011; Beesley *et al.*, 2017). Thus, if just a few snails are used as the source of metacercariae for experimental trials they will not accurately represent liver fluke populations in the field. This is particularly important as we start to confirm cases of field resistance to non-TCBZ drugs where resistance alleles are likely to be at low frequency as resistance emerges. With this is mind, we highlight several important considerations when conducting CET.

- *Inherent variation in infection amongst hosts highlights the need for multiple animals per treatment group.* For example, in Sanabria *et al.* (2013), the CEDIVE isolate was shown to be ABZ-R and TCBZ-S. This study had a different control group for the two drugs tested and, despite the control sheep for ABZ and TCBZ being given the same batch and number of metacercariae, there was a statistical

Table 7.5. Controlled efficacy test (CET) to characterize drug resistance in *Fasciola hepatica* in sheep.

Year	Country (isolate)	Infection	No. and source of metacercariae	Mean recovery rate (%)[a]	Drug (dose)	Stage of parasite at time of treatment (wpi)	No. of sheep per group	Weeks post infection at PM	Efficacy (%)	Resistance status as defined by authors	Reference
1989	Australia (multiple)	Exp	Lab[c] maintained or naturally infected snails	Not cited[c]	RAFOX (7.5 mg/kg)	Late immature (6)	4–5 × T 4–5 × C	Not specified	0–76	RAFOX-R	Boray and De Bono, 1989
					CLOS (7.5 mg/kg)	Late immature (6)	4–5 × T 4–5 × C		9–64	CLOS-R	
1995	Australia	Exp, as FECRT follow-up	~200/sheep Naturally infected snails	12.5	TCBZ (10 mg/kg)	Early immature (4)	3 × T 3 × C	12 weeks	TCBZ–, 25 ± 15.5 TCBZ+, 11 ± 7.8	TCBZ-R	Overend and Bowen, 1995
2001	The Netherlands (Dutch)	Exp, as FECRT follow-up	~500/sheep Lab maintained isolate	37.66	TCBZ (10 mg/kg)	Late immature (8)	6 × T 6 × C	16 weeks	10.78	TCBZ-R	Gaasenbeek *et al.*, 2001
2001	UK	Exp	~200/sheep Lab maintained isolate	38.85	TCBZ+LEV (10 mg/kg)	Mature adult (12)	7 × C 7–8 × T	13 weeks 5 days to 14 weeks 1 day	0	TCBZ-R	Coles and Stafford, 2001
2004	Australia (Oberon)	Exp	~300/sheep Lab maintained isolate	20.1	TCBZ (10 mg/kg)	Early immature (2,4)	8 × T 8 × C	16 weeks	2wpi=0 4wpi=5	TCBZ-R	Walker *et al*., 2004
2009	Bolivia Farm 1	Nat[b]	N/A	55–96 fluke at PM	ABZ TCBZ (10 mg/kg)	+ve FEC confirmed at treatment	10 × T 10 × C	8 weeks post-treatment	13.6 98	ABZ-R TCBZ-S	Mamani and Condori, 2009
	Bolivia Farm 2	Nat[b]	N/A	53–157 fluke at PM	ABZ TCBZ (10 mg/kg)	+ve FEC confirmed at treatment	10 × T 10 × C		2.3 36.3	ABZ-R TCBZ-R	
2011	Australia (Oberon)	Exp	~200/sheep Lab maintained isolates	11	TCBZ (10mg/kg)	Mature adult (12)	6 × T 7 × C	16 weeks	0	TCBZ-R	Flanagan *et al*., 2011

Continued

Table 7.5. Continued.

Year	Country (isolate)	Infection	No. and source of metacercariae	Mean recovery rate (%)[a]	Drug (dose)	Stage of parasite at time of treatment (wpi)	No. of sheep per group	Weeks post infection at PM	Efficacy (%)	Resistance status as defined by authors	Reference
2012	Scotland, UK	Exp	~150/sheep Field isolate with history of treatment failure	N/A	TCBZ	Mature adult (15)	6 × T No control group	17 weeks	Not calculated, 19–70 adult fluke/liver at PM	TCBZ-R	Gordon *et al.*, 2012b
2012	New Zealand	Nat	N/A	N/A	TCBZ (10 mg/kg) TCBZ (10 mg/kg) MOX	Immature and adult[d] (>3)	10 × T 10 × T 16 × C	8 weeks post-treat-ment	21 30	TCBZ-R	Hassell and Chapman, 2012
2013	Argentina (CEDIVE)	Exp	~200/sheep Lab maintained isolate	36.15	ABZ (7.5 mg/kg)	Mature adult (16)	4 × T 4 × C	18 weeks and 1 day	29	ABZ-R	Sanabria *et al.*, 2013
2013	Peru (Cajamarca)	Exp	~200/sheep Field isolate	2.5–24	TCBZ (10 mg/kg)	Mature adult (15)	6 × T 5 × C	17 weeks and 1 day	25.2	TCBZ-R	Ortiz *et al*., 2013
2017	Australia (Oberon)	Exp	~200 or 44/ sheep Lab maintained isolate	55 and 22.7	TCBZ (10 mg/kg)	Late immature(6) and adult (10)	3 × T 3 × C	13 weeks	6wpi=71.8 10wpi=30	TCBZ-R	George *et al.*, 2017
2019	Uruguay (Uru-mon)	Exp	~75/sheep Lab maintained isolate	13–21 fluke at PM	ABZ (7.5 mg/kg)	Mature adult (16)	4 × T 4 × C	18 weeks	1.1%	ABZ-R	Ceballos *et al.*, 2019

[a]Recovery calculated based on number of parasites from untreated controls as a % of metacercariae used for infection; [b]naturally infected sheep returned to infected pasture post-treatment; [c]cited that protocol from Boray *et al*. (1969) was used, which cites range of 200-4000 metacercariae/sheep and a 28-44% recovery rate; [d]sheep removed from grazing for 3 weeks and recorded a positive FEC.

C, control (untreated) sheep; PM, post mortem; N/A, not applicable; T, treated sheep; wpi, weeks post-infection.

difference in the number of parasites when comparing control groups alone. This does not detract from the overall conclusion of the resistance status of the CEDIVE isolate, but it does highlight the importance of using multiple animals per group. Similarly, in Ortiz *et al*. (2013) all sheep were given 200 metacercariae from a common pool, but the numbers of parasites in untreated controls were 48, 22, 8, 6 and 5, whilst in TCBZ-treated (TCBZ+) sheep the numbers were 33, 18, 10, 9, 5 and 2, so there are large confidence intervals with both groups.

- *Inherent variation in infection and applying statistical tests.* Some papers only cite an overall efficacy as a percentage based on comparison of the mean number of parasites in treated versus untreated hosts, although others follow best practice and report the numbers of parasites for each animal within a group, along with the mean (both arithmetic and geometric) and standard deviation (SD). When statistical analyses have been performed, a range of different tests have been used. For example, Ceballos *et al*. (2019) compared liver fluke counts in treated and untreated groups by a non-parametric unpaired test (Mann–Whitney), Ortiz *et al*. (2013) used the Student's *t*-test and Gaasenbeek *et al*. (2001) used a paired *t*-test and the method of least-squares.
- *Cut-off values for reporting resistance will need to be tailored for each drug based on established efficacy.* The majority of CETs have focused on determining drug resistance to TCBZ (Table 7.5), which is easier to interpret due to its high efficacy against all life-cycle stages. Some reports of 'resistance' could be regarded as within the normal efficacy range of several drugs, especially the non-TCBZ drugs such as ABZ, CLOS and OXYCLO. When establishing drug efficacy, it is important that pioneer products are used.
- *CETs conducted with naturally infected animals require careful interpretation*. Two studies reported CETs on naturally infected animals. Hassell and Chapman (2012) removed lambs from pasture for 3 weeks prior to treatment, at which point they were confirmed as FEC positive and the authors subsequently showed that TCBZ-R parasites were present post-treatment. Similarly, Mamani and Condori (2009) demonstrated TCBZ resistance using naturally infected lambs, from two farms, that continued to graze infected pasture for a further 8 weeks pt, prior to necropsy. During this timeframe, reinfection may have taken place, although it is unlikely to have reached patency. However, the lack of efficacy of ABZ against pre-patent fluke is an important issue, as it is not possible to distinguish between eggs from parasites that are resistant, eggs shed by surviving mature adult parasites and eggs from parasite stages that were not killed by ABZ and have subsequently matured to egg-producing adults. This is less of an issue with the TCBZ-treated group, given its high efficacy, but is an important consideration for other drugs if relying on naturally infected hosts.
- *Provenance of parasite material and recovery rates are important*. The liver fluke undergoes clonal expansion with infected snails shedding thousands of genotypically identical cercariae, potentially reducing the number of different genotypes used for infection (Hodgkinson *et al*., 2018); this could be compounded by giving low doses to each animal (< 100 metacercariae). Indeed, the early work of Boray (1969) specified criteria for the propagation and infection of snails to ensure population representation, and the provenance of liver fluke isolates was the feature of a review in 2011 (Fairweather, 2011a). In the majority of CETs, ≥ 200 metacercariae were used for experimental infection of sheep (see Fig. 7.1 and Table 7.5), but very few provided any details about their provenance. It is important to provide context for the parasite material used: how many infected snails were metacercariae derived from, what the total number of cercariae shed by each snail was and how many metacercariae from each snail were used for infection. Assessing viability of metacercariae prior to infection is essential to achieve the high percentage recovery rates that allow effective evaluation of drug efficacy.

Despite their obvious advantages it is not always practicable or economic to perform CETs in

the field and, overall, a CET was carried out in less than one-fifth of drug resistance studies to date.

7.5.1.2 FECRT

Traditionally, fluke infections have been diagnosed by detecting eggs in faeces. In parallel, the FECRT has become the test most widely used for the determination of drug efficacy and drug resistance. Of the field reports of drug resistance in sheep, 23 (88.5%) were primarily based on FECRT, although several employed other diagnostic tests to support FECRT observations (Table 7.6). In cattle, all field reports of drug resistance in *F. hepatica* and *F. gigantica* were determined by FECRT, none of which were confirmed by CET, but a small number of studies provided additional diagnoses (Fairweather *et al.*, 2020) (Table 7.7). The majority of these studies were conducted on only one or two farms or restricted to geographical regions, so there is a lack of large-scale prevalence studies.

There is no standard protocol for *Fasciola* spp. FEC, for example whether a flotation (in zinc sulfate) or sedimentation (in water) method should be used, or whether individual or composite samples should be examined. Published FECRT studies cite multiple sedimentation methods (e.g. Dorsman, 1956; Thienpont, 1979; Dixon and Westcott, 1987), often with minor modifications, whilst others describe bespoke methods (Olaechea *et al.*, 2011). There has been some focus on developing and applying the FLOTAC technique for large-scale on-farm surveys and for the assessment of drug efficacy (Cringoli *et al.*, 2010, 2017; Rinaldi *et al.*, 2015). A validated composite FECRT (cFECRT) for TCBZ resistance in sheep flukes, which involves collection of two × 10 samples for preparation of a composite FEC at the time of treatment and 21 days later, was published by Daniel *et al.* (2012) and later employed on the largest field trial to date, conducting a cFECRT on 26 farms in England and Wales (Kamaludeen *et al.*, 2019).

The majority of studies have used the WAAVP efficacy values defined for nematodes (Coles *et al.*, 2006), where drug treatment is regarded as successful if there is a ≥ 90–95% reduction in fluke FECs. Generally, FECRTs are conducted 21 days pt particularly for TCBZ, although many studies have carried out several FECs, at 7, 14, 28 and 56 days pt, particularly for non-TCBZ drugs. However, it can be very time consuming for both farmers and laboratory staff to collect and process multiple samples. In addition, the FECRT suffers from a number of limitations that must be taken into account.

- *FECRT only detects patent infections.* Flukes do not lay eggs until they are ~9–10 weeks of age in the definitive ruminant host.
- *Timing of post-treatment FECs are likely to differ for each flukicide.* More than three-quarters of all resistance studies relate to TCBZ resistance. In TCBZ-susceptible populations, all stages from the first week post-infection will be effectively killed and it will be many weeks before eggs will be shed in the faeces, either through reinfection or through maturation of days-old flukes. For other flukicides, it is more complex and important to navigate the range of efficacies that they demonstrate; a proportion of adult parasites may survive treatment due to reduced efficacy (e.g ABZ) or they may mature to egg-producing adults within the post-treatment time-frame. For example, if considering CLOS, the post-treatment timepoints at which samples showed > 95% FEC reduction were different depending on the study: at day 7 (Mooney *et al.*, 2009) or day 14 (Elitok *et al.*, 2006), whilst for others it was day 21 (Moll *et al.*, 2000; Hanna *et al.*, 2015b). Importantly, egg shedding can be irregular, and eggs may be stored for some time in the host gall bladder and their delayed release could lead to false positive FECs post-treatment, even when the flukes have been removed (for further detail see Flanagan *et al.*, 2011a,b).
- *Cut-off values for reporting resistance may need to be tailored for individual drugs.* Percentage efficacy information from the 39 entries in Tables 7.6 and 7.7 (the majority of which are FECRT) has been collated in Table 7.8. A 95% threshold was used in seven studies, 90% in another nine and no level mentioned for the rest (23 studies). As stated above for the CET, some 'resistance' reports (e.g. 71–80% and 81–90% efficacy) could be regarded as within the normal efficacy range of the drug, especially for non-TCBZ drugs such as ABZ and OXYCLO.

Table 7.6. Field reports of drug resistance in *Fasciola hepatica* in sheep.

Year	Country	Drug	Number of farms[a]	CET	FEC/FECRT	Sero-diagnosis	CRT	EHT	Molecular	Histology	Ref.
1995	Australia	TCBZ	1/1	X	X						1
1998	Scotland	TCBZ	1/1		X	X					2
2000	The Netherlands	TCBZ	1/1		X						3
2000	Wales	TCBZ	1/1		X						4
2005	The Netherlands	TCBZ	1/1		X	X					5
2006	Spain	ABZ, TCBZ	1/1		X						6
2008	Brazil	TCBZ	1/1		X						7
2009	Ireland	TCBZ	1/1		X	X					8
2009	Bolivia	ABZ, TCBZ	2/2	X	X	X					9
2010	Northern Ireland	TCBZ	1/12		X		X				10
2010	Spain	TCBZ	1/1		X	X					11
2011	Scotland	TCBZ	1/1		X						12
2012	Wales, Scotland	TCBZ	7/25		X						13
2012	Scotland	TCBZ	n/s	X	X	X	X				14
2012	Scotland	TCBZ	2/2		X	X	X				15
2012	New Zealand	TCBZ	1/1	X	X						16
2012	Sweden	ABZ	1/1		X		X				17
2013	Peru	TCBZ	1/1	X							18
2013	Spain	ABZ, CLORS	2/2		X		X		X		19
2013	Argentina	ABZ	1/1	X							20
2014	Spain	ABZ, CLORS	1/1		X						21
2015	Northern Ireland	TCBZ	5/13		X		X			X	22
2016	Sweden	ABZ	2/2		X	X		X			23
2019	England, Wales	TCBZ	21/26		X						24
2019	Uruguay	ABZ	n/s	X				X			25
2019	Argentina	ABZ	4/4		X			X			25

[a]Number of farms on which resistance detected/total number of farms surveyed.

ABZ, albendazole; CET, controlled efficacy test; CLORS, clorsulon; CRT, coproantigen reduction test; EHA, egg hatch assay; FEC/FECRT, faecal egg counts/faecal egg count reduction test; n/s, not stated; TCBZ, triclabendazole.

References: 1, Overend and Bowen (1995); 2, Mitchell *et al*. (1998); 3, Moll *et al*. (2000); 4, Thomas *et al*. (2000); 5, Borgsteede *et al*. (2005); 6, Álvarez-Sánchez *et al*. (2006); 7, Oliveira *et al*. (2008); 8, Mooney *et al*. (2009); 9, Mamani and Condori (2009); 10, Flanagan (2010); 11, Martínez-Valladares *et al*. (2010); 12, Sargison and Scott (2011); 13, Daniel *et al*. (2012); 14, Gordon *et al*. (2012a); 15, Gordon *et al*. (2012b); 16, Hassell and Chapman (2012); 17, Novobilský *et al*. (2012); 18, Ortiz *et al*. (2013); 19, Robles-Pérez *et al*. (2013); 20, Sanabria *et al*. (2013); 21, Martínez-Valladares *et al*. (2014); 22, Hanna *et al*. (2015b); 23, Novobilský *et al*. (2016); 24, Kamaludeen *et al*. (2019); 25, Ceballos *et al*. (2019). *After Fairweather et al. (2020)*.

Table 7.7. Field reports of drug resistance in *Fasciola hepatica* and *Fasciola gigantica* in cattle.

Year	Country	Drug	Number of farms[a]	FEC/FECRT	Sero-diagnosis	CRT	EHT	Molecular	Histology	Ref.
(a) *F. hepatica*										
2000	The Netherlands	TCBZ	1/1	X						1
2006	Turkey	ABZ, RAFOX	1/1	X	X					2
2011	Argentina	TCBZ	1/1	X	X					3
2012	Peru	ABZ, TCBZ	n/s	X						4
2012	Peru	TCBZ	3/5	X						5
2013	Peru	TCBZ	1/1	X						6
2014	Australia	TCBZ	5/8	X		X				7
2015	Australia	TCBZ	1/6	X		X				8
2015	Sweden	CLOS	2/3	X		X				9
2019	Chile	TCBZ	1/1	X						10
(b) *F. gigantica*										
2008	Tanzania	ABZ, OXYCLO	1/1	X						11
2013	Egypt	ABZ, RAFOX	n/s	X						12
2015	The Philippines	ABZ, TCBZ	n/s	X	X					13
2018	Tanzania	ABZ	n/s	X						14

[a]Number of farms on which resistance detected/total number of farms surveyed.

ABZ, albendazole; CET, controlled efficacy test; CLORS, clorsulon; CLOS, closantel; CRT, coproantigen reduction test; EHA, egg hatch assay; FEC/FECRT, faecal egg counts/faecal egg count reduction test; n/s, not stated; OXYCLO, oxyclozanide; RAFOX, rafoxanide; TCBZ, triclabendazole.

References: 1, Moll *et al.* (2000); 2, Elitok *et al.* (2006); 3, Olaechea *et al.* 2011; 4, Chávez *et al.* (2012); 5, Rojas (2012); 6, Ortiz *et al.* (2013); 7, Brockwell *et al.* (2014); 8, Elliott *et al.* (2015); 9, Novobilský and Höglund (2015); 10, Romero *et al.* (2019); 11, Keyyu *et al.* (2008); 12, Shokier *et al.* (2013); 13, Venturina *et al.* (2015); 14, Nzalawahe *et al.* (2018). *After Fairweather et al. (2020)*.

Table 7.8. Efficacy percentage reports of drug resistance in *Fasciola hepatica* and *Fasciola gigantica*.

Efficacy (%)	TCBZ	ABZ	CLOS	CLORS	RAFOX
91–100					
81–90	(R13)^{S}; (R21)^{C}	(R28)^{C}			(R20)^{C}
71–80	(R4)^{S}; (R10)^{S}; (R21)^{C}; (R26)^{C}	(R20)^{C}; (R26)^{C}	(R25)^{C}	(R4)^{S}; (R18)^{S}	
61–70	(R6)^{S}; (R13)^{S}; (R16)^{C}	(R5)^{C}; (R18)^{S}; (R27)^{S}			(R5)^{C}
51–60	(R7)^{S}; (R13)^{S}; (R31)^{C}				
41–50		(R4)^{S}; (R28)^{C}; (R29)^{S}		(R22)^{S}	
31–40	(R2)^{C}; (R8)^{S}; (R12)^{C}; (R13)^{S}; (R14)^{S}; (R17)^{C}; (R24)^{S}				
21–30	(R1)^{S}; (R15)^{S}; (R17)^{S}	(R19)^{S}; (R29)^{S}			
11–20	(R2)^{S}; (R9)^{S}; (R13)^{S}; (R14)^{S}; (R21)^{C}	(R8)^{S}			
0–10	(R2)^{C}; (R3)^{S}; (R11)^{C}; F^{2} (R13)^{S}; F^{2} (R16)^{C}; (R23)^{C}; F^{4} (R24)^{S}; F^{9} (R30)^{S}	(R8)^{S}; (R12)^{C}; (R22)^{S}; F^{2} (R29)^{S}	(R25)^{C}		

^{C}cattle; ^{S}sheep.
ABZ, albendazole; CLORS, clorsulon; CLOS, closantel; F^{n}, number of farms involved (where F not included, *n*=1). RAFOX, rafoxanide; (Rn), reference number; TCBZ, triclabendazole; %, percentage.
References: R1, Overend and Bowen (1995); R2, Moll *et al.* (2000); R3. Borgsteede *et al.* (2005); R4, Álvarez-Sánchez *et al.* (2006); R5, Elitok *et al.* (2006); R6, Oliveira *et al.* (2008); R7, Mooney *et al.* (2009); R8, Mamani and Condori (2009); R9, Flanagan (2010); R10, Martínez-Valladares *et al.* (2010); R11, Olaechea *et al.* (2011); R12, Chávez *et al.* (2012); R13, Daniel *et al.* (2012); R14, Gordon *et al.* (2012b); R15, Hassell and Chapman (2012); R16, Rojas (2012); R17, Ortiz *et al.* (2013); R18, Robles-Pérez *et al.* (2013); R19, Sanabria *et al.* (2013); R20, Shokier *et al.* (2013); R21, Brockwell *et al.* (2014); R22, Martínez-Valladares *et al.* (2014); R23, Elliott *et al.* (2015); R24, Hanna *et al.* (2015), McMahon *et al.* (2016); R25, Novobilský and Höglund (2015); R26, Venturina *et al*. (2015); R27, Novobilský *et al*. (2016); R28, Nzalawahe *et al*. (2018); R29, Ceballos *et al*. (2019); R30, Kamaludeen *et al*. (2019); R31, Romero *et al*. (2019). *After Fairweather et al. (2020)*.

- *A minimum pre-treatment FEC.* Some studies report very low FEC (1–2 eggs/g), which presents a challenge when detecting a reduction in egg shedding post-treatment. Moreover, when fluke burdens are small and FECs low, inaccuracies of sampling could have a large effect on the outcome, with a miscounting of just one or two eggs having an unbalanced influence on the pre- or post-FEC.

In addition, factors such as host age, faecal water content and the number of aliquots tested per sample can all affect the sensitivity of the FEC (reviewed by Alvarez Rojas *et al*., 2014). Nevertheless, FECRTs are used because they are simple, convenient and are applicable to all anthelmintic classes, but at the very least the FECRT should be validated for each anthelmintic class (Brockwell *et al.,* 2014; Novobilský and Höglund, 2015).

7.5.1.3 Egg hatch test

The egg is probably the most accessible stage in the *Fasciola* spp. life cycle for collection and experimentation and they are known to be sensitive to drug action (e.g. McConville *et al*., 2012; Hanna, 2015b). As a result a number of egg hatch tests (EHTs) have been carried out with BZs: TCBZ/TCBZ.SO, OXF, MBZ and in particular ABZ/ABZ.SO (see Fairweather *et al*., 2020 for further details). The value of the EHT for detecting TCBZ resistance is not clear. Whilst two studies suggested that TCBZ had an ovicidal effect (Fairweather *et al*., 2012; Arafa *et al*., 2015), others showed that an EHT was not able to detect resistance to TCBZ (Robles-Pérez *et al*., 2015); hence an EHT is not widely used to detect TCBZ resistance in the field. The EHT methods lack consistency, such as the location from which the eggs are sourced (e.g. the faeces, gall bladder or directly from the fluke itself) and whether the eggs have been exposed to treatment *in vivo* or *in vitro* without previous drug exposure, which has made it difficult to compare EHT studies. Recently, Ceballos *et al*. (2019) highlighted how crucial the length of incubation with the drug is to the outcome of the test, with a longer incubation (8–15 days versus 12 h) rendering a drug-resistant isolate to behave as

if it was drug sensitive. Other variables include the range of concentrations used and which form of the drug is used, either the commercial drench diluted with a solvent such as DMSO, or the parent drug or its metabolite(s).

Solana *et al.* (2016) determined that *F. hepatica* eggs exposed to CLOS i*n vivo* showed reduced development 36 h pt (but not at 12 h and 24 h); however, when eggs from untreated hosts were exposed to CLOS *in vitro* there was a lack of ovicidal activity (Ceballos *et al.*, 2017). NITROX was reported to show ovicidal activity *in vitro* (Hegazi *et al.*, 2018), whilst OXYCLO was shown to have no effect on the development and hatching of *F. gigantica* eggs (Arafa *et al.*, 2015). EHTs are perhaps most appropriate for BZs, as they discriminate between eggs from drug-susceptible or drug-resistant fluke isolates, particularly for ABZ (e.g. Fairweather *et al.*, 2012; Canevari *et al.*, 2014; Novobilský *et al.*, 2016; Ceballos *et al.*, 2019). Recently, a well-validated EHT for detecting ABZ resistance has been developed by the authors (Alvarez *et al.*, 2020). This method was validated using isolates known to be either ABZ-S or ABZ-R and, as such, forms a valuable *in vitro* diagnostic for ABZ resistance that can replace the costly and time-consuming CET (Ceballos *et al.*, 2019; Alvarez *et al.*, 2020). Not only does this EHT provide a standardized *in vitro* protocol for the detection of ABZ resistance; it also raises important considerations for validation of the EHT when reviewing existing reports in the literature and when developing EHTs for other flukicides.

- *A minimum egg development of 70% in untreated eggs is required prior to assessing ovicidal activity.* To avoid ambiguous or erroneous results, it is important that eggs are sufficiently viable to detect the effects of the drug. It is also important to establish a baseline (maximum 10%) of egg development to reduce the impact of development during storage.
- *Only the parent drug should be used in the EHT.* Use of commercial products is not appropriate, as the excipients could interfere with egg development.
- *Ovicidal activity and statistical significance should be determined.* The percentage of developed eggs should be reported as the arithmetic mean ± SD and subjected to parametric (unpaired *t*-test) or non-parametric (Mann–Whitney test) analysis for the discriminating dose, with a significance of $P < 0.05$. Ovicidal activity should be expressed as a percentage (Alvarez *et al.*, 2020). For example, for ABZ resistance to be confirmed, it is important that egg development in untreated eggs did not significantly differ ($P < 0.05$) between ABZ-treated eggs (discriminating dose of 0.5 µM ABZ) and untreated eggs.
- *To estimate ovicidal activity, a critical concentration of drug should be determined.* Identifying a discriminating dose and the percentage ovicidal activity for a given drug is an essential part of the validation process and should be defined using isolates of known resistance status. For example, for ABZ resistance, the discriminating concentration is 0.5 µM ABZ. If ovicidal activity is ≥ 70%, the isolate is considered to be ABZ-S; if the activity is between 40% and 70%, the result is inconclusive; and if ovicidal activity is ≤ 40%, the isolate is considered to be ABZ-R.
- *The minimum time-frame for incubation with the drug should be established.* As mentioned above, leaving the eggs in the presence of ABZ for 15 days, rather than the recommended 12 h incubation (with subsequent washing of the eggs to remove the drug), made a known ABZ-R isolate appear to be ABZ-S.

7.5.1.4 Coproantigen Reduction Test

The MM3 COPRO test, first described by Mezo *et al.* (2004), is based on the MM3 MoAb that binds to both CatL1 and CatL2 proteases and has been commercialized by BIO-X Diagnostics (La Jemelle, Belgium) (see also Chapter 10, this volume). Kajugu *et al.* (2012, 2015) showed that this test has a high diagnostic specificity (100%) and showed no cross-reaction when tested with soluble fractions of homogenates from *Paramphistomum cervi* and *Taenia hydatigena* or in co-infections with paramphistomes, coccidians and/or gastrointestinal nematodes. However, the sensitivity of the coproantigen ELISA (cELISA) can sometimes be compromised by the high variability in the concentration of cathepsin proteinases in faecal samples and by differences in the between-batch performance of peroxidase-labelled

anti-mouse IgG polyclonal antibodies (Martínez-Sernández *et al.*, 2016). Brockwell *et al.* (2013) and Palmer *et al.* (2014) improved the sensitivity of the MM3 COPRO ELISA using a customized cut-off for sheep and cattle whilst maintaining the specificity above 99%. However, the test had poor diagnostic sensitivity in horses (Palmer *et al.*, 2014). Recently, a new version of the coproantigen test using a streptavidin-polymerized horseradish peroxidase conjugate has been evaluated and was sufficiently sensitive to detect infection with a single fluke (Martínez-Sernández *et al.*, 2016).

The cELISA is highly sensitive, reported to be capable of detecting infections of as few as one fluke in sheep and cattle (Mezo *et al.*, 2004; Martínez-Sernández *et al.*, 2016). The antigens only persist for the lifetime of the infection, so are indicative of current infection. Other advantages of the cELISA are that it is non-invasive and farmers can submit samples without requiring a visit from the vet. In experimentally infected sheep and cattle, coproantigens can be detected from 5–6 weeks pi, a time that corresponds to the entry of the flukes into the bile ducts (Mezo *et al.*, 2004; Flanagan *et al.*, 2011a,b; Brockwell *et al.*, 2013; Calvani *et al.*, 2018). Although this is later than the reported sero-antibody tests or detection by molecular methods, it is earlier than the detection of eggs at patency. The difference may be 2 weeks (Flanagan *et al.*, 2011a,b; Brockwell *et al.*, 2013; Calvani *et al.*, 2018) but can be longer – up to 5 weeks (Valero *et al.*, 2009; Martínez-Pérez *et al.*, 2012). The cELISA has proved to be a convenient alternative to FECs and there is a strong correlation between cELISA data and fluke burdens. The assay has been validated in experimental infections (Flanagan *et al.*, 2011a,b; Brockwell *et al.*, 2013; Robles-Pérez *et al.*, 2013; George *et al.*, 2017; Calvani *et al.*, 2018), and also under natural (field) conditions (Gordon *et al.*, 2012a; Novobilský *et al.*, 2012, 2016; Robles-Pérez *et al.*, 2013; Brockwell *et al.*, 2014; Elliott *et al.*, 2015; Hanna *et al.*, 2015b; Novobilský and Höglund, 2015; Arifin *et al.*, 2016). The assay is suitable for use with sheep, cattle and deer (French *et al.*, 2016), but not horses (Palmer *et al.*, 2014).

In contrast with studies in experimentally infected sheep and cattle, there is no evidence that the cELISA detects natural infection any earlier compared with FECs. This is probably due to the trickle infection of sheep and cattle naturally exposed to *F. hepatica* relative to the timing of sample collection (Gordon *et al.*, 2012b; Martínez-Pérez *et al.*, 2012; Novobilský *et al.*, 2012; Brockwell *et al.*, 2014; Elliott *et al.*, 2015; Hanna *et al.*, 2015b; Arifin *et al.*, 2016). Similarly some studies suggest that the cELISA is less sensitive when compared with FECs when used in field studies (Gordon *et al.*, 2012b; Novobilský *et al.*, 2012; Hanna *et al.*, 2015b; Kajugu *et al.*, 2015).

It is preferable to use the cELISA with individual samples. A limited number of studies have investigated its use for bulk faecal samples (Martínez-Pérez *et al.*, 2012; Brockwell *et al.*, 2013; Elliott *et al.*, 2015), but there has been no comprehensive field validation of the cELISA for use with bulk faecal samples compared with bulk FECs.

The cELISA is increasingly being used as a diagnostic tool to determine drug efficacy and, by extension, for the diagnosis of drug resistance. While (understandably) the test has been used mainly for the determination of TCBZ efficacy, it has also been used to evaluate the efficacy of ABZ, CLOS, CLOR, NITROX, OXYCLO (Flanagan *et al.*, 2011a,b; Novobilský *et al.*, 2012, 2016; Brockwell *et al.*, 2013, 2014; Robles-Pérez *et al.*, 2013; Elliott *et al.*, 2015; Hanna *et al.*, 2015b; Novobilský and Höglund, 2015; George *et al.*, 2017, 2019). It has also assisted in the diagnosis of resistance to TCBZ (Table 7.6), ABZ (Novobilský *et al.*, 2012, 2016; Robles-Pérez *et al.*, 2013) and CLOS (Novobilský and Höglund, 2015).

Modifications and improvements have been made to the technique in individual laboratories; they include overnight antigen extraction and host species-specific cut-off values (Brockwell *et al.*, 2013, 2014; Palmer *et al.*, 2014; Elliott *et al.*, 2015; Martínez-Sernández *et al.*, 2016). Further work is required to optimize the protocol for field use. A recent study has raised concerns about the reliability of the cELISA under field conditions, in situations where mixed-age infections are likely to be present, and especially if the fluke population is largely immature (George *et al.*, 2017). This potential limitation can be overcome by carrying out a second test at least 6 weeks after the first one; this would allow the immature flukes to develop and be recognized. A recent field investigation by George *et al.* (2019) has shown that the diagnostic sensitivity for epidemiological

studies can be increased if the cELISA and FEC methods are used together and ideally in parallel.

In some studies, the coproantigen reduction test has been favoured, but still requires further validation in field studies (Kelley *et al.*, 2016) and, as with the FECRT, it is time-consuming and lacks the precision to directly detect drug resistance alleles as they emerge in parasite populations. The assay does offer some important advantages over the FECRT for use with flukicides that are only effective against late immature and adult fluke, as evidence suggests that coproantigens disappear within 7 days of treatment. This takes away the complication of susceptible flukes maturing and being conflated with the presence of resistant flukes in a FEC 21 days after treatment.

In recommending the coproantigen reduction test as a method to detect resistance, there are several aspects of its use that must be considered.

- *The cut-off used in the cELISA requires standardization and the diagnostic sensitivity established in comparison with FEC.* In field studies, using the manufacturer's cut-off, the cELISA appears to be less sensitive than FEC; however, where cut-offs have been altered in-house, sensitivity appears to be high. Similarly, there are differences between studies in how samples are stored and then prepared for analysis in the cELISA. For optimal use of the cELISA, full comparative validation of sample preparation is needed.
- *More information is needed describing the sensitivity of the cELISA in field studies.* Whilst it is clear from experimental infections that the cELISA is very effective in detecting infection earlier than FECs, Gordon *et al.* (2012b) showed that both FEC and cELISA came positive at the same time in first-season grazing sheep on Scottish farms. Kajugu *et al.* (2015) showed that, in naturally infected sheep and cattle, the cELISA was less sensitive in animals with low FEC. It may be possible to resolve this if a lower cut-off in the cELISA is established for naturally infected sheep and cattle.
- *No full validation studies have been reported comparing bulk faecal samples tested by cELISA and FECs.* Brockwell *et al.* (2013) compared single versus bulk samples in the cELISA using samples from experimentally infected cattle. They made pools with combinations ranging from 1/3 to 1/5 positives. In every case, if a positive sample was included in the pool, the pool was positive in the cELISA. However, the optical density (OD) values of each individual sample were high (ranging from 0.6 to 2.097; negative cut-off was 0.014) and pools were made from three or five samples. Similarly, Elliott *et al.* (2015) also investigated pools from naturally infected cattle. The pools under investigation were tested first and only those that tested positive were further evaluated. In our experience on farms (D. Williams, unpublished observations; P. Skuce, personal communication), in pools made from ten sheep or cattle and compared with FEC using the same composite, the cELISA is invariably less sensitive compared with FEC, particularly where prevalence is low.
- *Use of cELISA as a means of detecting drug efficacy in fluke populations is likely to be a valuable tool, especially for non-TCBZ medicines, but full validation in the field is needed.* Hanna *et al.* (2015b) showed that six farms out of 13 had FEC sufficient to do a reduction test and that there was agreement between the two tests on only three out of six farms. Some authors considered the coproantigen reduction test to be a 'robust alternative' to the FECRT as OD values fell within 7 days of treatment (Brockwell *et al.*, 2014) whilst Gordon *et al.* (2012b), in a field study, found that the cELISA (CLOS and TCBZ) was no better than the FECRT in an evaluation of efficacy. Clearly more validation is required, particularly in field settings.

7.5.2 Other methodologies

A number of other methods have been assessed for their ability to detect resistance. These include serodiagnosis, a sensitive means of detecting infection but unsuitable for efficacy and resistance testing due to antibody persistence. Recently, pilot studies using recombinant molecular biomarkers, calreticulin (CRT) and triose phosphate isomerase (TPI), showed that they are recognized in the sera of *F. hepatica*-infected

sheep and may provide a novel molecular test platform to measure TCBZ efficacy in future (Collett *et al*., 2020).

It has been suggested that molecular techniques can detect infection earlier than that achieved with other methods (e.g. FECs and cELISA), but this may not always be the case (Calvani *et al.*, 2018). Sensitivity of molecular methods is greater than that of conventional methods (Martínez-Pérez *et al*., 2012; Robles-Pérez *et al*., 2013; Cabada *et al*., 2017) but there have been some inconsistent results (Arifin *et al*., 2016; Calvani *et al*., 2018). To date, only one molecular study has been used to detect drug resistance in *F. hepatica* and this was with naturally infected sheep (Robles-Perez *et al.*, 2013). On the basis of all three tests, the authors concluded that sheep harboured liver fluke susceptible to TCBZ, but resistant to ABZ and CLOR, although it is important to distinguish this from parasite persistence and continued shedding of eggs post-treatment, given the lower efficacy levels of ABZ and CLOR. Loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) have been developed for *F. hepatica* infection in faecal samples from sheep and cattle, but not yet for detecting resistance (Martínez-Valladares and Rojo-Vázquez, 2016; Cabada *et al*., 2017). It is clear that whilst molecular methods may offer promise, they are not yet widely employed for detecting drug resistance.

Although histology by itself cannot be used to establish a diagnosis of drug resistance, it can be used to complement and support the results of other methods (e.g. FECRT and CRT) and has been of value in complementing ultrastructural data to understand drug actions and mechanisms of resistance (Fairweather *et al.*, 2020).

7.6 Mechanism(s) of Resistance

If effective drug control is to be implemented, we need to understand the genetic and molecular basis of resistance to flukicides in *F. hepatica* populations, especially to TCBZ, and we require an appreciation of the factors that influence the emergence and spread of drug resistance alleles in liver fluke populations. For example, our current lack of understanding of the mechanisms involved in TCBZ resistance raises several questions. Is there a single origin of TCBZ resistance or multiple origins and is a common pathway involved in the expression of a TCBZ-R phenotype? Is TCBZ resistance a dominant or recessive trait? Is the same mechanism employed by both adult and immature parasites? All of these questions comprise important knowledge gaps that limit our ability to mitigate the impact of TCBZ-R liver fluke infections.

Our current knowledge of mechanisms of drug resistance in liver flukes is extensively covered in by Fairweather *et al.* (2020) and we refer the reader there for a detailed review. To identify the mechanism(s) of resistance to TCBZ, studies have centred on candidate mechanisms and more recent omics-based approaches. Crucial to these studies is the availability of characterized liver fluke isolates, with which to explore drug-resistance mechanisms. Efficacy data has been reported for eleven isolates of either *F. hepatica* or *F. gigantica*, four of which were shown to be TCBZ-R, eight ABZ-R and three CLOS-R (see Table 3 in Fairweather *et al*., 2020). Four of these isolates were resistant to two or more drugs (Cajamarca, Santillán de la Vega, RA, Corrulón). In addition, three clonal TCBZ-R *F. hepatica* isolates were derived from single miracidium–snail infections and subsequent phenotyping by CET (Hodgkinson *et al*., 2018).

7.6.1 Candidate gene studies

Candidate gene studies exploit our knowledge of drug targets within the parasite and our knowledge of how parasites might mitigate drug action. For *F. hepatica* the focus has been on altered tubulin binding, altered drug uptake and modified drug metabolism. As TCBZ is a BZ, the presumed target is β-tubulin, although given that *F. hepatica* is refractory to other BZs, the nature of TCBZ action is presumed to be distinct from other BZs (Robinson *et al*., 2004b; Ranjan *et al*., 2017) (Fig. 7.2). Morphological changes typical of microtubule inhibition seen in TCBZ-S but not TCBZ-R flukes are consistent with involvement of β-tubulin (for references, see previous reviews by Fairweather, 2005, 2009, 2011b). Sequence comparisons of β-tubulin isotypes between TCBZ-S and TCBZ-R flukes showed that there were no sequence differences in β-tubulin isotypes and no differences in expression levels

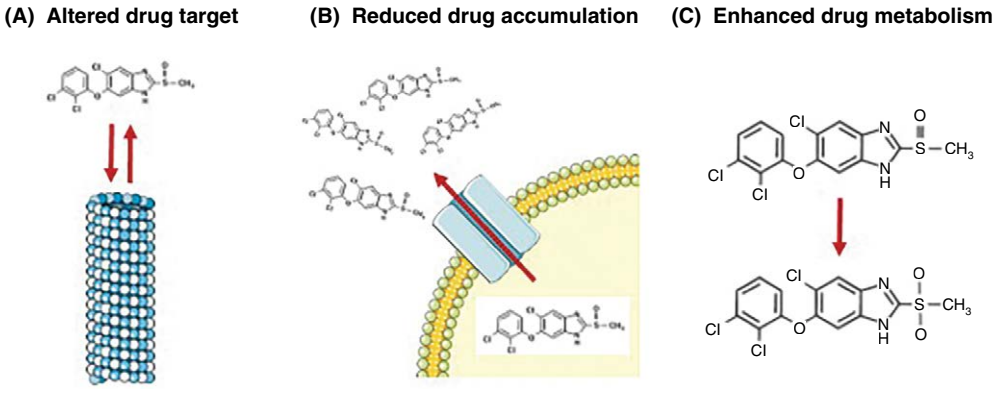

Fig. 7.2. Proposed mechanisms of TCBZ resistance in *Fasciola hepatica*. **(A)** Initial studies focused on the putative target of TCBZ, namely β-tubulin, although no mutations conferring resistance have been identified. **(B)** Several studies suggest that TCBZ is a substrate for membrane transporters such as P-glycoprotein. Their activity is increased in TCBZ-resistant flukes which may reduce the intracellular concentration of the drug at its site of action. **(C)** Metabolism of active forms of TCBZ to comparatively inert metabolites (e.g. TCBZ sulfoxide to TCBZ sulfone as shown here) is increased in TCBZ-resistant flukes. Medical art provided by Les Laboratories Servier (https://smart.servier.com/). After Fairweather *et al.*, 2020.

between isolates on exposure to TCBZ and its metabolites (Robinson *et al.*, 2002; Chemale *et al.*, 2010; Fuchs *et al.*, 2013).

The focus of attention has shifted to exploring other potential resistance mechanisms, most notably in relation to altered drug uptake and drug metabolism. It is known that the uptake of TCBZ and TCBZ.SO by TCBZ-R fluke isolates is significantly less than that by TCBZ-S flukes (Alvarez *et al.*, 2005; Mottier *et al.*, 2006). This suggests that P-glycoprotein (Pgp)-linked drug efflux pumps may be involved in resistance. Resistance can be reversed by co-incubation *in vitro* with ivermectin (IVM) (Mottier *et al.*, 2006) and R(+)-verapamil (a Pgp inhibitor) has been shown to potentiate TCBZ action *in vitro* in TCBZ-R, but not TCBZ-S, flukes (Meaney *et al.*, 2013; Savage *et al.*, 2013). Wilkinson *et al.* (2012) found a single nucleotide polymorphism (SNP), $T^{687}G$, in a Pgp gene from small numbers of *F. hepatica*; this SNP was not found in TCBZ-R and TCBZ-S isolates from Australia (Elliott and Spithill, 2014), Latin America (Solana *et al.*, 2018) or Scotland (P. Skuce, 2016, unpublished observations) and it is now widely accepted that this SNP could not be used as a marker for TCBZ resistance. This does not discount the involvement of Pgp, not least because there are known to be multiple Pgp genes in *Fasciola* spp. (Maule *et al.*, 2020, personal communication) and a number of flukicides have been shown to inhibit Pgp-mediated rhodamine 123 transport in a recombinant cell line (LLC-PK1 cells) over-expressing Pgp (Dupuy *et al.*, 2010).

With regard to drug metabolism, it is known that metabolism of TCBZ to TCBZ.SO and TCBZ.SO to $TCBZ.SO_2$ is greater in TCBZ-R than TCBZ-S isolates (Robinson *et al.*, 2004a; Alvarez *et al.*, 2005), suggesting that drug metabolism is up-regulated in TCBZ-R flukes. In a series of morphological studies, co-incubation of TCBZ and TCBZ.SO with FMO and CYP450 inhibitors led to a potentiation of drug action in TCBZ-R flukes that was not seen in TCBZ-S isolates (see Devine *et al.*, 2012 and references therein). Activity of glutathione *S*-transferase (GST) is greater in TCBZ-R than TCBZ-S flukes (Scarcella *et al.*, 2012; Fernández *et al.*, 2014, 2015b) and the mu type, specifically, is differentially expressed at elevated levels in TCBZ-R flukes (Fernández *et al.*, 2014). A comparison of the GST mu gene in a TCBZ-S and in a TCBZ-R isolate of *F. hepatica* revealed an amino acid change in the TCBZ-R fluke, where threonine is replaced by serine at position 143 (Fernández *et al.*, 2015a). A GST has been cloned, expressed as a recombinant protein and shown to bind TCBZ.SO, indicating that it might have a role to play in resistance to

TCBZ (Chemale *et al.*, 2010). Elevated levels of GST activity have also been linked to salicylanilide resistance in *F. hepatica* (Miller *et al.*, 1994).

7.6.2 Omics approaches to understanding drug resistance

Genetic linkage mapping of heritable traits, such as drug resistance genes, has proved very successful for protozoa, for example *Plasmodium falciparum* (Su *et al.*, 2007); for trematodes, for example mapping oxaminiquine resistance in the human blood fluke, *Schistosoma mansoni* (Valentim *et al.*, 2013); and in our understanding of anthelmintic resistance in gastrointestinal nematodes (Doyle and Cotton, 2019; Doyle *et al.*, 2019). A genetic linkage mapping exercise has been carried out for *F. hepatica* and TCBZ resistance (for full explanation, see Hodgkinson *et al.*, 2013), made possible due to sequencing of the *F. hepatica* genome (Cwiklinski *et al.*, 2015). The approach controlled for the complex reproductive biology and demography of *F. hepatica* by first producing TCBZ-S and TCBZ-R clones of *F. hepatica*, carrying out an F2 cross of one TCBZ-S and TCBZ-R clone and subsequently mapping TCBZ-R genes in phenotyped recombinant F2 populations, using a panel of genome-wide SNPs (Fig. 7.3). Whole-genome resequencing was used to support discovery of ~9 million SNPs, followed by pooled genotyping on a number of replicates. Analysis of allele frequencies to associate SNPs (and hence genomic regions) to TCBZ resistance was performed on F2 populations by comparing the frequency of SNP alleles derived from the resistant parental clone and linked to the TCBZ resistance locus (or loci) in TCBZ-exposed parasites (TCBZ-R parasites only) relative to untreated controls (a mixture of TCBZ-R and TCBZ-S parasites). This has successfully mapped TCBZ resistance genes to six scaffolds within the *F. hepatica* genome, which were subsequently shown to be in linkage disequilibrium (Fig. 7.4) (Hodgkinson *et al.*, 2020, unpublished data). This suggests that TCBZ resistance is conferred due to inheritance of this single genomic locus, which contains about 30 genes. Functional analyses to characterize these genes and RNAi follow-up (McGonigle *et al.*, 2008) will reveal which gene(s) can confer TCBZ resistance. This then provides the basis on which to establish if this is the only

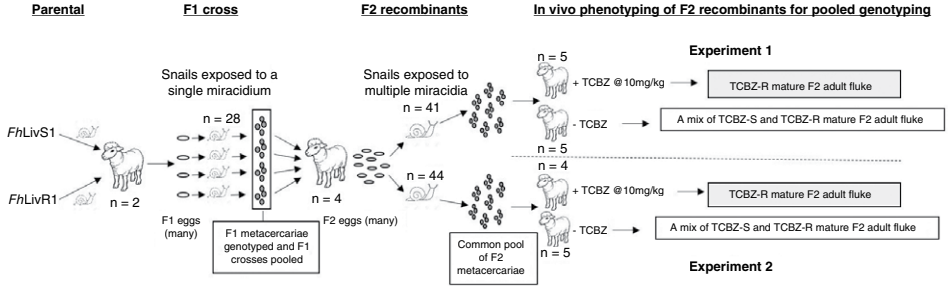

Fig. 7.3. Schematic of the *in vivo* work to produce an F2 cross from *Fh*LivS1 (triclabendazole (TCBZ)-susceptible clone) and *Fh*LivR1 (TCBZ-resistant clone). Clonal parental parasites *Fh*LivS1 and *Fh*LivR1 were used to co-infect two sheep to allow cross-fertilization of adult parasites. F1 derived from cross-fertilization were identified following clonal infection of snails and subsequent genotyping of F1 metacercariae (n = 28). The F1 metacercariae were mixed to make a common pool and used to infect sheep (n = 4). Following this, F2 recombinants (eggs) were harvested from adult F1 parasites, subsequently passaged through snails, and F2 metacercariae were produced by multiple miracidia infections of snails (Experiment 1, n = 41 snails; Experiment 2, n = 44 snails). Two groups of sheep (Experiment 1, n = 10; Experiment 2, n= 9) were infected with F2 metacercariae and allowed to reach patency (confirmed by positive faecal egg count). At this point sheep from each experiment were randomly separated into two groups: one group was treated with TCBZ (10 mg/kg) and the other group remained untreated. For each experiment this provided two populations of adult F2 parasites: TCBZ-R only adults (from treated sheep) and a mixture of TCBZ-R and TCBZ-S adults (from untreated sheep). This work was funded by UKRI, UK (BBSRC) BB/P001912/1 and BB/1002480/1.

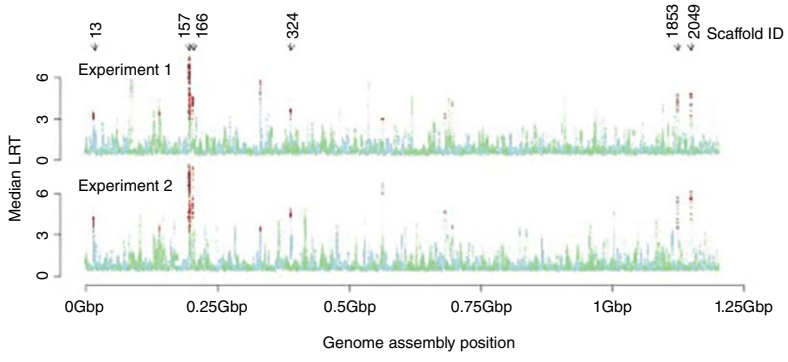

Fig. 7.4. Genome scan for regions associated with resistance to triclabendazole. Data show the median likelihood ratio test (LRT) statistic from generalized linear models within moving windows of 1000 informative SNPs. Red dots indicate where the median LRT appears in the top 1% quartile for each experiment. Scaffold order on the *x*-axis is arbitrary and does not imply physical proximity. Results of the two replicate crossing experiments are shown (Experiment 1 and Experiment 2). Position of scaffolds under greatest selection 13, 157, 166, 324, 1853 and 2049 is indicated by arrows. This work was funded by UKRI, UK (BBSRC) BB/P001912/1 and BB/1002480/1.

mechanism involved in TCBZ resistance, or whether it confers TCBZ resistance in different regions and geographical locations.

A transcriptomic approach to understanding TCBZ resistance was adopted by Radio *et al.* (2018). Comparisons were made between three isolates with different susceptibilities to TCBZ and ABZ: one TCBZ-R/ABZ-R; a second ABZ-R/TCBZ-S; and the third ABZ-S/TCBZ-S. The levels of expression of cytoskeleton-related proteins were lower in the TCBZ-R and ABZ-R isolate than in the other two: the proteins were α- and β-tubulin, kinesins and dyneins (Radio *et al.*, 2018). Moreover, there was a downregulation of a number of drug metabolism enzymes in this isolate, although the GST mu protein was upregulated (Radio *et al.*, 2018). Upregulation of an ABC transporter-like protein was also observed in the double-resistant isolate. Interestingly, there was a downregulation of adenylate cyclase (AC); inhibition of AC by TCBZ has been described in yeast (Lee *et al.*, 2013). The AC enzyme in *F. hepatica* is one of the most active in eukaryotes and is likely to play a significant role in fluke biology through its established roles in cell signalling mechanisms (Fairweather, 2004; Kelley *et al.*, 2016). The impact of TCBZ on fluke AC activity warrants further study. A recent study reported the transcriptomic response of TCBZ-R and ABZ-R liver fluke (from rabbits) pre- and post-drug exposure *in vitro* to TCBZ or ABZ, respectively (Miranda-Miranda *et al.*, 2021). Whilst this provides a useful resource, with identification of novel genes and splice variants, it requires further analysis to identify which genes are responding to drug treatment.

At the protein level, TCBZ has been reported to induce a broad range of effects, with several potential modes of action (Chemale *et al.*, 2010). Much of the work cited above on a key role for GSTs in the detoxification of TCBZ has been revealed by proteomics. Although it is less clear about the role of GSTs in TCBZ resistance, proteomic analyses were responsible for revealing the significantly increased expression of GSTs in TCBZ-R compared with TCBZ-S flukes (Scarcella *et al.*, 2012; Fernández *et al.*, 2014, 2015a). Recent advances in the ability to incorporate individual flukes in proteomic and sub-proteomic analyses may offer us a more effective way to monitor TCBZ resistance in future (Stuart *et al.*, 2021).

7.7 Concluding Remarks

The ultimate goal of any parasite control programme is to reduce parasite transmission and infection, maximize animal welfare and mitigate the economic impacts of parasitism (Kelley *et al.*, 2016; Fairweather *et al.*, 2020) (see Chapter 11). For *Fasciola* spp., this will be best achieved via integrated control measures, utilizing disease forecasting and environmental control, but

there will always be a need for safe, effective anthelmintics. For livestock farmers it is vital to know if their livestock harbour drug-resistant parasites and if the drug they are using will be efficacious; conversely, if they avoid use of drug due to unwarranted concerns of drug resistance, this could lead to suboptimal control. Therefore, accurate diagnosis of flukicide resistance is vital and it is important that initial indication or suspicion of reduced drug efficacy or treatment failure should be followed up, ideally by more than one test. In reality, the necessary time or resources may not be available to carry out multiple tests. Either way, with so much at stake, it is crucial that the tests are well designed, appropriately analysed and accurately interpreted.

A number of reviewers have raised important questions and identified research needs to stimulate and direct future research (Kelley *et al*., 2016; Beesley *et al*., 2018; Sabourin *et al*., 2018). They have stressed the need to determine the true prevalence of resistance to TCBZ and other flukicides in different areas of the world and to what extent resistance has permeated human populations. Perhaps one of the most important aspects is standardization of techniques and production of guidelines for investigating drug resistance on farms. It is not a simple task, as many factors must be taken into account and we have tried to highlight those we consider the most important. This is particularly pertinent as widespread TCBZ resistance (real or perceived) is leading to greater reliance on alternative flukicides and, whilst the detection methods may be the same, the timings of post-treatment sampling, efficacy cut-off thresholds and choice of test may differ, so identifying TCBZ resistance will rely on different criteria to those for CLOS resistance, for example. The prospect of multidrug-resistant *Fasciola* spp. populations looms, so accurate identification of resistance is key to improving control and advice to stakeholders.

Acknowledgement

Joe Boray was co-author of the flukicide chapter in the first edition of this book. Sadly, Joe died in 2018. If we are to learn from his example and focus on improving disease management at the farm level, it is important that any advances made in academic or industry laboratories are communicated down to veterinarians and advisers so that they are better informed. After all, it is they who are in the best position to ensure that farmers make the right decisions to deal with the disease and conserve the efficacy of the anthelmintics at our disposal for as long as possible. This would be a fitting legacy for a truly great parasitologist. An account of his life and career appeared in *Veterinary Parasitology* 261, 104–105. This revised and updated chapter is dedicated to Joe.

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8 Metabolism

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8.1 Introduction

Parasitic helminths, including *Fasciola hepatica*, have many interesting properties in their biochemistry which have stimulated research not only to enlarge our fundamental biochemical knowledge in general, but also for the development of new chemotherapeutics. In contrast to infections with bacteria, viruses and protozoa, a continued helminth infection in the host is not dependent on replication of the parasite. Potential targets for anthelmintic drugs are therefore the metabolic pathways of the parasite and attention has to be especially focused on differences in metabolism between the host and the parasite. Another aspect, which has stimulated research on parasite biochemistry, is the adaptation of the metabolism of parasitic helminths to the changing environment during their life cycle.

There are several unresolved difficulties in the study of the biochemistry of *F. hepatica*. The parasitic stages have to be studied isolated from their host. Therefore, the interaction between host and parasite is lost and, consequently, essential requirements for proper physiological functioning might be lacking. Most studies have been performed on liver flukes obtained from infected final hosts, as it is too difficult to obtain the parasitic stages (inside the intermediate snail host) pure and in quantities large enough for biochemical experiments. Another limitation to biochemical investigations is size; even the size of adult liver flukes is so small that intact or homogenized parasites are used instead of isolated organs or tissues. The results obtained will, therefore, represent the overall activity of the entire parasite. This will mask typical metabolic processes of specific organs or tissues of the parasite that are so far unknown. As in other animals, distinct differences in metabolism will exist between the cells of different tissues. These questions can only be answered when culture methods for certain cell types of *F. hepatica* are established. In this chapter, an overview is given of some selected aspects of the biochemistry of *Fasciola*, with emphasis on its energy metabolism and related subjects.

8.2 Nutrition

8.2.1 Free-living stages

F. hepatica has, as all other parasitic helminths, various developmental stages in its life cycle and the availability of substrates varies widely during this life cycle. When the flukes reside inside their definitive mammalian or intermediate snail host, *Fasciola* can, and will, obtain food from the host,

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as the term 'parasite' itself signifies. The free-living stages, (meta)cercariae, eggs and miracidia, on the other hand, are vehicles to go from one host to the next one. These free-living stages do not multiply and, apart from the eggs, they possess limited or no biosynthetic capacities. The free-living stages are self-supporting; they do not obtain substrates other than oxygen from the environment and are completely dependent on the endogenous energy reserves they stored in their previous parasitic stage. Glycogen is present in the free-living stages and is used to span the time before a new host is entered and new substrates become available. As these glycogen reserves are limited, the free-living stages are either short-lived and in a frantic search for a new host (miracidia), or they are a dormant stage, waiting to be reactivated by external triggers (metacercariae and eggs). The vitelline cells inside the eggs of *F. hepatica* function as food reserve and these eggs, which embryonate outside the mammalian host, require no extra nutrients for the developing embryo.

8.2.2 Parasitic stages

The parasitic stages inside the snail and the mammalian host need substrates for energy as well as for the biosynthesis of structural elements. These substrates are taken from the host. Seen from the perspective of the parasite, substrates are plentiful once inside the host. The only concern of these parasitic stages is to produce offspring and avoid being eliminated by the immune system of the host. During their stay in the host, which will last for the rest of their lives, the host provides food *ad libitum* and a sheltered place to live. *F. hepatica,* as all other parasitic helminths, has very limited anabolic capacities (see section 8.4, below). Therefore, they need to obtain complex building blocks from the host for their biosynthetic machinery, for maintenance and repair as well as for reproduction.

Infection of the mammalian host happens by ingestion of vegetation contaminated with the encysted stage, the metacercariae, from which the newly excysted juveniles emerge and penetrate through the intestinal wall and migrate to the liver (Gonzalez-Miguel *et al.*, 2021). Within the liver, the parasite's growth advances rapidly, doubling in size approximately every 2 weeks, alongside the development of parasite digestive and reproductive structures. To facilitate this rapid growth and development the parasite feeds on liver tissue and blood while it migrates through the liver. The adult *F. hepatica* lives in the bile ducts of its host and uses the oral sucker both for attachment and for feeding on the blood of its host. Obviously, the major life-cycle stages of *F. hepatica* in the final host, namely the newly excysted juveniles, immature flukes and adult flukes, occupy very different niches within the host. This is reflected in the proteins they secrete in these three stages (Cwiklinski *et al.*, 2021).

The adult flukes inside this final host possess two surfaces that can potentially absorb nutrients: the external surface, called the tegument, and the intestinal epithelium of the gut (Halton, 1997). The tegument of *F. hepatica* plays an important role in the uptake of nutrients and consists of a standard lipid bilayer from which a thin glycoprotein coat, the glycocalyx, extends. Small substrates such as glucose, amino acids and lipids can be absorbed via the tegument. The relative involvement of the two surfaces in uptake of various nutrients is still unclear, but both intestine and tegument are considered to be important in the uptake of substrates by *F. hepatica.*

The digestive tract of *F. hepatica* is blind-ended, with a single opening that functions both as entrance and as exit. With its oral sucker, the liver fluke can browse the bile-duct epithelium and the underlying tissues. The cul-de-sac gut is first filled with nutrients and after digestion the undigested remains are regurgitated through the same oral opening. The intestine of the fluke is covered with a cellular single layer of epithelial cells through which the nutrients are absorbed after degradation by digestive enzymes. Proteins of the host, including haemoglobin, are degraded by proteolytic enzymes, after which the resulting amino acids can be taken up by the parasite.

8.3 Energy Metabolism

8.3.1 Substrates of energy metabolism

In contrast to the host, in *F. hepatica* a clear distinction exists between substrates for ATP production and substrates for biosynthetic purposes. The mammalian host can adapt its

metabolism to the availability of various substrates. Depending on the supply, carbohydrates, lipids or proteins can be the main source of energy. *F. hepatica,* on the other hand, is completely dependent on carbohydrates for its energy metabolism. Because of the very limited aerobic capacity of adult flukes (see later), their metabolism has to be mainly fermentative. Carbohydrates are suitable substrates for fermentation, as both oxidation and reduction of this substrate can occur. Lipids are too reduced to be fermented and therefore cannot be used for the production of ATP by parasitic stages of helminths in general. Most free-living stages of parasitic flatworms have a classical aerobic metabolism; they are therefore not restricted to a fermentative metabolism and could in principle be able to utilize lipids for the production of ATP. Very little research has been done on the lipid metabolism of these stages of parasitic flatworms but, up to now, no evidence has been found for the degradation of lipids for energy generation by any of them. Furthermore, genomic analyses have shown that *F. hepatica* and *Schistosoma mansoni* do not possess the genes for enzymes essential for β-oxidation, which implies that even their free-living stages are unable to use fatty acid oxidation for the production of ATP (Bexkens *et al.*, 2019; Coghlan *et al.*, 2019).

Amino acids are necessary as precursors of protein synthesis for normal growth and reproduction in *F. hepatica,* but oxidation of amino acids is considered to contribute little to the overall energy metabolism. A possible role for amino acids in energy metabolism could be an anaplerotic one, as in schistosomes, where degradation of amino acids, and especially glutamine, provides Krebs cycle intermediates (Foster *et al.*, 1989).

8.3.2 Energy metabolism in free-living versus parasitic stages

Energy metabolism relates to the biochemical reactions that occur in living cells to harness, store, transfer and release the energy taken up by organisms from their surroundings. A central issue is the means by which energy from the nutrients is coupled to the energy-requiring reactions in the cell. The chemical energy in the nutrients has to be converted into forms of energy useful for the organism, such as motion, concentration and electrical gradients, and the chemical energy in ATP, the universal energy currency. To convert the chemical energy in the carbon fuels into the energy in ATP, these nutrients are oxidized. Two completely different mechanisms exist to generate ATP: via substrate-level phosphorylation and via the use of ion gradients. In substrate-level phosphorylations, a kinase enzyme transfers a phosphate group from a substrate with a high phosphoryl-transfer potential to ADP, producing ATP. The other mechanism that exists to regenerate ATP from ADP makes use of a gradient of protons across the mitochondrial inner membrane and is called oxidative phosphorylation. In this process, the electrons obtained by oxidation of the carbon fuels flow through a series of large protein complexes embedded in the inner mitochondrial membrane, the electron transport chain, also known as respiratory chain. The flow of electrons through the complexes powers the pumping of protons from the inside of the mitochondria to the intermembrane space, establishing a proton gradient over the mitochondrial inner membrane. The final phase of oxidative phosphorylation is carried out by the ATP synthase complex, which regenerates ATP from ADP and phosphate in a reaction that is driven by the flow of protons back to the mitochondrial matrix. In both these ways to produce ATP, the high-energy electrons extracted from the carbon fuels are carried by NADH through the cell and are donated in another intermediate reaction in metabolism, or to the electron transport chain.

The electrons extracted from the nutrients, the electron donors, have to leave the cell, which implies that also an electron acceptor is necessary. In a classical aerobic energy metabolism, oxygen functions at the end of the electron transport chain as the final electron acceptor and is reduced to water in aerobic class 1 mitochondria, which are characterized by ATP production via an electron transport chain that uses oxygen as the final electron acceptor (Müller *et al.*, 2012). When oxygen is not used as the final electron acceptor, another compound has to be reduced. When an endogenously produced electron acceptor is used, the process is called

a fermentation. An example is the use of endogenously produced pyruvate, acting as an electron acceptor in lactate fermentations. Many eukaryotes living (from time to time) in an anaerobic or hypoxic environment possess class 2 mitochondria, which are characterized by ATP production via an electron transport chain that can function anaerobically, using compounds other than oxygen as the final electron acceptor (Müller *et al.*, 2012).

The free-living stages of *F. hepatica* (eggs, miracidia, cercariae and metacercariae) are self-supporting: they do not obtain food or substrates other than oxygen from the environment and are completely dependent on their endogenous energy stores acquired in the previous host. Glycogen is present as storage carbohydrate in these stages of the life cycle of *F. hepatica* and it is used to span the gap in food supply until the next host is reached. Miracidia will die if the glycogen reserves are exhausted before the next host (a snail) is entered. Metacercariae can be considered, biochemically speaking, to be a dormant stage and therefore their substrate reserves are not as rapidly exhausted as those of miracidia. Metacercariae are dormant, but certainly not dead (Cwiklinski *et al.*, 2018; Xu *et al.*, 2020). A *Fasciola* metacercaria is like a sleeping beauty, waiting to be kissed and revived by components in the gut of the new host, and is fully prepared to start living in the new environment.

It is generally accepted that free-living stages of parasitic helminths have an aerobic energy metabolism, although this has only been studied in very few cases. For *Schistosoma mansoni* it has been shown that cercariae, as well as miracidia, degrade their stored glycogen reserves via the classical glycolytic pathway to pyruvate. Inside their mitochondria, this pyruvate is then broken down to carbon dioxide via the Krebs cycle (Tielens *et al.*, 1991, 1992). The main part of ATP is generated via the mitochondrial respiratory chain and oxidative phosphorylation. The energy metabolism in these free-living stages is not different from the standard aerobic metabolism in mammals. Metabolic experiments show that *F. hepatica* miracidia are bioenergetically very comparable to those of *S. mansoni* and are also mainly dependent on the aerobic degradation of glycogen to carbon dioxide (Boyunaga *et al.*, 2001). The consumption of oxygen by these miracidia, as well as their motility, was strongly reduced in the presence of cyanide, an inhibitor of complex IV of the electron transport chain, which indicates that ATP production via electron transfer to oxygen plays an essential role in their energy metabolism.

Adult parasitic helminths have an energy metabolism strikingly different from that in the free-living stages. Although the pattern of their end products varies greatly between different species of adult trematodes, none of them degrades carbohydrates completely to carbon dioxide, as the free-living stages do. In general, adult parasitic helminths do not use oxygen as final electron acceptor, but have a fermentative metabolism instead. When oxygen cannot function as the final electron acceptor, the degradation of substrates will still have to be in redox balance: the number of NADH-producing reactions always has to be equal to the number of NADH-consuming ones. In adult parasitic helminths, two pathways exist to maintain this redox balance without the use of oxygen: anaerobic glycolysis and malate dismutation. The classical adaptation to metabolism without oxygen, fermentation of carbohydrates to lactate (so-called anaerobic glycolysis), is used to a certain extent by parasitic helminths. However, most of them mainly use a different pathway to ferment carbohydrates: malate dismutation.

8.3.3 Malate dismutation

Most parasitic worms, but also many marine invertebrates, use malate dismutation as fermentation variant (Müller *et al.*, 2012). Malate dismutation is also the main pathway used by adult *F. hepatica* to produce ATP. Malate dismutation is a fermentation that synthesizes ATP not only via substrate-level phosphorylations, but also via oxidative phosphorylation. In malate dismutation, carbohydrates are first degraded to phosphoenolpyruvate (PEP) via the classical glycolytic pathway. This PEP is then carboxylated to oxaloacetate by an ATP-linked phosphoenolpyruvate carboxykinase (PEPCK). This oxaloacetate is subsequently reduced to malate by malate dehydrogenase (Fig. 8.1). This part of the pathway occurs in the cytosol and is comparable to the formation of lactate: it is in redox balance and yields two ATP per glucose degraded. The malate

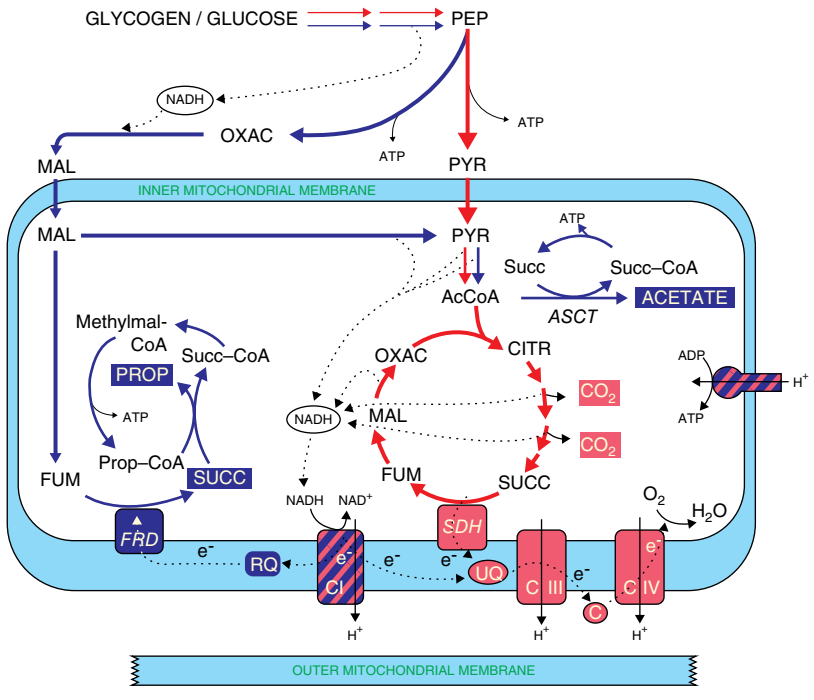

Fig. 8.1. Main pathways of the aerobic and anaerobic carbohydrate degradation in *F. hepatica.* The aerobic degradation by newly excysted juveniles and free-living stages is indicated by red arrows; the anaerobic pathway of the adult (malate dismutation) is indicated by blue arrows. AcCoA, acetyl-CoA; ASCT, acetate:succinate CoA-transferase; C, cytochrome *c*; CI–CIV, complexes I–IV of the electron transport chain; CITR, citrate; FRD, fumarate reductase; FUM, fumarate; MAL, malate; Methylmal-CoA, methylmalonyl-CoA; OXAC, oxaloacetate; PEP, phosphoenolpyruvate; PROP, propionate; Prop-CoA, propionyl-CoA; PYR, pyruvate; RQ, rhodoquinone; SDH, succinate dehydrogenase; SUCC, succinate; Succ-CoA, succinyl CoA; UQ, ubiquinone.

produced in the cytosol is not excreted like lactate, but is transported into the mitochondria for further degradation.

In a branched pathway, a portion of the substrate (malate) is oxidized to acetate and another portion of it is reduced to succinate (Fig. 8.1). In the oxidative branch of malate dismutation, malic enzyme catalyses the oxidative decarboxylation of malate to pyruvate, which is then further oxidized to acetyl-CoA by the pyruvate dehydrogenase complex. Parasitic helminths such as *F. hepatica* use an acetate:succinate CoA-transferase (ASCT) for the production of acetate from acetyl-CoA (van Vugt *et al.*, 1979; Saz *et al.*, 1996; van Hellemond *et al.*, 1998; van Grinsven *et al.*, 2009). ATP is formed by substrate-level phosphorylation when subsequently the succinyl-CoA, which is produced during acetate formation by ASCT, is recycled to succinate by succinyl-CoA synthetase (SCS), an enzyme usually participating in the Krebs cycle (Fig. 8.1). The produced acetate is excreted.

The production of acetate from a portion of the incoming malate results in the formation of the reduced co-factor NADH and is balanced by the reduction of another portion of the malate to succinate. This reduction of malate to succinate occurs via two reactions that reverse part of the Krebs cycle (Fig. 8.1). First, malate is converted to fumarate by the Krebs cycle enzyme fumarase, running in reverse. The resulting fumarate is then reduced by the membrane-bound fumarate reductase to succinate, and in this way fumarate serves as the final electron acceptor for the electrons extracted in the oxidative branch to acetate.

Many parasites, including *F. hepatica*, metabolize succinate further to propionate, which is then excreted. This conversion of succinate to propionate occurs via a reversal of the reactions that mammals use during the formation of succinyl-CoA from propionate. One additional ATP is generated in the process of the decarboxylation of succinate to propionate. The CoA moiety from propionyl-CoA is transferred to succinate thanks to a dual substrate specificity of ASCT (van Grinsven *et al.*, 2009) (see also section 8.3.8). This reaction generates propionate as a metabolic end product. A cycle consisting of the vitamin B_{12}-dependent methylmalonyl-CoA mutase, methylmalonyl-CoA epimerase and propionyl-CoA carboxylase regenerates propionyl-CoA. This decarboxylation of succinate to propionate is coupled to substrate-level phosphorylation during the decarboxylation of methylmalonyl-CoA to propionyl-CoA, a reaction catalysed by propionyl-CoA carboxylase (Köhler *et al.*, 1978; Pietrzak and Saz, 1981). As can be seen in Fig. 8.1, redox balance inside the mitochondria is maintained when twice as much propionate as acetate is produced, because, in the oxidative branch to acetate, two reactions result in NADH formation, while in the reduction of malate to succinate only one reaction consumes NADH: the reduction of fumarate to succinate.

The reduction of fumarate to succinate is coupled to an electron transport linked phosphorylation of ADP at site I of the respiratory chain (Figs 8.1 and 8.2). Apart from the electron transport associated ATP formation in the reduction of fumarate, this so-called malate dismutation is accompanied by substrate-level phosphorylations (Fig. 8.1). In total, the anaerobic degradation of glucose to propionate and acetate yields about five ATP per glucose degraded, which compares favourably with the two ATP produced during lactate fermentation.

All parasitic worms capable of anaerobic mitochondrial metabolism favour malate as the primary mitochondrial substrate and the oxidative decarboxylations of first malate and then pyruvate generated intramitochondrial reducing power in the form of NADH (Fig. 8.1). In contrast, the pathways used to reoxidize this intramitochondrial NADH are quite diverse and depend on the stage or species of parasite under examination, but, in all cases, redox balance is maintained and electron transport associated ATP is generated by the NADH-reduction of fumarate to succinate. Three examples of these different variants of the reductive branch of malate dismutation are as follows.

1. In the cestode *Hymenolepis diminuta*, succinate and acetate are the major end products of anaerobic malate dismutation and are excreted in the predicted 2:1 ratio.
2. As discussed earlier, in *F. hepatica*, succinate is decarboxylated to propionate with an additional substrate-level phosphorylation. *F. hepatica* forms primarily propionate and acetate as end products, again in a ratio of 2:1 to maintain redox balance.
3. In the parasitic nematode *Ascaris suum*, acetyl-CoA and propionyl-CoA are not only converted to acetate and propionate, respectively, but mostly they are also condensed and reduced to the branched-chain fatty acids 2-methylbutanoate and 2-methylpentanoate via a pathway similar to the reversal of β-oxidation (Komuniecki *et al.*, 1989).

8.3.4 Selected aspects of glycolysis

Several glycolytic enzymes in parasitic helminths have been purified and studied in great detail. These studies were stimulated by the early observation that the chemotherapeutic action of antimonials on schistosomes was associated with an inhibition of its phosphofructokinase (PFK), a glycolytic enzyme with a high flux control. Helminth PFKs appear to be much more sensitive to these anti-schistosomal drugs than the PFK of the mammalian hosts. Attacking the ATP-producing systems of parasites is considered to be one of the more rational and promising ways of combating parasitic diseases, as ATP is one of the few things parasites cannot directly obtain from the host. Special attention has, of course, been directed towards enzymes that are absent in the host or where differences are observed between host and parasite enzymes.

PFK is usually the main regulatory site of glycolysis and *F. hepatica* PFK has been extensively studied. In general, the activity of PFK is regulated by several effectors. The most potent activators of mammalian as well as *Fasciola* PFK are fructose 2,6-bisphosphate and AMP, whereas ATP is inhibitory (van Schaftingen *et al.*, 1981;

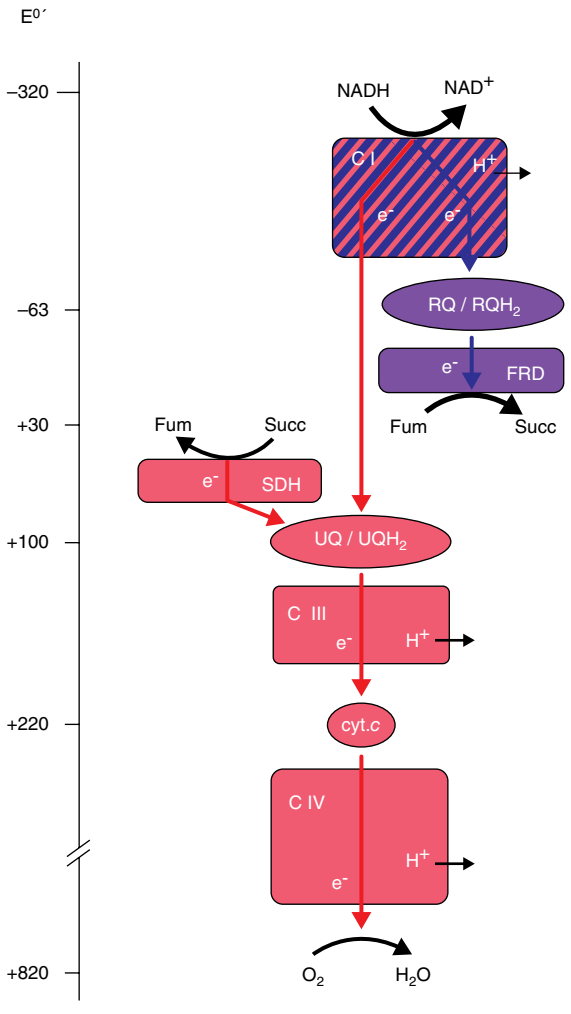

Fig. 8.2. Schematic representation of the mitochondrial respiratory chain of *F. hepatica*. Boxes indicate electron transport chain complexes, whereas ovals represent the pools of the electron transporters ubiquinone, rhodoquinone and cytochrome *c*. The colours red and blue are used in the same way as in Fig. 8.1. Components involved in electron transport in aerobic metabolism are in red, whereas blue is used for the components involved in anaerobic metabolism (malate dismutation). Complex I and the ATP synthase are involved in both types of metabolism and are therefore shown in red plus blue. The vertical bar represents a scale for the standard redox potentials in mV. Translocation of protons by the complexes is indicated by $H^+\rightarrow$. CI, CIII and CIV, complexes I, III and IV of the electron transport chain; cyt *c*, cytochrome *c*; FRD, fumarate reductase; Fum, fumarate; SDH, succinate dehydrogenase; Succ, succinate; RQ, rhodoquinone; UQ, ubiquinone.

Kamemoto *et al.*, 1987). In contrast to mammalian PFKs, the PFK enzyme of *Fasciola* is relatively insensitive to inhibition by citrate (Kamemoto *et al.*, 1987). However, when compared with mammalian PFKs, the most remarkable difference is that the kinetic properties of *Fasciola* PFK are strongly influenced by phosphorylation. Phosphorylation by a cAMP-dependent protein kinase results in activation of PFK, and it was proposed that this phosphorylation plays, at least in part, a functional role in the liver fluke regulation of PFK *in vivo* (Kamemoto *et al.*,

1989). *Fasciola* PFK is phosphorylated by a cAMP-dependent protein kinase to between 0.2 and 0.3 phosphate per protomer (Kamemoto *et al.*, 1987). The enzyme is sensitive to small changes in phosphorylation: a threefold activation was observed on an increase of 0.1 phosphate per protomer. This phosphorylation occurs at a threonine residue in a phosphorylation site which shows no homology to the phosphorylation site of PFK of *Ascaris,* an enzyme also known to be stimulated by phosphorylation (Kulkarni *et al.*, 1987; Mahrenholz *et al.*, 1991). Further structural data to determine the differences between mammalian PFKs and those of parasitic helminths are not yet available.

As in other parasitic helminths, PEPCK functions in *F. hepatica* as a CO_2-fixing enzyme. This is remarkably different from the decarboxylating anabolic role of PEPCK in mammalian gluconeogenesis. However, parasitic helminths are not unique in this respect, because PEPCK functions as a CO_2-fixing enzyme in the glycolytic degradation of glucose in many other invertebrates, such as some marine organisms (Müller *et al.*, 2012). PEPCK activity appears to be controlled primarily by the concentrations of enzyme, substrates and products, in contrast to the activity of pyruvate kinase (PK), which is under tight allosteric control (Behm and Bryant, 1982).

PEPCK is a key enzyme in the cytosolic production of malate, an important process in the anaerobic energy metabolism of *F. hepatica* adults, whereas PK is a key enzyme in the glycolytic pathway to pyruvate, which is operative in the aerobically functioning juvenile liver fluke (Fig. 8.3). Therefore, during the change from an aerobic to an anaerobic energy metabolism that occurs during the development of *F. hepatica* in the final host, a change in the relative importance of PK and PEPCK may be expected (see section 8.3.9). Such a change was indeed observed, as the development of the fluke in the host liver is

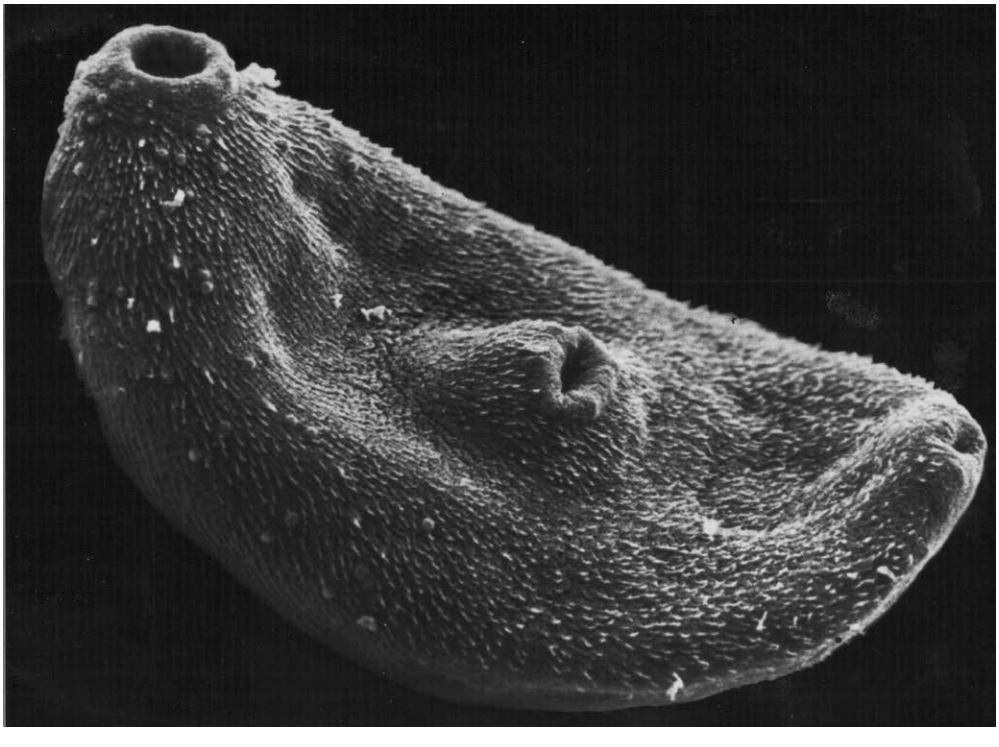

Fig. 8.3. Scanning electron micrograph of a newly excysted juvenile liver fluke, *F. hepatica*. A method developed for large-scale *in vitro* excystment of metacercariae and subsequent isolation of juvenile liver flukes was used for the preparation of this specimen (Tielens *et al.*, 1981b). (Photograph© A.G.M. Tielens and C.J.A.H.V. van Vorstenbosch.)

accompanied by an almost complete disappearance of PK expression and activity (Tielens *et al.*, 1987; Cwiklinski *et al.*, 2015).

One should bear in mind, however, that the fate of PEP at the PK/PEPCK branch point cannot simply be estimated by measuring the ratio of PK and PEPCK activities *in vitro,* as the actual use of PEP in each reaction is determined by K_m values and substrate concentrations, by the extent of phosphorylation of PK, by the presence of (in)activators, and by the rate of the subsequent reactions.

8.3.5 Selected aspects of mitochondrial processes

Inside the mitochondria, Krebs cycle activity is used for the final part of carbohydrate catabolism by juvenile liver flukes, by miracidia and, most likely, also by cercariae. The adult liver flukes, on the other hand, use malate dismutation as the final pathway of carbohydrate degradation, a mitochondrial adaptation to anaerobic functioning. Parasitic worms utilizing anaerobic malate dismutation produce a range of metabolic end products of carbohydrate metabolism, but their mitochondrial pathways are surprisingly similar given their diverse phylogenetic origins. As discussed earlier, these organisms typically use only part of the Krebs cycle (from malate to succinate) and in some cases β-oxidation, in a direction opposite to that found in aerobic organisms.

Malate, the major end product of the cytosolic carbohydrate degradation, is transported into the mitochondria for further degradation. A portion of the malate is oxidized to pyruvate via malic enzyme, which is NADP-linked in *F. hepatica* (Prichard, 1978; Tielens *et al.*, 1987). However, NADPH cannot be used by the enzyme fumarate reductase (FRD) in the reductive branch of malate dismutation. Cestodes are known to possess an active NADPH:NAD transhydrogenase associated with the inner mitochondrial membrane (Fu *et al.*, 2019). This enzyme couples the malic enzyme reaction to fumarate reduction. The gene for this transhydrogenase is now annotated in the genome of many parasitic worms, but not yet in *F. hepatica* (Coghlan *et al.*, 2019).

Pyruvate is oxidized and decarboxylated to acetyl-CoA by pyruvate dehydrogenase. The dihydrolipoyl dehydrogenase (E3) subunit of this large mitochondrial enzyme complex was discovered in the excretory/secretory (ES) products of adult *F. hepatica* from sheep. The abundance of the E3 subunit in the ES products is low, but the protein is highly immunogenic (Walsh *et al.*, 2021). The pyruvate dehydrogenase complex has not yet been well characterized in *F. hepatica,* but the complex from *A. suum* was shown to be specially adapted to anaerobic functioning (Song and Komuniecki, 1994). Its activity is controlled by phosphorylation and dephosphorylation, effected by associated kinase and phosphatase activities. Because of an unusual E3-binding protein, the high NADH/NAD^+ ratio, characteristic for these anaerobically functioning mitochondria, does not result in inactivation of the pyruvate dehydrogenase complex (Harmych *et al.*, 2002).

Compared with typical aerobic class 1 mitochondria, the three main distinctions of these anaerobic class 2 mitochondria are: (i) the presence of a special enzyme catalysing the reduction of fumarate to succinate; (ii) the quinone that connects this electron transfer to the enzyme complex in the electron transport chain; and (iii) the presence of ASCT, which converts acetyl-CoA into acetate (Müller *et al.*, 2012). These characteristic features of anaerobically functioning mitochondria are further discussed below.

8.3.6 Succinate dehydrogenase versus fumarate reductase

In juvenile liver flukes (Fig. 8.3) and in miracidia, a respiratory chain up to cytochrome *c* oxidase (complex IV) is active, and all evidence obtained so far indicates that this electron-transport chain is not different from the classical one, present in mammalian mitochondria. In these aerobically functioning stages of *F. hepatica*, electrons are transferred from NADH to ubiquinone via complex I, and also from succinate to ubiquinone via complex II (succinate dehydrogenase) (Figs 8.1 and 8.2). Subsequently, these electrons are transferred from the formed ubiquinol to oxygen via complexes III and IV of the respiratory chain.

In the anaerobically functioning mitochondria of the adult liver fluke, however, this electron transport chain is different, as oxygen cannot be

used as the final electron acceptor. Instead, endogenously produced fumarate functions as the final electron acceptor during malate dismutation. In this case, electrons are transferred from NADH not to oxygen, but to fumarate, and this happens via complex I and fumarate reductase (Figs 8.1 and 8.2). This difference implies that during the development of *F. hepatica* in its final host, a transition occurs from succinate oxidation via succinate dehydrogenase in the Krebs cycle of the juvenile liver fluke to the reverse reaction: reduction of fumarate to succinate in the adult. Bacteria contain two homologous but distinct membrane-bound enzyme complexes for these reactions: one to oxidize succinate (succinate dehydrogenase) and one to reduce fumarate (fumarate reductase). Accordingly, in *Escherichia coli* the change between aerobic and anaerobic metabolism is accompanied by differential expression of two different enzymes for these reactions (Ackrell *et al.*, 1992). Succinate dehydrogenase is expressed under aerobic conditions, whereas fumarate reductase is expressed under anaerobic conditions. *In vitro*, the interconversion of succinate and fumarate is reversible for both enzymes. However, *in vivo,* distinct enzyme complexes are needed for these opposite reactions as the electron flow through the complex is reversed (Fig. 8.2), which implies differences in the affinity for electrons (standard redox potential, $E^{0'}$) of the electron-binding domains of these two enzyme complexes (Ackrell *et al.*, 1992; van Hellemond and Tielens, 1994).

In eukaryotes, the succinate dehydrogenase and fumarate reductase enzymes consist of four subunits: a flavoprotein (Fp)-containing subunit; an Ip subunit that contains iron–sulfur clusters; and two subunits which form the anchor, binding the complex to the mitochondrial inner membrane. As in *E. coli*, for these two enzyme complexes, distinct variants have been described in the parasitic nematodes *Haemonchus contortus* (Roos and Tielens, 1994) and *A. suum* (Saruta *et al.*, 1995; Sakai *et al.*, 2012). These were shown to be differentially expressed during the life cycle of the parasites and are suggested to function either as a succinate dehydrogenase or as a fumarate reductase. Evidence for differential expression of two different enzyme complexes in *F. hepatica* is lacking, as up to now only one gene coding for the Fp subunit has been found in the *F. hepatica* genome, and one for the Ip subunit (Coghlan *et al.*, 2019). It has been shown that the kinetic properties of the complex from adult liver flukes resemble those of other organisms known to reduce fumarate (van Hellemond *et al.*, 1995). Mitochondria of adult *F. hepatica* had a low succinate dehydrogenase/fumarate reductase activity ratio, which corresponds to their main *in vivo* function. However, significant differences in activity ratios were not detected between different stages of *F. hepatica* (adult and metacercariae), although *in vivo* the adult reduces fumarate, whereas the free-living stage oxidizes succinate. Apparently, the change in the activity ratio that was observed between free-living and parasitic stages of *A. suum* does not occur in *F. hepatica* (Saruta *et al.*, 1995; van Hellemond *et al.*, 1995; Sakai *et al.*, 2012).

8.3.7 Ubiquinone versus rhodoquinone

In addition to distinct enzyme complexes for succinate oxidation and fumarate reduction, distinct quinones are involved in these two processes in parasitic helminths. *In vivo*, the oxidation of succinate and the reduction of fumarate run efficiently only when electrons are transferred to an acceptor with a higher affinity for electrons, i.e. a higher standard redox potential (Fig. 8.2). When fumarate is the final electron acceptor, the electron transport chains of many bacteria, including *E. coli*, employ menaquinone, which has a lower standard redox potential than ubiquinone (Maklashina *et al.*, 2013). In parasitic helminths, however, the presence of rhodoquinone was demonstrated and, because rhodoquinone is present mainly in anaerobic, fumarate-reducing stages, it was suggested that rhodoquinol functions as electron donor in fumarate reduction, similar to menaquinol in fumarate reduction by bacteria (Allen, 1973). The sole difference between rhodoquinone and ubiquinone is that rhodoquinone has an amino group at the 5-position of the benzoquinone ring instead of a methoxy group, and this structural difference results in a difference in redox potential. Rhodoquinone with its relatively low standard redox potential ($E^{0'} = -63$ mV) is comparable to menaquinone in this respect and transfers electrons via fumarate reductase to fumarate ($E^{0'} = 30$ mV), whereas ubiquinone

($E^{0\prime}$ = 100 mV) preferentially accepts electrons from succinate and donates them to complex III of the electron transport chain (Fig. 8.2). It was shown that rhodoquinone is an indispensable component for efficient electron transport in the anaerobic electron transport chain of all eukaryotic organisms known to reduce fumarate during anoxia (van Hellemond *et al.*, 1995). The essential function of rhodoquinone in the anaerobic energy metabolism during the entire life cycle of *F. hepatica* was demonstrated, as the amount of rhodoquinone present reflects the importance of fumarate reduction in various stages (van Hellemond *et al.*, 1995; van Hellemond *et al.*, 1996).

It had been suggested that rhodoquinone biosynthesis in eukaryotes would start from ubiquinone (Powls and Hemming, 1996), as is the case in the α-proteobacterium *Rhodospirillum rubrum* (Brajcich *et al.*, 2010). Parasitic helminths have a very limited repertoire of lipid biosynthesis and obtain from the host, for instance, cholesterol and fatty acids, which they can then modify. Could the same be true for quinones? It was demonstrated that this is not the case: *F. hepatica* synthesizes ubiquinone and rhodoquinone *de novo*, and rhodoquinone is not produced by modification of ubiquinone obtained from the host (van Hellemond *et al.*, 1996, 1997).

Later it was shown that also in the free-living nematode *Caenorhabditis elegans*, ubiquinone is not a required precursor in rhodoquinone biosynthesis (Jonassen *et al.*, 2001). Recently it has been uncovered independently by Del Borrello *et al.* (2019) and Roberts Buceta *et al.* (2019) that in *C. elegans* the rhodoquinone biosynthetic pathway is indeed different from the one in *R. rubrum* and does not involve ubiquinone as substrate. The difference between ubiquinone and rhodoquinone biosynthesis occurs very early in the biosynthetic pathway, at the level of prenylation of an aromatic precursor by the *C. elegans* polyprenyltransferase COQ-2. Both investigations showed that if the *C. elegans* COQ-2 uses 4-hydroxybenzoate as substrate, which is derived from tyrosine in the mevalonate pathway, this will lead to the synthesis of ubiquinone. If, on the other hand, 3-hydroxyanthranilate is used, which is derived from tryptophan in the kynurenine pathway and contains the characteristic amino group, this will ultimately yield rhodoquinone. Further investigations showed that the *C. elegans* COQ-2 polyprenyltransferase has two distinct forms: COQ-2a and COQ-2e. COQ-2a is homologous to COQ-2 from other eukaryotes, whereas COQ-2e is only found in species that can synthesize rhodoquinone (Tan *et al.*, 2020). These two forms of COQ-2 are the result of alternative splicing of two internal exons (6a and 6e), resulting in remodelling of the core of COQ-2, which changes the substrate specificity of this polyprenyltransferase (Tan *et al.*, 2020). Inclusion of exon 6a results in the COQ-2a enzyme which uses 4-hydroxybenzoate as substrate and thus produces ubiquinone precursors. Switching to the alternative exon 6e yields COQ-2e which primarily uses 3-hydroxyanthranilate as substrate, producing rhodoquinone precursors. All eukaryotes possess a gene homologue of COQ-2a, but only organisms known to synthesize rhodoquinone, including *F. hepatica*, have the specific exon 6e in their genome (Tan *et al.*, 2020). The mechanism by which the alternative splicing is regulated has not been revealed yet.

8.3.8 Acetate:succinate CoA-transferase

As discussed earlier, one of the main end products of the carbohydrate metabolism in *F. hepatica* is acetate, just as it is in many other parasites. This acetate formation does not occur in the parasite's mammalian hosts; therefore, it could be a good target for the development of new anti-parasitic drugs. In parasites such as parasitic worms, trypanosomatids and parabasalids, acetate is produced from acetyl-CoA by an organellar acetate:-succinate CoA-transferase/succinyl-CoA synthase cycle (Müller *et al.*, 2012).

CoA-transferases catalyse the reversible transfer of coenzyme A groups from CoA-thioesters to free acids. Three unrelated protein families of CoA-transferases exist (I, II and III), which vary in substrate specificity and subunit composition (Heider, 2001). These enzymes are found in aerobic and anaerobic prokaryotes, as well as in many eukaryotes where they are located in their ATP-producing organelles, mitochondria or hydrogenosomes. In fact, analysis of the pathway of acetate production in trypanosomatids and *F. hepatica* identified the first metabolic pathway common to mitochondria and hydrogenosomes: the acetate:succinate CoA-transferase/succinyl-CoA synthase cycle

(van Hellemond *et al.*, 1998). At that time, it was welcome additional evidence in support of a common origin of mitochondria and hydrogenosomes, as the evolutionary origin of hydrogenosomes was still under debate.

The CoA-transferases involved in acetate production in parasites had long been known to be succinate-dependent and were therefore called acetate:succinate CoA-transferases (ASCT). They are all CoA-transferases of family I, because the CoA moiety is not transferred directly from one substrate to the next, but is first covalently bound to a glutamate residue in the active site of the enzyme (Heider, 2001). Therefore, the reaction follows a ping-pong mechanism where the first product (a free carboxylate) is released before the second substrate (the other carboxylate) enters the active site. This process is fully reversible. Based on work on the ASCTs of several parasites, the existence of at least three subfamilies of enzymes within the CoA-transferase family I was suggested (Tielens *et al.*, 2010). Enzymes of these three subfamilies (A, B and C) catalyse the ASCT reaction via the same mechanism, but the subfamilies share little sequence homology.

The *F. hepatica* ASCT enzyme belongs to the subfamily IB of the CoA-transferases (reviewed in Tielens *et al.*, 2010). Analysis of the available databases indicated that among other parasitic helminths *S. mansoni* also contains an ASCT that belongs to subfamily IB. Database searching showed that there are currently no mammalian homologues of this subfamily. Studies on the substrate specificity of the *F. hepatica* ASCT showed that it can transfer a CoA moiety from acetyl-CoA to acetate, propionate, butyrate and succinate (van Grinsven *et al.*, 2009). This broad substrate specificity explains why the *F. hepatica* ASCT is involved in two different reactions in malate dismutation: the formation of acetate via the ASCT-SCS cycle, as well as the formation of propionate where succinate accepts the CoA-moiety from propionyl-CoA (Fig. 8.1).

8.3.9 Transitions in energy metabolism

In the different stages of its life cycle, *F. hepatica* has to adapt to different environments. Among other chemical and physical parameters, the availability of oxygen and substrates varies widely during the life cycle. Therefore, transitions have to occur, for instance, from the fully aerobic functioning of the juvenile liver fluke to the almost completely anaerobic functioning of the adult parasite in the bile duct. Most likely a comparable switch from an aerobic to an anaerobic energy metabolism will also occur during the development of miracidia into sporocysts and rediae, but this switch has not yet been studied at the metabolic level. It should be realized that these transitions in *F. hepatica* are definitive, as the same organism will never encounter its previous environment again; only its offspring does. The change to a fermentative metabolism is permanent and is not forced by a burst-type of exercise or a temporarily hypoxic environment; therefore, these metabolic switches are not comparable to the transient switches that occur in, for instance, skeletal muscle and tidal marine organisms such as the common mussel, *Mytilus edulis* (Müller *et al.*, 2012).

After emergence from the metacercarial cyst, the aerobically functioning juvenile liver fluke (Fig. 8.3) develops gradually into a fermenting adult that lives in the bile ducts of its definitive host (Tielens, 1994). During this development, three different pathways of glucose breakdown successively provide the major part of ATP (Fig. 8.4). Krebs cycle activity, which is by far the main energy-yielding pathway of the newly excysted juvenile, is gradually replaced by the production of acetate. If acetate is produced without the concomitant production of twice as much propionate, formation of acetate requires oxygen for the reoxidation of NADH (Fig. 8.1). This aerobic formation of acetate is the most important source of energy for the developing fluke in the liver parenchyma. Finally, in the bile ducts, the adult type of energy metabolism develops when the anaerobic production of propionate and acetate provides essentially all the ATP required (Tielens *et al.*, 1984). Apparently, the aerobic capacity of *F. hepatica* decreases during its development in the liver of the host.

The observed decrease in Krebs cycle activity per milligram of total protein cannot be explained by a slow decrease in the number of remnant mitochondria from an earlier, more aerobic, stage. It was shown that, when calculated per fluke, the Krebs cycle activity actually increased immensely during development and that this increase was directly proportional to

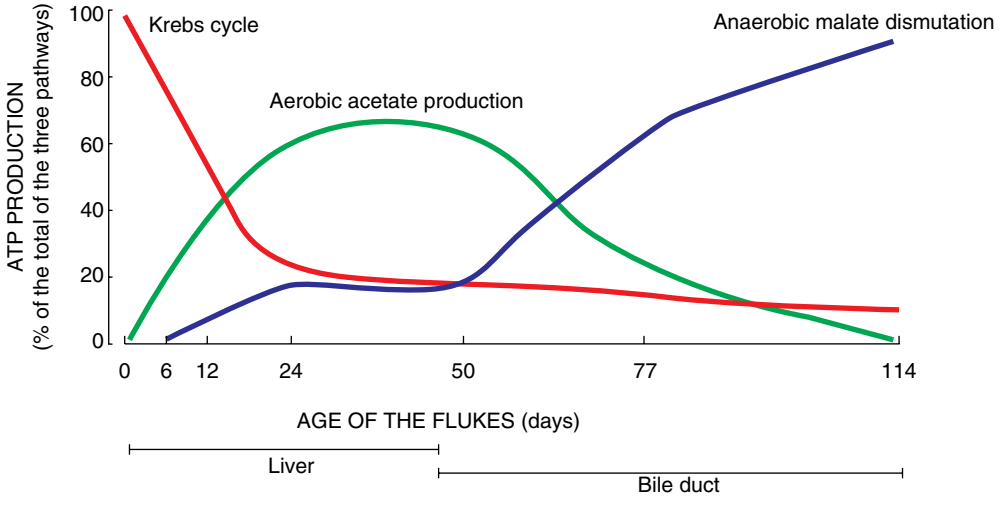

Fig. 8.4. Changes in energy metabolism during the development of *F. hepatica* in the final host. Contribution of the three pathways of glucose breakdown to ATP production is shown: Krebs cycle (red), aerobic acetate production (green) and anaerobic malate dismutation (blue). (After Tielens, 1984.)

the surface area of the fluke (Tielens *et al.*, 1984). This correlation holds true for the entire development from the newly excysted juvenile to the mature fluke in the bile ducts. These observations support the view that an aerobic metabolism is limited by the diffusion of oxygen and occurs only in the outer layer of the parasite (Fig. 8.5). Parasitic helminths possess neither respiratory organs nor a circulatory system. Therefore, the availability of oxygen inside the tissues is dependent on its slow diffusion, and growth of the parasite thus limits its aerobic capacity. In the adult stage, the aerobically functioning outer layer is far less than 1% of the total volume, making the metabolism of *Fasciola* appear to be completely anaerobic, even when incubated under aerobic conditions *in vitro*. However, throughout its development in the final host, *F. hepatica* retains Krebs cycle activity; it is only restricted to a very thin outer layer (Fig. 8.5). Furthermore, oxidative phosphorylation is essential for the functioning of adult flukes, as they are sensitive to uncouplers like the anthelmintic closantel (Skuce and Fairweather, 1990; Fairweather and Boray, 1999) (see also Chapter 7, this volume).

The metabolism of the developing liver fluke is thus altered by a lack of oxygen. This happens in two steps. First, as outlined above, in the aerobic liver parenchyma of the host the limited diffusion of oxygen in the growing fluke will result in a lack of oxygen in the innermost tissues of the parasite. Secondly, after arrival in the bile ducts, the entire fluke will have to function anaerobically because of the very low oxygen content of the bile. The prolonged stay in this anaerobic environment will ultimately result in metabolic adaptations both in the cytosol and in the mitochondria (Lloyd, 1986; Tielens *et al.*, 1987). A striking example of such an adaptation is the change in quinone composition that occurs during the development of *F. hepatica* (van Hellemond *et al.*, 1995, 1996). During the entire life cycle of *F. hepatica*, the amounts of ubiquinone and rhodoquinone reflect the type of energy metabolism. The free-living stages, miracidia and metacercariae, contain predominantly ubiquinone, which correlates with their aerobic energy metabolism. The adult parasitic stage, on the other hand, contains almost exclusively rhodoquinone. Therefore, throughout the development of the liver fluke in the final host, a strong correlation is found between the quinone composition and the type of metabolism: the amount of ubiquinone correlates with the use of the aerobic electron transport chain, and the amount of rhodoquinone with the use of fumarate reduction (van Hellemond *et al.*, 1995,

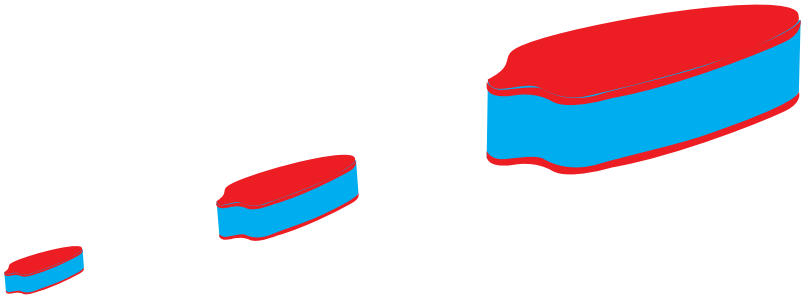

Fig. 8.5. Impression of the tissue layers with an aerobic (red) or anaerobic (blue) energy metabolism in *F. hepatica* while the size of that parasite increases during its development from newly excysted juvenile (left) to adult (right). The thickness of the aerobic (red) layer remains constant during growth throughout this development. Hence, the thickness of the anaerobic functioning layer (blue) increases (see text for further details) (Tielens, 1994).

1996). The amount of both quinones is thus adapted to the energy metabolism during the life cycle, and as both quinones are synthesized *de novo* by *F. hepatica,* the rates of synthesis of both quinones are adapted to their use.

Cytochemical staining of cytochrome oxidase in tissues of parasitic nematodes indicated that in these parasites a metabolic gradient exists, as in *F. hepatica:* an aerobic energy metabolism near the outside and a more anaerobic one inside (Fry and Beesley, 1985). Furthermore, it is known that nematode species with a large diameter have a more anaerobic metabolism than the thinner ones (Atkinson, 1980). Apparently, the diffusion of oxygen is a limiting factor for an aerobic energy metabolism in many parasitic helminths.

During the development of *Fasciola,* changes also occur in the cytosolic metabolism. In the adult, the cytosolic degradation of glucose via PEPCK is the most important route, whereas in the juvenile a large part is degraded via PK. However, the adult probably does not exclusively use the PEPCK pathway, nor does the juvenile exclusively use the PK pathway (Tielens *et al.*, 1987). In both stages, a mixture of malate and pyruvate is the substrate for the mitochondria (Tielens *et al.*, 1981a, 1987; Kane and Bryant, 1984). The presence of malic enzyme, both in the cytosol and in the mitochondria, enables the use of this mixture in any ratio as substrate for the mitochondrial production of acetate and propionate.

The pH profiles of PK and PEPCK of *Fasciola* show that metabolism at the PK/PEPCK branch point could be regulated by the pH (Tielens *et al.*, 1987). Such a pH effect possibly complements regulation by (de)phosphorylation of PK during the aerobic/anaerobic transitions of, for instance, bivalve molluscs (de Zwaan and Dando, 1984). When, during the development of *Fasciola,* the inner layers of the parasite will gradually be forced to anaerobic functioning, the acid end products will lower the cytosolic pH. After arrival in the bile ducts, the excretion of these end products together with the restricted flow of bile and the limited buffer capacity will acidify the environment of the fluke and, as ^{31}P-NMR studies have shown, this will contribute to the decrease in intracellular pH (Tielens *et al.*, 1982). This lower internal pH will favour a partitioning of the flux towards malate formation at the PK/PEPCK branch point. Eventually, a lasting predominance of the PEPCK pathway occurs as PK activity almost completely disappears (Tielens *et al.*, 1987). The expression of key enzymes of metabolism also reveals that the parasite switches from an aerobic to an anaerobic energy metabolism during the development from the juvenile to the adult stage (Cwiklinski *et al.*, 2015).

Although the juvenile liver fluke degrades glucose to carbon dioxide in the presence of oxygen, it is already fully equipped for anoxic functioning: in the absence of oxygen, the newly excysted juvenile produces – just like the adult – propionate and acetate, in a molar ratio of 2:1 (Tielens *et al.*, 1981a). This readiness for anoxic functioning, and hence fumarate reduction, is also revealed

by the observed presence of rhodoquinone in the metacercariae.

8.4 Biosynthetic Capacities

In accordance with its opportunistic way of living as a parasite, *F. hepatica* has limited biosynthetic capacities. As described above, the liver fluke obtains many simple substrates from the host. More complex molecules that the parasite needs but cannot obtain directly from the host are synthesized from these simpler building blocks. The parasite has to synthesize complex structures like proteins and DNA by itself and, in general, the biosynthetic pathways of parasitic helminths bear a close resemblance to those of their mammalian hosts. The enzymes of these pathways, however, often possess different kinetic properties and in some cases, where parasites produce unique end products, certain distinct parts of a pathway – and thus the enzymes involved – are absent in the host.

Carbohydrates such as glucose are not synthesized *de novo* by *F. hepatica*. Gluconeogenesis has never been demonstrated in parasitic helminths, except from intermediates at the level of triose phosphates. Simple carbohydrates are obtained from the host and are then used by the parasite to synthesize the complex ones. Examples include the glycoproteins of the surface glycocalyx and glycogen, the storage carbohydrate used by *F. hepatica*.

Lipids, such as fatty acids and cholesterol, are not synthesized *de novo* by *F. hepatica* but are obtained directly from the host. More complex lipids, such as phospholipids and triacylglycerols, are synthesized from the building blocks obtained from the host (fatty acids and head groups of phospholipids).

Purine and pyrimidine nucleotides are essential components of many biochemical molecules, from DNA and RNA to ATP and NADH. The pyrimidine and purine metabolisms of parasitic helminths have been extensively investigated (reviewed in Carter *et al.*, 2003), because of differences with the pathways in the mammalian host which make them potential targets for chemotherapeutic attack. Trematodes do not synthesize purines *de novo* but obtain them from the host. Analysis of its genome first seemed to indicate that *F. hepatica* is an exception, with a complete biosynthetic pathway apparently present, but inspection of individual hits revealed that the pathway was provided by the recently reported *Neorickettsia* endosymbiont (McNulty *et al.*, 2017; Coghlan *et al.*, 2019). Parasitic helminths possess elaborate purine salvage pathways for a more economical management of this resource. Pyrimidines, on the other hand, are synthesized *de novo* by many parasites, including *F. hepatica*, but this capacity seems to be absent in some nematodes and tapeworms (El Kouni, 2017; Coghlan *et al.*, 2019).

Polyamines such as putrescine, spermidine and spermine are low-molecular-weight di- and triamines. They have important functions in cell multiplication and differentiation and are abundant in rapidly proliferating cells. They are present in parasitic helminths in amounts comparable to those in vertebrate cells. In platyhelminths, the enzymes necessary for their synthesis are lacking and these compounds are obtained from the host (Cazzulo, 2003). It is now known that the gene for the key enzyme, ornithine decarboxylase, is not present in their genome (Coghlan *et al.*, 2019). In *F. hepatica*, a polyamine *N*-acetyltransferase has been characterized and is suggested to play a major role in the polyamine metabolism of this parasite by inactivating excess amines (Aisien and Walter, 1993). Mechanisms of parasitic acquisition of polyamines are different from those of mammalian cells and, in addition, interference with this key pathway could have more severe consequences for the parasite than for the host (Müller *et al.*, 2001). Blockade of parasitic biosynthesis of polyamines has been reported to be a valid chemotherapeutic approach and various polyamine derivatives with anti-parasitic activities have been synthesized or isolated from natural sources (Labadie *et al.*, 2004).

8.5 Lipid Metabolism

Lipids such as phospholipids, triacylglycerols and cholesterol play various important roles in living organisms. Phospholipids and cholesterol are the main components of biological membranes, which not only form the boundaries of the various compartments inside cells and organisms but also act as the interface

between organism and environment and, in the case of *F. hepatica*, the outer membrane is therefore the site of interaction between parasite and host.

Very little of the lipid metabolism of *F. hepatica* has been studied, although it is in several aspects distinctly different from that of the mammalian host. Lipids such as phospholipids, triacylglycerols and cholesterol are not synthesized *de novo* by parasitic flatworms, but are obtained directly from the host (cholesterol) or are synthesized from building blocks obtained from the host (fatty acids and, in case of phospholipids, also the head groups). A schematic overview of the main pathways of lipid metabolism in *F. hepatica* is shown in Fig. 8.6.

Fatty acids are not degraded and thus are not used for ATP production by *F. hepatica* (see section 8.3.1). *De novo* fatty acid synthesis also does not occur; therefore, fatty acids must be obtained from the host. *F. hepatica* contains fatty acid-binding proteins (FABPs) (Hillyer, 2005) and proteomic and secretome analyses indicated that they have a role in the uptake of fatty acids from the blood of the host by the adult flukes (Cwiklinski *et al.*, 2021).

Although *F. hepatica* cannot synthesize fatty acids *de novo*, comparison of host and parasite fatty acids revealed the presence of some fatty acids in adult *F. hepatica* that are virtually absent in the host (Oldenborg *et al.*, 1975). These parasite-specific fatty acids are indeed not synthesized *de novo*, but are produced by modification of fatty acids obtained from the host. *F. hepatica* can use acetate for elongation of host-derived fatty acids (Oldenborg *et al.*, 1975). This chain elongation has specificity for certain fatty acids, of which the elongation of 18:1 to 20:1 is quantitatively the most important. In general, two different pathways exist for the elongation of fatty acids, one being a reversal of β-oxidation and in the other acetyl-CoA is carboxylated to malonyl-CoA and subsequently added to pre-existing fatty acids. It has not yet been fully established which pathway occurs in *F. hepatica*, but acetyl-CoA carboxylase is present in the parasite genome (Coghlan *et al.*, 2019), whereas there is no evidence for the occurrence of a reversal of β-oxidation. The reason for the persistence of chain elongation during evolution, while all other synthesizing and catabolic pathways of fatty acids are lost, is unclear.

Fatty acids, with or without prior chain elongation and desaturation, are incorporated into phospholipids and triacylglycerols (Fig. 8.6). Phosphatidylcholine and phosphatidylethanolamine are the major phospholipid classes present in *F. hepatica*, and both the head groups and the fatty acids are obtained from the host and used as precursors. Pathways

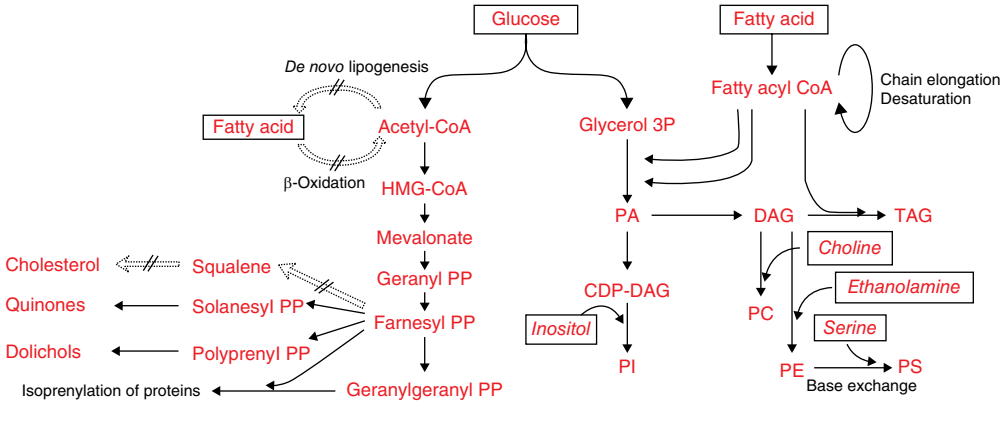

Fig. 8.6. Schematic representation of the main pathways in the lipid metabolism of *F. hepatica*. Boxed substrates are supplied by the host. Pathways present in mammalian systems but absent in *F. hepatica* are shown by open arrows. CDP-DAG, cytidine diphosphodiacylglycerol; DAG, diacylglycerol; Farnesyl PP, farnesyl pyrophosphate; Geranyl PP, geranylpyrophosphate; Geranylgeranyl PP, geranylgeranylpyrophosphate; HMG-CoA, hydroxymethylglutaryl-CoA; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; TAG, triacylglycerol.

for the synthesis of other phospholipids have not been thoroughly investigated in parasitic flatworms. Generally, the phospholipid classes present in the host are also found in parasitic helminths, although sometimes in different ratios.

Sterols such as cholesterol are not synthesized *de novo* by parasitic trematodes, but the so-called mevalonate pathway (Fig. 8.6) is active in parasitic helminths (Coppens and Courtoy, 1996). One of the branches from farnesyl pyrophosphate leads to the formation of quinones, essential lipids in electron transport chains where they carry electrons from one protein complex to the next. This mevalonate pathway was shown to be used by *F. hepatica* for the synthesis of rhodoquinone and ubiquinone (see sections 8.3.6 and 8.3.7) (van Hellemond *et al*., 1996).

As mentioned above, fatty acids obtained from the host are to some extent modified. In addition, cholesterol obtained from the host is not only directly used as a component of the membranes of *F. hepatica,* but is also used as a substrate for the synthesis of other compounds, such as ecdysteroids (Foster *et al*., 1992). Sphingomyelin synthesis in *F. hepatica* occurs probably by a pathway similar to that found in mammals (Bankov *et al*., 1998).

Apparently, *F. hepatica* has discarded some main pathways of synthesizing lipids *de novo,* but has selectively retained several biosynthetic pathways, mainly to modify lipids obtained from the host. Lipids such as specific unsaturated fatty acids, eicosanoids, ecdysteroids and quinones, which are difficult to obtain because the concentration in the host is very low, are synthesized by the parasite by modification of substrates that are easier to obtain from the host. In this way, the lipid metabolism of *F. hepatica* is adapted to an opportunistic way of living, just like its energy metabolism (Brouwers *et al*., 1996).

8.6 Protein Metabolism

Amino acids are not an important energy substrate for *F. hepatica,* but it should be realized that they are of course essential for normal development, just as they are for the host. Amino acids in parasitic helminths are mainly used for biosynthetic purposes. The basic features of the protein and amino acid metabolism in parasitic helminths, including *F. hepatica,* resemble those of their mammalian hosts (for review see Cazzulo, 2003). *F. hepatica* obviously needs to synthesize many different proteins, not only to function as enzymes in the various metabolic pathways but also, for instance, for structural elements like tubulin of which different isotypes are expressed in different tissues and at different life-cycle stages (Fuchs *et al*., 2013).

Protein synthesis also plays an important role in the process of egg-laying of *F. hepatica.* Large amounts of proteins have to be synthesized by adult flukes not only for the proteins of their offspring, the prospective miracidia, but also for the formation of the eggshells. As mature liver flukes produce thousands of eggs per day, a large proportion of their total biosynthetic capacity (and energy budget) is devoted to egg production. Each egg is enclosed in a tough and chemically resistant shell made of tanned proteins. Eggshell precursor proteins are synthesized and stored within the extensive vitellaria of the adult fluke.

Fasciola spp. obtain amino acids by hydrolysis of proteins from the host, utilizing proteases excreted by the parasite. Several types of proteases have been described in *F. hepatica*: they are not only used in protein degradation to provide substrates for biosynthetic purposes, but are also involved in host–parasite interactions, the invasion of host tissues and the evasion of immune attack mechanisms (see also Chapter 11, this volume).

8.7 Concluding Remarks

F. hepatica has many interesting properties in its biochemical processes and especially in its energy metabolism. In the different stages of its life cycle, *F. hepatica* has to adapt its metabolism to the different environments it encounters. Free-living stages do not gather food and are therefore completely dependent on the endogenous stores they acquired in the previous host. Oxygen, on the other hand, is present in the aqueous environments of the freeliving stages (cercariae and miracidia) as well as in that of the newly excysted juvenile, in the tissues of the liver. This enables these stages to live very economically: in their class 1 aerobic mitochondria, they use Krebs cycle activity and oxidative phosphorylation to obtain as much energy as possible from the breakdown of carbohydrates. In the free-living stages, this thrifty metabolism serves

their only mission very well: becoming a parasitic stage in the next host before their glycogen storage is depleted.

The adult parasitic stage in the final host (and most likely also that in the intermediate host) uses mainly a fermentative process for the generation of energy. They use anaerobic class 2 mitochondria and produce ATP via an electron transport chain that can function anaerobically, using fumarate as the final electron acceptor (Müller *et al.*, 2012). An uneconomical energy metabolism is not detrimental for these stages, as the host provides the food. Adult liver flukes produce mainly propionate and acetate as end products of the fermentative degradation of carbohydrates. These end products are formed via malate dismutation, a fermentative pathway that is common in parasitic helminths and occurs also in animals such as freshwater snails, mussels, oysters and other marine organisms.

It should be realized that all parasitic helminths have an anaerobic (at least partly), and hence wasteful, energy metabolism when parasitic, but a thrifty (aerobic) one when they become free-living and external substrates are no longer freely available. It is not yet known whether the specialized anaerobic pathways of parasites have evolved as a reaction to the hypoxic conditions that occur in many of their habitats, or whether the anaerobic energy metabolism itself has certain unidentified advantages. The excretion of anaerobic end products could be important for the parasite either in its continuous struggle with the defence mechanisms of the host or by the effect of these products on the habitat of the parasite. Otherwise, when glucose is abundant and free, it could be advantageous for an organism to use highpower, low-efficiency pathways. Thus, anaerobic functioning is not a defect – as it is often regarded – but a positive property, made possible by the literal meaning of parasitism: eating the food of others.

F. hepatica has also, in other biochemical pathways, interesting features that reflect its parasitic way of living. It has lost several main anabolic pathways, for instance the ability to synthesize fatty acids, cholesterol and purines *de novo.* Therefore, many substances have to be obtained from the host, but *F. hepatica* is then able to modify them to suit its own needs. *F. hepatica* is apparently adapted in many ways to the opportunistic way of living of a parasite.

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9 Immunological Interaction Between *Fasciola* and its Host

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9.1 Introduction

The helminth parasite *Fasciola hepatica* infects a wide range of mammalian hosts, including humans, cattle, sheep, goats, as well as rats, horses, pigs, dogs, rabbits and hedgehogs (Raue *et al.*, 2017; Beesley *et al.*, 2018). Indeed, it infects almost every mammal it has the opportunity to encounter and therefore has evolved a universal mechanism of infection, which is linked to the parasite's ability to modulate immune pathways that are common in all species.

F. hepatica, like other helminths, has a complex life cycle. Within the definitive host, the metacercariae, the infectious stage of the life cycle, excyst to form newly excysted juvenile flukes (NEJs) that penetrate the intestinal wall and migrate for several days in the peritoneal cavity prior to entering the liver capsule. Here they develop and grow before moving into the bile duct to mature fully. Within the bile duct they produce eggs that are released with bile from the gallbladder via the common bile duct into the gastrointestinal tract, from where they are excreted with faeces (Mas-Coma *et al.*, 2019). The parasite, therefore, migrates through different anatomical locations, encountering distinct immunological compartments. Unlike other trematode infections, for example schistosomes, eggs released by mature flukes do not cause extensive pathology, although some studies have reported immune responses to egg antigens during chronic infection in cattle (Moxon *et al.*, 2010). Immunological studies therefore focus upon immune responses associated with the flukes as they migrate from the gut mucosa through the peritoneal cavity and liver tissue and then the bile duct and gallbladder (Martínez-Moreno *et al.*, 1997; McCole *et al.*, 1998; Pérez *et al.*, 1998; Molina and Skerratt, 2005; Ruiz-Campillo *et al.*, 2018).

The immune responses elicited during infection are the result of extensive tissue damage caused by the migrating flukes and a plethora of molecules they release; some of these molecules exhibit a range of immune modulatory properties (Dalton *et al.*, 2013; Molina-Hernández *et al.*, 2015). Studies examining the immune response associated with infection have mainly focused upon experimental rodent models but increasingly the interest is turning to cattle, sheep and goats. There are many similarities in the immune response among these species, as infection is associated with initial mixed T helper immune cells Th1/Th2, followed by biased Th2 and then the induction of T-regulatory (Treg) or tolergenic/anergenic T cells (O'Neill *et al.*, 2000; Molina and Skerratt, 2005; Walsh *et al.*, 2009; Fu *et al.*, 2017; Ruiz-Campillo *et al.*, 2018).

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Innate immune cells associated with infection, such as macrophages, exhibit regulatory phenotypes that resolve the inflammatory process or are involved in tissue repair (Mendes *et al.*, 2013; Japa *et al.*, 2015; Fu *et al.*, 2017; Valero *et al.*, 2017). These immune outcomes are mediated by the various molecules secreted by the parasite, including proteins, glycans and miRNAs. Unlike the intestinal nematodes, Th2 immune responses against *Fasciola* are not correlated with parasite expulsion, but are primarily important for tissue repair (Donnelly *et al.*, 2005; Pesce *et al.*, 2009; Donnelly *et al.*, 2010). The consensus from studies in several host species is that the suppression of Th1-type 'normal' protective responses occurs during infection and that activation of this arm of the immune response (as shown in some vaccine trials) is associated with immune protection (Clery *et al.*, 1996; Hansen *et al.*, 1999; O'Neill *et al.*, 2000). However, immune protection is impaired by the potent immune suppression established by the parasite during infection. As a result, there is no evidence of acquired immunity established from a primary infection and thus there is a need for better understanding of the immune mechanisms associated with all stages of infection.

In this chapter, we examine the immune responses in ruminants naturally infected with *F. hepatica* and compare the findings from these studies with those obtained using experimental rodent models. We also know that infection with *F. hepatica* can have bystander immune suppressive effects in the host, such as increasing the susceptibility of rodents and ruminants to microbial infections that require a Th1 immune response for protection (Aitken *et al.*, 1978a; Brady *et al.*, 1999; Miller *et al.*, 2009). Paradoxically, this parasite-induced suppression can provide protection against non-communicable pro-inflammatory diseases such as arthritis, asthma, multiple sclerosis, type 1 diabetes and colitis. The molecules released by the parasite can mimic immune responses associated with *F. hepatica* infection and similarly exhibit therapeutic effects in mouse models of inflammatory disease. This chapter examines the latest findings describing the immune-modulating properties of these released molecules. Finally, it overviews the future directions required to progress our understanding of the immune response mechanisms associated with resistance and susceptibility to *F. hepatica* infection, which is critical for the development of an effective vaccine.

9.2 Infection in the Natural Host

Despite an ability to infect all mammals, including humans, *F. hepatica* is considered a veterinary parasite because of the extent of infection in animals of agricultural importance (Beesley *et al.*, 2018). Studies examining host–parasite interactions have primarily focused on sheep and cattle because of their global economic importance and thus consideration as targets for vaccine development. Infection in 'minor' livestock such as goat and buffalo, however, can have a huge impact on local economies, for example in Southeast Asia (Molina and Skerratt, 2005; Zafra *et al.*, 2010). As with other helminths of veterinary importance, infection with *F. hepatica* results in biased Th2 immune responses, with evidence of Th1 responses in the early stages of infection, and the development of regulatory responses and tolerance in the later stages (Clery *et al.*, 1996; Fu *et al.*, 2017). Studies examining innate immune cells have focused upon macrophages and eosinophils, suggesting a role in tissue repair and resolution of inflammation (Duffus *et al.*, 1980; Rodriguez *et al.*, 2017; Ruiz-Campillo *et al.*, 2018). Due to the large number of studies in livestock, we have a good understanding of the antibody responses observed during the course of infection, with IgG1 antibody responses dominating in both experimental and natural infection (Clery *et al.*, 1996). There are some studies examining T cell and B cell responses, which are consistent with what is known about these cells in other helminth infections (McSorley and Maizels, 2012).

9.2.1 Cattle

The majority of fluke infections in cattle are sub-clinical and chronic with an adverse effect on growth, food conversion and hence milk and meat production (Howell *et al.*, 2015). Studies examining immune responses in cattle infected naturally or experimentally show consensus in that animals exhibit immune responses modulated towards a non-proliferative type 2

response, characterized by an increased production of IL-4, IL-5 and eosinophilia (Oldham and Williams, 1985; Oldham *et al.*, 1985; Brown *et al.*, 1994; Clery *et al.*, 1996; Clery and Mulcahy, 1998; McCole *et al.*, 1998, 1999; Bossaert *et al.*, 2000; Sachdev *et al.*, 2017; Graham-Brown *et al.*, 2018; Garcia-Campos *et al.*, 2019). This skewed Th2 cytokine profile provides the necessary signals to activate immunoglobulin class switching in B cells towards a dominant IgG1 isotype. After natural infection, both chronically infected and naive cattle exposed to a trickle infection produced a dominant IgG1 antibody isotype within 4–5 weeks (Clery *et al.*, 1996). Although calves infected with a single heavy infection elicited a greater initial antigen-specific IgG1 response when compared with a trickle infection, over the course of 24 weeks antibody responses reached a similar plateau (Clery *et al.*, 1996). In contrast, while IgG2 and IgM were detected in sera, the levels of these antibodies were not significantly different to those in uninfected animals, most likely due to the suppression of Th1 cytokines. In vaccine trials in cattle, the induction of IgG2a correlates with protection and therefore it is to the advantage of the parasite to suppress these responses. While fluke-specific IgA was absent in sera (Clery *et al.*, 1996), there is evidence for the presence of IgA in the bile and liver of infected cattle (Hughes *et al.*, 1982; Molina and Skerratt, 2005). Of particular interest is the emerging role for eosinophils in the maintenance of mucosal IgA (Travers and Rothenberg, 2015), as these cells are also present in increased numbers in colon and liver tissue during *F. hepatica* infection in cattle, which may explain the co-localization of parasite-specific IgA and eosinophils in the gut (McCole *et al.*, 1998).

Eosinophils are associated with Th2 immune responses and while originally considered as effector cells of the Th2 response, they are now acknowledged as multifunctional cells that contribute to the induction, development and resolution of immune responses, including tissue repair (Spencer and Weller, 2010). Infection with *F. hepatica* results in a significant increase in the number of eosinophils and transcriptional analysis of these cells in serum suggests a greater degree of activation in response to infection (Garcia-Campos *et al.*, 2019; Bossaert *et al.*, 2000). A graded eosinophil infiltration of the colon is observed during *F. hepatica* infection, leading to the suggestion that eosinophils might be activated to kill NEJs as they migrate through the gut (McCole *et al.*, 1998). Indeed, evidence from *ex vivo* studies indicates that eosinophils isolated from infected cattle have the capacity to kill NEJs via antibody dependent cellular cytotoxicity (ADCC). This process requires engagement of *Fasciola*-specific antibody on the parasite surface where the Fc domain binds with the Fc receptor on the surface of effector immune cells (Duffus and Franks, 1980; Duffus *et al.*, 1980). However, evidence also suggests that the proteases secreted by the parasite have the capacity to cleave antibodies at the hinge region, which would prevent the immune cell recognition of the antibody attached to the parasite, thus minimizing the killing effect (Carmona *et al.*, 1993).

While the infiltration of eosinophils is also observed within the liver at day 21 post-infection (pi), there is no associated evidence of adult parasite killing (Doy and Hughes, 1984), which suggests that their role at this stage of infection is to mediate repair to the tissue damage caused by the migration through the liver. The possibility that eosinophils may have efficacy only against juvenile worms is further supported by the observation of a relationship between the magnitude of blood eosinophilia in cattle with parasite length, but not burden, suggesting that eosinophils may play a role in specifically combating NEJs by either stunting their growth or slowing their migration (Bossaert *et al.*, 2000).

Macrophages are just as efficient as eosinophils at mediating ADCC, as they express all classes of Fc receptors (Nimmerjahn and Ravetch, 2008) and, similarly, bovine macrophages from uninfected donors cultured *in vitro* with serum isolated from infected cattle effectively killed NEJs (Sulaiman *et al.*, 2016). However, NEJs have evolved strategies to combat host defence mechanisms, as they express a TGF-like molecule (FhTLM) that significantly reduces ADCC-mediated killing *in vitro* by inducing an M2-like macrophage phenotype (Japa *et al.*, 2015; Sulaiman *et al.*, 2016). Circulating arginase-1 positive macrophages were observed during pasture-derived *F. hepatica* infection in cattle at 4 weeks pi and the cell number reached significant levels at 12 weeks pi (Golden *et al.*, 2010). However, when compared with vaccine-protected animals, levels of circulating arginase-1 positive macrophages were significantly reduced, suggesting that

they do not participate in protective immunity. The presence of large numbers of circulating arginase-1 positive macrophages at 12 weeks coincides with the production of significant liver damage, suggesting a role for these macrophages in tissue repair. Indeed, macrophages in the liver of infected cattle are found in close proximity to collagen-producing myofibroblasts (Golbar *et al.*, 2013). Unlike macrophage studies in humans and mice, the repertoires of bovine macrophage phenotypes are not yet fully characterized (Sulaiman *et al.*, 2016).

Treatment of a bovine macrophage cell line with adult excretory/secretory products (FhES) induced a macrophage phenotype that produced significant amounts of both arginase-1 and IL-10 (Flynn and Mulcahy, 2008a). The extent of IL-10 produced was dependent upon the parasite strain, as a wild isolate elicited greater IL-10 compared with an in-house Weybridge isolate, although, irrespective of that difference, the cells were unresponsive to pro-inflammatory ligands (Flynn and Mulcahy, 2008a; Bąska *et al.*, 2013). Furthermore, bovine macrophages exposed to FhTLM resembled a regulatory cell phenotype as they expressed high levels of IL-10 and PDL-1 and moderate arginase-1 activity. While it was proposed that these macrophages might be involved in the promotion of Th2 immune responses during infection, more evidence is required to support this suggestion (Sulaiman *et al.*, 2016).

By 13 weeks post *F. hepatica* infection in cattle there is a notable lack of mitogen- or parasite-specific lymphocyte proliferation despite evidence of parasites residing in the liver and bile duct. While this is associated with an upregulation of regulatory cytokines IL-10 and TGFβ (Flynn and Mulcahy, 2008b; Mendes *et al.*, 2013), there was no evidence of CD4 Foxp3+ regulatory T cells in the mesenteric or hepatic draining lymph nodes (Sachdev *et al.*, 2017). However, the $CD4^+$ pool of cells from both sites showed high levels of expression of anergenic markers such as CTLA4 and PD-1, suggesting a conversion to an anergenic state. Th2 immune responses typically activate fibroblasts and macrophages that initiate the process of wound healing. This process also engages negative feedback loops to restrain the immune response to ensure that excessive tissue scarring does not occur, as this would impair organ function (Barron and Wynn, 2011). Therefore, at 13 weeks post *F. hepatica* infection it is likely that regulatory T cells limit organ damage. Evidence from studies of liver fibrosis following infection with *Schistosoma mansoni* suggests that the presence of arginase-1 producing macrophages signals that the suppression and resolution of fibrosis is under way (Pesce *et al.*, 2009). In addition to competing with Th2 cells and fibroblasts for l-arginine, and thus preventing the ongoing production of collagen, these macrophages induce the differentiation of regulatory T cells, which control the activation of Th2 cells and thus fibrosis (Murray and Wynn, 2011).

Considering all of this evidence, we speculate that, in cattle, the host immune response attempts to combat the parasite during the early stages of infection by activating eosinophils and macrophages with killing capacity. However, equally, the NEJs have evolved to thwart these host defences, allowing at least a portion of the infective dose of parasite to reach the liver. Although characterized as inducing a skewed Th2-type immune response, parasite-specific IFN-γ is also produced in hepatic lymph nodes (HLNs) and peripheral blood mononuclear cells (PBMCs) during the first 3 weeks of infection (Oldham and Williams, 1985; Clery and Mulcahy, 1998). Such a mixed inflammatory response immediately after infection also suggests an attempt by the host to induce protective immune responses. However, IFN-γ production ends with the entry of parasites into liver tissue and/or the onset of egg production (Oldham, 1985; Oldham and Williams, 1985; Bossaert *et al.*, 2000). Although natural infection of cattle is also characterized by the presence of a non-proliferative Th2 immune response, there is no evidence of an early pro-inflammatory host response (Graham-Brown *et al.*, 2018). It is possible that this was missed due to sampling times, or that the induction of parasite-specific IFN-γ is related to the infectious dose of parasite, which is typically much higher in experimental compared with natural infections. Once in the liver, the feeding and migratory activities of the parasite forces the host to switch from anti-parasite responses to mechanisms of tissue repair, which supports the maturation of the parasite to adulthood. Finally, the regulation of pathology and inflammation, by the induction of T cell

anergy, establishes a scenario in which the adult parasite in the bile duct is tolerated by the host as chronic infection ensues.

9.2.2 Sheep

There are many similarities between *F. hepatica* infection in sheep and cattle but the clinical presentation can be very different, with one study showing approximately 10% of infected sheep at risk of acute fasciolosis (NADIS, n.d.; Piedrafita *et al.*, 2001) which results in death from extensive liver damage and haemorrhage. This percentage can vary according to a number of factors, including location and season, and intensity of worm infection. Acute fasciolosis is linked to the migration of large numbers of immature parasites through the liver, suggesting that sheep are more susceptible to infection. This idea is reinforced by one study that directly compared infection in cattle and sheep. The average recovery of flukes from the livers of cattle was 3.4% as compared with 57.4% for sheep, although these percentage can vary with fluke strain used (Phiri *et al.*, 2006).

From the use of transcriptomic approaches, it is now evident that there are additional systemic differences in how sheep respond to infection compared with cattle. Evaluating the number of differentially expressed genes (DEGs) in the PBMC of these hosts revealed striking differences (Fu *et al.*, 2017; Garcia-Campos *et al.*, 2019). In cattle, 5% of the total DEGs occurred during the acute phase of infection, as compared with 70% in sheep (Alvarez Rojas *et al.*, 2015; Fu *et al.*, 2017). Furthermore, the fold change in expression was much higher in sheep (up to 22-fold) than in cattle (up to threefold), suggesting a significantly greater change during the acute phase of infection in sheep, consistent with the difference in disease phenotype between these two species.

As with cattle, sheep showed a reduced proliferative response to concanavalin A and phytohaemagglutinin in PBMC from week 2 to week 20 pi (Martínez-Moreno *et al.*, 1997). Analysis of the PBMC of sheep infected with *F. hepatica* has shown that, as in cattle, there is an initial and transient expression of a Th1-type immune response, as characterized by the production of IFN-γ (Moreau *et al.*, 1998; Zhang *et al.*, 2005), followed by a sustained expression of IL-10. In addition, functional and pathway analysis of PBMCs and liver tissue revealed changes in gene expression related to T cell activation. These changes indicated the establishment of a biased Th2 immune response accompanied by the expression of TGFβ (Alvarez Rojas *et al.*, 2015; Rojas *et al.*, 2016; Fu *et al.*, 2017). Unlike the situation in cattle, $Foxp3^+$ Tregs increased significantly in the acute and chronic stages of infection in hepatic lesions (HLs) while these cells were only observed in the HLN in the acute phase of infection (Escamilla *et al.*, 2016). Transcriptomic data also indicated that sheep infected with *F. hepatica* had reduced numbers and activity of Th17 cells, which may benefit parasite survival and may also underlie the immune-protective capacity of infection with this helminth (Fu *et al.*, 2017). Ultimately, the acute nature of disease in sheep, inflammatory and often fatal, may need to be offset by the rapid induction of immunoregulation via non-antigen specific Foxp3-driven mechanisms contrasting with antigen-specific exhaustion seen in chronic bovine infection.

As in cattle, IgG1 is the predominant antibody in sheep, increasing in serum from 4 weeks pi, reaching a peak at 12–13 weeks of infection to a level that is then sustained (Phiri *et al.*, 2006). The production of parasite-specific IgM mirrors this kinetic profile, but at a much lower titre (Phiri *et al.*, 2006). In parallel with the early increase in Th1-type immune response, there is evidence of a slight increase in parasite-specific IgG2, 2 weeks after infection, which is quickly suppressed (Hansen *et al.*, 1999; Phiri *et al.*, 2006). In sheep, there is also evidence for the production of IgA both in serum and in bile (Ferre *et al.*, 1997), although in serum this appears as a biphasic response, peaking at 6 weeks and 18–20 weeks pi (Phiri *et al.*, 2006). As in cattle, a likely role for eosinophils in the production of IgA antibody emerges from the observation that eosinophilia during infection in sheep also occurs in a biphasic pattern (Zhang *et al.*, 2005).

Eosinophilia is evident as early as 9 days pi, when eosinophils represent the predominant immune cell in the peritoneal cavity (Ruiz-Campillo *et al.*, 2017; Pérez-Caballero *et al.*, 2018). However, proteomic analysis of the peritoneal fluid of infected sheep failed to identify

any evidence of eosinophil degranulation (Ruiz-Campillo *et al.*, 2018). This suggests that the peritoneal eosinophils are not activated or that an alternative undefined phenotype is induced and therefore, just like in cattle, is not part of the protective immune response (Ruiz-Campillo *et al.*, 2017, 2018). This alternative eosinophil phenotype could be involved in inflammatory resolution, which is important in sheep given the susceptibility of this host to acute *F. hepatica* infection and the high number of flukes that transverse the peritoneal cavity to the liver (Strandmark *et al.*, 2016). Indeed, their presence in the liver correlates with the parasite's migratory tunnels, which supports a role in tissue repair (Chauvin and Boulard, 1996).

The macrophage is the next most predominant immune cell after eosinophils in the peritoneal cavity during early infection (Pérez-Caballero *et al.*, 2018). As described above, macrophages from cattle are capable of mediating ADCC against newly excysted juvenile flukes via a mechanism that is dependent upon, and directly proportional to, the production of nitric oxide (NO) (Sulaiman *et al.*, 2016). In contrast, macrophages isolated from sheep lack the ability to produce NO in the presence of immune sera and a range of stimuli (Piedrafita *et al.*, 2001). This lack of effective defence mechanisms has also been noted *in vivo*, with peritoneal macrophages from infected sheep showing no increase in the expression of the gene encoding inducible NO synthase (iNOS), the enzyme that catalyses the conversion of NO from l-arginine (Piedrafita *et al.*, 2001; Ruiz-Campillo *et al.*, 2018). This observation was supported by a study that showed species-specific transcriptional regulation of genes involved in NO production and arginase (ARG) expression in macrophages. The response of macrophages to lipopolysaccharide (LPS) stimulation was not conserved across species, as cattle, mice and rat but not sheep macrophages expressed iNOS and ARG in response to LPS (Young *et al.*, 2018). While sheep macrophages may have an inherent defect in the production of NO, parasite-secreted products further drive the phenotype of these macrophages away from a protective M1 phenotype towards a non-protective, anti-inflammatory M2 phenotype (Flynn *et al.*, 2007).

Assembling all of these observations infers the same scenario as in cattle: that in sheep the host immediately responds with a typical protective immune response, which is somehow regulated by the parasite to prevent its expulsion, and then once the liver is penetrated, the immune response is switched to mediate tissue repair mechanisms. However, studies in sheep indicate that the early Th1-type immune response initiated by the host may not be sufficiently potent to reduce the numbers of flukes migrating to the liver. Consistent with this proposition are studies showing that, like in cattle, induction of an early Th1 immune response in sheep through vaccination correlates with an ability to resist liver fluke infection (Pleasance *et al.*, 2011). Therefore, the suppression of Th1 immune responses by the parasite is critical to its survival and many studies have shown the Th1-suppressive properties of *F. hepatica*-secreted molecules that target different immune cell types using diverse mechanisms (Donnelly *et al.*, 2005; Dalton *et al.*, 2013; Ramos-Benitez *et al.*, 2018; Tanaka *et al.*, 2018).

9.2.3 Goats

Goats are regarded as a 'minor' livestock (AAVP, 1983) and therefore less targeted for drug development; as a consequence this has meant less investigation into the immune response of goats after infection with *F. hepatica.* However, *F. hepatica* infection in goats can be considered to be more closely aligned to sheep rather than cattle. Following an experimental infection protocol, goats exhibited typical acute disease with some mortalities following weekly infection with 50 *F. hepatica* metacercariae on four occasions, and with higher doses of 200 *F. hepatica* metacerariae (Reddington *et al.*, 1986; Martínez-Moreno *et al.*, 1997; Raadsma *et al.*, 2007). At post-mortem, examination of the liver and migratory tracts demonstrated infiltration of eosinophils, plasma cells and $CD3^+$ lymphocytes, as well as the development of calcified granulomas and abundant lymphocyte infiltrates on a background of fibrosis, bile duct hyperplasia and loss of lobular architecture in the chronic stages (Martínez-Moreno *et al.*, 1997). Similar studies have shown elevated CD2, CD8 and CD4 T cells in HL and HLN of goats infected with *F. hepatica* whereas γ/δ T-lymphocytes and B cells (IgM+,

lambda-IgG$^+$ or CD79$^+$) were present in HL but significantly less than compared with HLN (Pérez *et al.*, 1998). *F. hepatica*-infected goats display lesions that are typical of chronic fasciolosis with inflammatory infiltration of plasma cells, macrophages and granulomatous foci. An infiltration of CD2$^+$, CD4$^+$ and CD8$^+$ T lymphocytes and IgG$^+$ plasma cells were observed in HL and HLN. IFNγ, although elevated, was much lower than that of IL-4 in the HLNs, suggesting a skewed Th2 response, while neither cytokine was detected in the hepatic tissue (Zafra *et al.*, 2010).

Escamilla *et al.* (2016) compared the expansion of Foxp3$^+$ Tregs in acute and chronic HL and HLN in sheep and goats. The number of both Foxp3$^+$ and CD3$^+$ T lymphocytes increased significantly in both species in the acute and chronic stages of infection in HL. While in HLN the number of cells became elevated for both species in the chronic stage, only goats had elevated numbers of cells in the acute phase (Escamilla *et al.*, 2016). Similar to sheep, infected goats also showed a reduced proliferative response to concanavalin A and phytohaemagglutinin in PBMCs from weeks 2–20 pi (Martínez-Moreno *et al.*, 1997). Like sheep, goats have a reduced oxidative response in polymorphonuclear leucocytes during *F. hepatica* infection and this was reduced further during secondary infection, which is in keeping with the mechanism of immune impairment during fasciolosis (Martínez-Moreno *et al.*, 2000).

9.2.4 Other definitive hosts

While other animals commonly infected with *F. hepatica* include horses, pigs, dogs, cats, rabbits and hedgehogs, immune studies in non-ruminants, other than experimental animals, are limited (Raue *et al.*, 2017). Studies have shown that there are differences between species with regard to susceptibility and resistance to infection (Spratt and Presidente, 1981; Valero *et al.*, 1998; Dracz *et al.*, 2016; Howell *et al.*, 2020). While rats are used as experimental models they are also a natural wild reservoir for *F. hepatica*, with reports of infection in Corsican black rat (*Rattus rattus*) and Australian bush rat (*Rattus fuscipes*), albeit with a low prevalence suggesting that these animals exhibit some resistance to infection (Spratt and Presidente, 1981; Valero *et al.*, 1998). In contrast, a number of studies have reported that the capybara *Hydrochoerus hydrocaeris,* a giant cavy rodent native to South America, is an exceptionally 'permissive' host that is highly susceptible to infection (Dracz *et al.*, 2016). Infection in horses is relatively common but the parasite is tolerated well, with low numbers of flukes recorded and low egg excretion levels (Quigley *et al.*, 2017; Howell *et al.*, 2020). Experimental infection of horses, even with doses of up to 1000 metacercariae, has not resulted in flukes detected in the liver (Quigley *et al.*, 2020). The horse is particularly interesting as a host, since studies suggest that the majority of parasites are eliminated or immobilized at an early stage of the infection, presumably before reaching the liver. This hypothesis was supported by the finding that about 15% of excysted larvae implanted intraperitoneally into two horses succeeded in reaching maturity in the bile ducts (Nansen *et al.*, 1975). Apparently, a similar outcome has been observed in *F. hepatica* infection in pigs (Ross *et al.*, 1967; Raue *et al.*, 2017). However, given the dearth of knowledge on immune responses, we are unable to speculate on the possible immune responses associated with susceptibility and resistance to infection in these different animal species. Understanding why some species are resistant or susceptible to infection could be an alternative strategy for developing therapeutic approaches. Studies in the natural host are lacking in information regarding the immune responses associated with early infection, as most studies have focused on the liver and late stages of infection. Therefore, experimental models such as mice are tractable to study this, particularly since the immune profile in the mouse appears to be close to that of natural infection in economically important hosts.

9.3 Infection in Experimental Hosts

Mouse, rat, hamster, guinea pig and rabbit are experimental mammalian models used to examine the interaction of *F. hepatica* with its host (Coles, 1975; O'Neill *et al.*, 2000; Tliba *et al.*, 2002b; Terasaki *et al.*, 2002; Jarujareet *et al.*, 2018). These models are commonly used to study parasite growth, development and behaviour; however, when examining immune responses associated with infection, rodents as models of

disease are the most commonly utilized, due to the close resemblance of their genetic and biological characteristics to those of higher mammals (Perlman, 2016). Furthermore, many of the clinical signs and stages of *F. hepatica* infection can be replicated in rodents. In addition, they provide many advantages, including their small size and ease of care, as well as the abundant immunological and genetic resources available (Bryda, 2013).

In mice, the duration of *F. hepatica* infection is generally limited to 21 days, as mortality from infection is high after 3–4 weeks because of severe liver damage, enlarged spleen and ascites (O'Neill *et al.*, 2000; Zhang *et al.*, 2020). Mice are therefore useful models for investigating immune responses during the very early stages of *F. hepatica* infection (Chung *et al.*, 2012a,b) and for examining immune protection in the very early stages of infection following vaccination (Jayaraj *et al.*, 2012; López-Abán *et al.*, 2012). The advantage of the rat model is that the course of infection resembles the complete infection cycle of the natural host (Paz-Silva *et al.*, 2002; Valero *et al.*, 2011). Unlike in mice, NEJs in rats can develop into adult stages, residing in the common bile duct to produce viable eggs for as long as its host's natural life, which is typically 2.5–3 years (Valero *et al.*, 1998, 2002). However, the number of immune studies in rats is significantly less than in mice, as the reagents for the rat model are not as readily available. Furthermore, rats are significantly more expensive to purchase and house compared with mice, particularly when infection can persist for the natural life of the host.

9.3.1 Mice

Like infection in ruminant hosts, mice can be infected by the oral delivery of metacercariae. These excyst in the duodenum and NEJs transverse the gut mucosa, migrating across the peritoneal cavity to the liver, where tracts formed from migrating NEJs can be observed macroscopically (O'Neill *et al.*, 2000; Stempin *et al.*, 2016). Because of differences in host size, this whole process occurs much more quickly in mice compared with cattle or sheep, with the parasites reaching the liver within 1 week of infection. However, the immune response observed in the mouse at 1–2 weeks is similar to the chronic stages of infection in the definitive host, illustrating the contribution that tissue damage makes to the development of host immune responses (O'Neill *et al.*, 2000; Walsh *et al.*, 2009; Stempin *et al.*, 2016). Like the ruminant hosts, once established in the liver, Th2 and T-regulatory immune responses are observed as measured by antigen-specific production of IL-4, IL-5 and IL-10 in isolated spleen and draining lymph nodes. In addition, reflecting this skewed immune phenotype, significant antigen-specific IgG1 titres, but not IgG2a, are found in serum 3 weeks pi (Brady *et al.*, 1999; O'Neill *et al.*, 2000; Chung *et al.*, 2012a).

As in sheep and cattle, regulatory T cell phenotypes are associated with infection in mice (Walsh *et al.*, 2009). Studies have shown that there is a slight increase in $CD4^+$, $CD25^+$, $Foxp3^+$ cells in the local mesenteric lymph nodes, indicating that only a small percentage of cells express regulatory markers at this site. However, in the peritoneal cavity there is significant expression of T-regulatory cells that express a panel of regulatory markers, including CD25, TGFβ, IL-10, IL-10R, CTLA-4, T1/ST2 and CCR5 (Walsh *et al.*, 2009). This observation suggests that regulatory cell phenotypes are important at the site of infection to control local inflammatory responses related to migrating NEJs in the peritoneal cavity.

At 2 weeks post *F. hepatica* infection only a small population of cells were lymphocytes (4–5%) while the 89–81% of peritoneal exudate cells (PECs) were macrophages with some neutrophils (5–6%). In contrast to the analysis of peritoneal cells in ruminants, no eosinophils were observed at this stage of infection (Miller *et al.*, 2009). However, as the liver damage increased at 3 weeks pi, eosinophils (Siglec F^+) appeared, accompanied by an increase in the percentage and absolute numbers of dendritic cells (DCs), macrophages ($F4/80^+$), neutrophils ($GR1^+$), and $CD4^+$ T cells (Walsh *et al.*, 2009). When the PECs from infected mice were co-cultured with $CD4^+$ T cells from DO11.10 OVA TCR Tg mice in the presence of Ova peptide, the T cells were driven to produce significantly less antigen-specific IL-10, IL-17 and IFNγ than PECs from control mice, suggesting a regulatory function. In contrast, APCs isolated from the spleen

of infected DO11.10 OVA TCR Tg mice showed enhanced IL-10, and IL-4 production, indicating the existence of differential populations of cells activated within distinct immunological niches of the host (Walsh *et al*., 2009).

Considering the predominance of macrophages in the peritoneal cavity during early infection in mice, a large amount of research has focused on characterizing the phenotype and functionality of these cells. They have been characterized as M2 macrophages due to the expression of markers such as arginase, YM1, Relm1α, IL-10, and TGFβ and Low MHCII (Donnelly *et al*., 2005; Miller *et al*., 2009; Walsh *et al*., 2009). Macrophages isolated from PECs of *F. hepatica*-infected mice also express PDL2. *F. hepatica* infection in PDL2 knockout mice elicited reduced arginase and IL-10 expression in macrophages isolated from PECs and resulted in reduced antigen-specific Th2 immune responses and enhanced Th1 immune responses. While the parasite burden was not examined in these studies, the mice displayed significant liver damage and shortened survival compared with infection in wild-type mice (Stempin *et al*., 2016). Such an outcome supports the notion that the parasite-activated macrophages drive Th2 immune responses that are critical for the management of tissue repair mechanisms.

Supporting this hypothesis is the observation that the macrophages associated with *F. hepatica* infection produced less pro-inflammatory cytokines, such as IL-12, that are necessary for the promotion of Th1 immune responses (Stempin *et al*., 2016). In addition, DCs isolated from PECs of *F. hepatica*-infected mice expressed a phenotype that also produced reduced IL-12. These cells expressed lower levels of CD80, CD40, MHC class II and CD86 and higher quantities of CCR5 as well as increased expression of IL-10 and LAP (Walsh *et al*., 2009). The lack of IL-12 is a common feature in *F. hepatica* infection and a number of the antigens secreted by the parasite actively inhibit the ability of innate immune cells to produce IL-12, thus preventing the development of protective Th1 immune responses (Hamilton *et al*., 2009; Miller *et al*., 2009; Donnelly *et al*., 2010; Martin *et al*., 2015). Importantly, both macrophages and DCs isolated from the peritoneal cavity of infected mice are hyporesponsive to stimulation with a range of inflammatory ligands (Miller *et al*., 2009; Walsh *et al*., 2009; Inclan-Rico and Siracusa, 2018; Smith *et al*., 2018). This suggests that these cells are broadly prevented from secreting antimicrobial mediators and inflammatory cytokines, which, as evidence from ruminant studies shows, are capable of killing the NEJs as they migrate to the liver.

There is currently only preliminary evidence to demonstrate that different breeds of ruminants are more susceptible or resistant *to F. hepatica* infection (Garcia-Campos *et al*., 2019). However, it is clear from murine studies that genotype affects the immune response and disease outcome of *F. hepatica* infection, providing a deeper understanding of the impacts and contribution of different components of the host immune response to disease pathogenesis (Takeyoshi *et al*., 1994; O'Neill *et al*., 2000; Chung *et al*., 2012a). Balb/c and 129Sv/Ev mice exhibit polarized Th2 immune responses during infection while presenting with high levels of liver pathology with 85% infection rate. In contrast, C57/BL6 infection induces more of a mixed Th1/Th2 immune response with lower levels of liver pathology and infection rate (45%) (O'Neill *et al*., 2000). Measurement of cytokines in serum and produced by spleen cells of Balb/c compared with C57/BL infected mice showed significant difference in cytokine levels. Balb/c mice exhibited increased IL-4 while C57/BL produced higher IL1β and TNFα; as infection progressed, both mice secreted increased levels of TGFβ. These observations also demonstrate that different strains have a genetic predisposition towards different immune profiles that can explain the differences in disease outcome (Chung *et al*., 2012a). In addition to these natural strains, the availability of knockout mice permits investigations into the role of specific immune mediators in the host response to *F. hepatica* infection. For example, using IFNγ$^{-/-}$ and IL-4$^{-/-}$ mice it was shown that the polarized Th1 immune response produced in IL-4$^{-/-}$ mice was protective, as mice displayed a reduced infection rate of 46% and exhibited less liver pathology, while in the absence of IFNγ mice are susceptible to higher infection rates with more extensive liver pathology (O'Neill *et al*., 2000). This study adds to the evidence from ruminant studies and vaccine trials that the presence of Th1 responses provides protective immunity (Clery *et al*., 1996).

These studies in mice support findings in the natural host demonstrating that a mixed

Th1/Th2 can be associated with early immune responses in mice while in the later stages of infection T-regulatory responses are induced. These studies demonstrate that the parasite is suppressing the typical host protective immune responses in the early stages of infection, particularly targeting innate cells such as macrophages and dendritic cells in order to facilitate the migration of NEJs towards the liver. On reaching the liver, the extensive tissue damage triggers immune cells that can regulate immune responses and are involved in tissue repair, both necessary for the host's survival. The main advantage of using mice as an experimental model is the availability of various strains and genetically modified mice, and the fact that results from these studies mirror the responses observed in the natural ruminant host. It is difficult to perform studies on the peritoneal cavity of ruminants given their size; therefore, murine studies are important to help us understand immune responses during this early stage of infection.

9.3.2 Rats

It is possible to examine both the acute and chronic stages of *F. hepatica* infection in the rat model (Valero *et al.*, 2002; Gironès *et al.*, 2007) where liver pathology in infected rats is similar to that seen in ruminant hosts (Beytut *et al.*, 2011). Similar to the mouse model, because of the size of a rat, the NEJs reach the liver within 7 days pi (Valero *et al.*, 2002; Gironès *et al.*, 2007) and by day 14 acute migratory tunnels infiltrated by eosinophils, neutrophils, macrophages and lymphocytes are observed (Tliba *et al.*, 2000; Beytut *et al.*, 2011). In the early stages of infection, similar to ruminants, there is a mixed Th1/Th2 immune response as hepatic mononuclear cells exhibit increased numbers of $CD4^+$ and $CD8^+$ populations that express enhanced IL-4, IL-10 and IFNγ transcripts when stimulated with a polyclonal activator (Tliba *et al.*, 2000, 2002a). A similar study examined NK cell populations in the early stages of infection demonstrating increased IL-2- and IFNγ-producing NK cells on day 7 of infection, indicating that these cells are another source of early IFNγ (Tliba *et al.*, 2002c). However, like infection in the natural host, IFNγ expression decreases over time, indicating that the parasite suppresses protective Th1 immune responses (Tliba *et al.*, 2002b,c). Decreased IFNγ coincides with inhibited cell proliferation in hepatic mononuclear cells on day 14 compared with the enhanced cell proliferation observed on day 7 (Tliba *et al.*, 2000). In similar studies, proliferation of splenocytes in response to LPS or ConA was suppressed compared with controls although no significant difference in the cellular composition was observed (Gironès *et al.*, 2007; Valero *et al.*, 2017).

There is a dearth of studies examining the phenotype of individual immune cells in the rat model and only limited studies have examined the profile of immune cells in the peritoneal cavity. During the first week of infection, the number of peritoneal cells increased compared with controls. The percentage of cells fluctuates over time, but there was a significant decrease in lymphocytes while macrophages, neutrophils and eosinophils, $CD4^+$, $CD8^+$ and NK cells increased during this period (Cervi *et al.*, 1998). This study reported immune changes consistent with mouse infections, as peritoneal exudate cells on day 7 expressed high levels of IL-10 and no nitrogen oxide. This observation corresponded to migratory flukes in the liver that were surrounded by eosinophils and polymorphonuclear cells, suggesting that in the early stages of infection these cells may already be switched to phenotypes associated with regulatory function and tissue repair (Cervi *et al.*, 1998). In contrast, other studies reported enhanced levels of nitric oxide and H_2O_2 in macrophages, neutrophils and eosinophils within 7 days pi (Jedlina *et al.*, 2011), suggesting that in the early stages, similar to infection in the natural host, these may be protective cell phenotypes that are involved in NEJ killing. However, further detailed studies are required.

Only a few studies have examined *F. hepatica* infection in rats in the chronic stages. At 2 months pi, fibrous connective tissue surrounds chronic migratory tunnels that are infiltrated with eosinophils, lymphocytes, macrophages, multinucleated giant cells and plasma cells (Beytut *et al.*, 2011). Serum from rats infected with *F. hepatica* for 22 days had high levels of IL-1β, IL-5, IL-13 and IFNγ. These elevated levels of cytokines correlated with metabolic changes in the liver as measured by enhanced levels of acute-phase proteins, including inflammatory

mediators such as transferrin, α-1-anti-trypsin and haptoglobulin that are secreted by hepatocytes in responses to tissue damage from migrating flukes (Saric *et al*., 2010). Raised white blood cells and eosinophils could be observed in the chronic stages of infection (Wesołowska *et al*., 2018) while enhanced expression of serum IL-4 and IFNγ was observed at weeks 7 and 10 but not at week 20 (Gironès *et al*., 2007). In a similar study examining *F. hepatica* infection in rats over a 60-day period, a significant decrease in proliferative responses was observed in the early stages of infection that correlated with supressed IL-2 and enhanced IL-4, IL-10 and IFNγ but no changes were observed compared with controls in the chronic stages of infection (Cervi *et al*., 1998).

Because there are no genetically modified rat species available, there is no strong evidence on the importance of cytokines during infection in rats. However, spleen cells from infected rats on day 7 that failed to proliferate when activated with polyclonal activators showed restored cell proliferation by the addition of IL-2 or antibodies to IL-4 and IL-10 in culture (Cervi *et al*., 1998). Another study showed that the administration of aminoguanidine, which opposes the effects on NO production in rats, caused a reduction in worm burden after 60 days. However, no differences in the inflammatory infiltrate in acute and chronic migratory tunnels were observed (Beytut *et al*., 2011). These studies suggest that *F. hepatica* suppresses the host's protective immune response to ensure that it can migrate to the bile ducts to complete its life cycle. Compared with mice studies there is still much to learn about infection in this model, in particular the phenotype of innate immune cells and immune responses associated with resistance and susceptibility to infection.

9.4 Bystander Effects of *Fasciola hepatica* Infection

As described, tissue repair and regulatory innate and adaptive immune cell phenotypes dominate in the host during chronic stages of *F. hepatica* infection. While this immune response ensures a successful infection by the parasite, a 'bystander effect' exists that affects the ability of the infected animal or human host to respond to concurrent microbial infections (Bentwich *et al*., 1996). Furthermore, studies have demonstrated reduced efficacy of Th1-inducing microbial vaccines in helminth-infected populations (Nash *et al*., 2017).

Prior to our current understanding of the immune response associated with *F. hepatica* infection, reports on the impact of this parasite on the course of subsequent disease emerged from studies in cattle. As early as 1976, Aitken and colleagues reported increased susceptibility of *F. hepatica*-infected cattle to subsequent infection with *Salmonella dublin* (Hall *et al*., 1981). Further investigation demonstrated that bacterial shedding, as well as *S. dublin*-associated disease and mortality, was altered by co-infection with *F. hepatica* (Aitken *et al*., 1978a). Post-mortem examination revealed a wider within-host dissemination of the bacteria, with a possibility for increased development of a carrier state (Aitken *et al*., 1978b). This initial observation is corroborated in a more recent study showing that *F. hepatica* infection leads to increased shedding of *E. coli* O157 in cattle (Howell *et al*., 2018). A recent study examining efficacy of PI-3, BRSV and *Mannheimia haemolytica* respiratory vaccination in calves reported no impact of *F. hepatica* infection on total antibody titre and specific neutralizing titre (Krump *et al*., 2014). However, a limitation of this study is that it did not examine antibody isotype or measure prevalence of disease and disease outcome over time.

The immune response to *Mycobacterium bovis* BCG (bacille Calmette–Guérin) in cattle is thought to mimic natural infection with *M. bovis*, and if used for vaccination it confounds the use of standard diagnostics. Tuberculin reaction size is thought to be a prediction of the extent and pathological progress of bovine tuberculosis (bTB) (Byrne *et al*., 2018). More specifically, delayed-type hypersensitivity (DTH) responses to purified protein derivative (PPD)-B and an *ex vivo* IFN-γ whole-blood assay are the foundation of the many bTB tests that influence policies on bTB screening and management. In an experimental co-infection of *F. hepatica*-infected cattle with BCG, both of these parameters were suppressed (Flynn *et al*., 2007; Byrne *et al*., 2018). Additionally, an effect on the DTH skin test response was evident in a study by Claridge *et al*. (2012) that used a modelling approach based on *F. hepatica* diagnosis and bTB reporting. This study demonstrated a significant negative effect of *F. hepatica* infection on bTB diagnosis in

the field, estimating a rate of under-diagnosis of 27–38% of farms (Claridge *et al.*, 2012). The extent to which these interactions impact upon the outcome and impact of co-infections between *Fasciola* spp. and *M. bovis* has been the topic of a recent systematic review (Howell *et al.*, 2019). Although the number of studies (n = 13) that met the inclusion criteria was relatively small, the balance of evidence suggested that liver fluke infection resulted in either no effect or a decreased response to four parameters relevant to the diagnosis of bTB, namely skin testing, IFNγ responses, lesion detection and mycobacterial recovery.

The exact immune mechanism(s) leading to suppression of *F. hepatica*-infected cattle to increased susceptibility to bacterial infection is unknown. Mycobacterial burden in cattle was reduced in animals co-infected with *F. hepatica*, in contrast to studies in *Salmonella* co-infected cattle that exhibited increased bacterial shedding (Aitken *et al.*, 1978a). However, PBMCs from cattle co-infected with *F. hepatica* and *M. bovis* displayed reduced *M. bovis*-specific Th1 immune response. *In vitro* examination of bovine macrophages derived from *F. hepatica*-infected animals challenged with *M. bovis* BCG *in vitro* demonstrated increased TLR2 and CD14 with a lower bacterial burden compared with controls (Garza-Cuartero *et al.*, 2018). When monocyte-derived macrophages were isolated from these co-infected animals, lower mycobacterial recovery and cellular uptake were observed. It was shown that these cells had a lower expression of pro-inflammatory cytokines compared with macrophages isolated from *M. bovis*-infected animals. Macrophage phenotype switched from an M1-like to an M2-like phenotype. The switch of macrophages to an M2 phenotype was also observed in helminth-infected human populations also exposed to pulmonary TB as lung damage correlated with increased serum arginase-1 activity (Monin *et al.*, 2015). These bystander effects of helminth-induced downregulation of pro-inflammatory responses adds new insights in our understanding of host–pathogen interactions during co-infection and highlights a need for further exploration.

Supporting the bystander effects of *F. hepatica* in cattle, studies using experimental mouse models reported the detrimental effect of *F. hepatica* infection in mice co-infected with *Bordetella pertussis*, a Gram-negative bacterium that causes whooping cough in children (Brady *et al.*, 1999). Co-infection in mice resulted in delayed clearance of bacterial infection from the lungs. This delayed clearance coincided with a suppression of *B. pertussis* antigen-specific Th1 immune responses, a cytokine critical for immunity. However, in mouse models, *F. hepatica* negatively affected the efficacy of a commercial *B. pertussis* whole-cell vaccine in mice; *F. hepatica*-infected mice exhibited delayed clearance of bacterial infection, which coincided with a reduction in vaccine-specific Th1 immune responses when compared with vaccinated mice free of *F. hepatica.* This suppression was partially dependent on IL-4, a key cytokine that drives Th2 immune responses. Moreover, the suppressive response could be mimicked by a cysteine protease secreted by the parasite in the adult stages of infection, pointing to the importance of parasite-secreted molecules in bystander effects observed during helminth infection (Brady *et al.*, 1999; O'Neill *et al.*, 2001).

In contrast, a study examining a co-infection with *Toxoplasma gondii*, an intracellular protozoan that causes severe disease in immune-deficient individuals, demonstrated that *F. hepatica* did not alter the course of infection. Instead, *T. gondii* supressed the *F. hepatica*-specific Th2 immune responses and switched the subset of peritoneal macrophages from M2a to M1 phenotype, which was observed irrespective of whether *F. hepatica* infection was established prior to or after *T. gondii* infection. This study used an Me49 strain that is most frequently associated with human infection and induces high levels of Th1-inducing cytokines during infection, as opposed to the Type I strain that induces M2 macrophages (Araujo and Slifer, 2003). It would be interesting to determine if this skewing of the immune response by Type I strain would affect the outcome of *F. hepatica* infection either by increasing worm burden or by pathology, as was demonstrated in IL-4$^{-/-}$ mice (Miller *et al.*, 2009). This study also bolsters the evidence showing that Th1 immunity is important to protect against *F. hepatica* infection.

9.5 Therapeutic Potential of *Fasciola hepatica*-derived Molecules

A number of clinical trials have reported the beneficial effects of helminth infections for the

treatment of immune-mediated inflammatory disorders that are a significant clinical and socio-economic burden globally (Fazal *et al*., 2018; Edwards *et al*., 2019; Janssen *et al*., 2020; Mattiuzzi and Lippi, 2020). Most immune-mediated diseases share similar underlying mechanisms, which involve dysregulation of Th1/Th17 pro-inflammatory cytokines such as TNF, IL-12p70, and IFN-γ, while, on the other hand, helminths share the common property of suppressing these immune responses (Walsh *et al*., 2009; Hübner *et al*., 2012; McSorley and Maizels, 2012; Weinstock and Elliott, 2014). Clinical trials predominantly involved the administration of either hookworm larvae (Feary *et al*., 2010; McSorley *et al*., 2011; Croese *et al*., 2015) or *Trichuris suis* ova (Summers *et al*., 2003; Fleming *et al*., 2011, 2019; Bourke *et al*., 2012; Sandborn *et al*., 2013; Schölmerich *et al*., 2017) as the therapeutic agent to provide the anti-inflammatory stimulus of a natural infection. These treatments have been proven to be safe with only mild side effects, unlike commonly used treatments, such as glucocorticoids, that have general immunosuppressive and sometimes serious side effects. The more recently discovered targeted treatments such as anti-TNF monoclonal antibodies (e.g. Enbrel, Humira or Remicade) also exhibit major side effects, including susceptibility to serious infections (Shivaji *et al*., 2019; Chan *et al*., 2020). The worm trials, however, have proved somewhat inconclusive, with results demonstrating beneficial effects for inflammatory bowel disease and multiple sclerosis but no clinical benefits for allergy.

Hookworms and *Trichuris suis* both reside in the intestine of their mammalian host and incur minimal pathological damage at low doses. In contrast, *F. hepatica* infection is not feasible as a live infection therapy to treat inflammatory disorders, due to the physiological and pathological damage the parasite causes in the liver. Therefore, as an alternative, the *F. hepatica* secretome has been interrogated in order to identify potential immune-modulatory molecules that could be produced recombinantly or synthetically to mimic the beneficial immune regulatory effects of a live worm infection. This approach has yielded successful outcomes in murine models of inflammatory disease, with beneficial effects in mouse models of allergy, arthritis, colitis, encephalomyelitis (EAE), sepsis and type I diabetes (TID) (Table 9.1). The therapeutic effects have been mediated by the entire native secretome and by single *F. hepatica* proteins such as cathepsin L, HDM, Fh15, Fh12 and GST (Donnelly *et al*., 2008; Robinson *et al*., 2012; Martin *et al*., 2015; Finlay *et al*., 2017; Ramos-Benitez *et al*., 2017; Aguayo *et al*., 2019). A number of observations in these studies included a significant decrease in clinical scores as a measure of disease severity (Table 9.1), while in the mouse model of sepsis, several molecules either reduced mortality or prolonged survival compared with untreated mice (Donnelly *et al*., 2010; Aguayo *et al*., 2019).

In most of these studies, protection was due to the preventive administration of the parasite molecules prior to, or at the onset of, disease induction (Table 9.1). However, when FhGST was administered after LPS-induced sepsis, the cytokine storm was significantly reduced, suggesting a therapeutic effect (Aguayo *et al*., 2019). Similarly, in a mouse model of house dust mite-induced asthma, the delivery of FhHDM (*F. hepatica* helminth defence molecule) at the time of challenge (after the sensitization phase) reduced lung inflammation and mucus production and improved measurements of lung function (Tanaka *et al*., 2018). This outcome contrasts with an ovalbumin (OVA) model of allergy, in which the administration of total FhES at the time of challenge was ineffective at reducing signs of allergy (Finlay *et al*., 2017). These differing outcomes illustrate the importance of testing the therapeutic properties of molecules before and after disease and pathology have developed, and that an understanding of immune-modulating mechanisms of each molecule can predict its therapeutic effect in the most appropriate disease indication.

A number of molecules that are released from the *F. hepatica* secretome exhibit immune modulatory properties with potential therapeutic benefits (Table 9.2). Studies mainly examined their interactions with mononuclear cells, macrophages and dendritic cells; that is, cells that are critical components in the development of immune-mediated diseases. These molecules share similar underlying immune properties, which include suppressing disease-specific cytokine and cellular responses, inducing regulatory macrophage phenotype, rendering innate immune cells hyporesponsive to TLR ligation and altering the processing and presentation of host

Table 9.1. Therapeutic properties of *F. hepatica* infection or its released molecules.

Disease	Treatment	Therapeutic property	Reference
Asthma	FhES	Protects mice against ovalbumin-induced asthma Protection is at the time of allergen sensitization but not allergen challenge. Reduced eosinophils and lymphocytes in alveolar lavage fluid. Supressed allergen specific Th2 immune responses and mucus secretion	(Finlay *et al*., 2017)
	FhHDM	Protects mice against experimental asthma Protection is at the time of allergen sensitization and challenge Reduced lung and tissue resistance Reduced lung inflammation and mucous secretion Reduced cellular infiltration	(Tanaka *et al*., 2018)
Arthritis	FhTE	Improved clinical score in mouse model of collagen-induced arthritis Reduced arthritis-specific Ig2a responses Inhibited IFNγ and IL-17 while it induced TGFβ, IL-10, and T-reg cells.	(Carranza *et al*., 2012)
	F. gigantica antigen	Ameliorated disease symptoms of experimental arthritis Suppressed serum TNF and IFNγ while it increased IL-4 and IL-5 MMPs and NO was inhibited in knee joint Decreased synovial hypertrophy and infiltration of polymorphonuclear cells	(Khan *et al*., 2015)
	FhHDM	Improved clinical score in mouse model of collagen-induced arthritis Protected against cartilage destruction Maintained bone mass and bone architecture of joints Suppressed CIA-induced expression of TNF, IL-17, and IFNγ in joints but not in serum levels. Did not alter CIA-induced suppression of T regulatory response.	(Khan *et al*., 2020)
Colitis	FhEVS	FhEVs supressed symptoms of DSS induced colitis.	(Roig *et al*., 2018)
EAE	FhES	Attenuated clinical symptoms of EAE Reduced Th1 and Th17 cells in brain tissue Protection dependent upon IL-33 and IL-5 while independent of IL-4, IL-10 and Tregs	(Finlay *et al*., 2017)
	FhHDM and FhCL1	FhCL1 did not ameliorate symptoms in an EAE mouse model. 50% of mice treated with FhHDM exhibited only one clinical attack while 30% were non-symptomatic.	(Lund *et al*., 2014)
	FhTE	Attenuated EAE by supressing pathogenic T-cell responses Reduced infiltration of neutrophils, inflammatory monocytes, γδ T-cells and Th17 cells	(Quinn *et al*., 2019)
Sepsis	FhHDM	Supressed LPS induced TNFα and IL-1β in sera and from peritoneal macrophages.	(Robinson *et al*., 2012)
	Fh12	Decreased cytokine storm as measured in sera (IFNγ, GM-CSF, IL-12p70, IL-3, IL-9, IL-10, IL-15, TNFα) in LPS induced sepsis in mice.	(Martin *et al*., 2015)
	Fh15	Decreased cytokine storm as measured in sera (IFNγ, IL-12p70, IL-3, IL-6, TNFα, IL-1β, MCP-1, MIP1α, MIP1β, KC) in LPS induced sepsis in mice. Peritoneal macrophages increased Reduced numbers of $CD38^{+}$ cells in the spleen.	(Ramos-Benitez *et al*., 2017)
	Cathepsin L1	Delayed mortality and reduced it by 30% Decreased cytokine storm as measure in sera and peritoneal lavage (IFNγ, IL-12p, IL-6, TNFα) in LPS induced sepsis in mice.	(Donnelly *et al*., 2010)
	GST (native)	Increased survival rate by 85% Suppressed cytokine storm (IFNγ, IL-12p70, TNFα, IL-2, IL-1β, MCP-1, MIP1α, KC, FGF basic, VEGF and IL-10) in LPS induced sepsis in mice	(Aguayo *et al*., 2019)
TID	FhHDM and FhCL1	FhCL1 did not prevent the development of type-1 diabetes in NOD mice. FhHDM protected 50% of NOD mice against Type-1 diabetes. Significant reduction of inflammation in the islets.	(Lund *et al*., 2014)

Table 9.2. The immune properties of purified native or recombinant *F. hepatica* antigens.

Molecule	Modulatory function	Reference
Cathepsin L1	Induced CD40, IL-6, IL-12p40, MIP2 and p38 expression in dendritic cells	(Dowling *et al*., 2010)
	Inhibited IL-23	
	Inhibited Th17 cells	
	Supressed Pro-inflammatory cytokines in macrophages	(Donnelly *et al*.,
	Blocked MYD88 TRF dependent signalling pathway	2010)
	Prevented the expression of and cleaved TLR3	
	Supressed Th1 immune responses to Th1 inducing vaccine	(O'Neill *et al*., 2001)
	Cleaved IgG antibody preventing antibody attachment of eosinophils	(Carmona *et al*., 1993)
Cathepsin L3	Induced NLRP3 inflammasome independent of caspase activation in dendritic cells	(Celias *et al*., 2019)
Fh12	Induced arginase, chitinase-3-like protein and IL-10 in human macrophages	(Figueroa-Santiago
	Inhibited nitric oxide synthase, TNFα, IL-12 and IL-1β	and Espino, 2014)
	In vitro: suppressed LPS and other TLR ligand induced cytokines secretion and expression of intracellular signalling molecules.	(Martin *et al*., 2015)
	Binds to CD14.	
Fh15	Decreased cytokine storm in LPS induced sepsis in mice	(Ramos-Benitez
	Increased peritoneal macrophages numbers	*et al*., 2018)
	Supressed CD38 expression on cells in the spleen	
	Suppressed TLR ligand stimulated TNFα and IL-1β production in murine macrophages and THP1 blue CD14 cells.	(Ramos-Benitez *et al*., 2017)
FhHDM	Inhibited activation of NLRP3 Inflammasome in macrophages by preventing acidification of the endolysosome	(Alvarado *et al*., 2017)
	Binds to LPS	(Robinson *et al*.,
	Protects mice against sepsis	2012)
	Anti-microbial activity	(Robinson *et al*.,
	Impeded macrophage antigen presentation	2012)
	Inhibited pro-inflammatory cytokines from macrophages	(Lund *et al*., 2014)
	Reduced immune cell infiltrate	
	Does not affect the proliferation of autoantigen-specific T-cells or production of cytokines	
	Macrophages had reduced TNF and IL-6	
	Inhibited RANKL-induced osteoclast formation	(Khan *et al*., 2020)
	Inhibited lysosomal acidification	
	Upregulation sequestome1/p62, a negative regulator of NF-κB expression	
	Suppressed RANKL production from osteoblasts	
	Suppressed ICAM-1 expression on macrophages	
FhTLM	Binds to TGF-β RII/R1 on macrophages and fibroblasts	(Sulaiman *et al*.,
	Induced regulatory phenotype in PBMCs (increased IL-10, arginase, PDL1 and reduced IL-12 and NO)	2016)
nGST (Native)	Suppressed TNFα and IL-1β in LPS primed macrophages	(Aguayo *et al*., 2019)
	Suppressed cytokine storm	
	THP1-blue CD14 supressed TLR intracellular molecules.	
rGST	Dendritic cells: ↑ CD40, IL-6, IL-12p40, MIP2 and ↑ PGE2	(Dowling *et al*., 2010)
	Suppressed IL-23	
	Inhibited Th17 cells	
	Induced JNK, NFκB, ERK and IRF5	
Kunitz	Suppressed pro-inflammatory cells from LPS activated DCs	(Falcón *et al*., 2014)
Mucin-Like Peptide	Promoted pro-inflammatory dendritic cells and Th1 immune responses	(Noya *et al*., 2017)
Peroxredoxin	Induced regulatory/M2 macrophages	(Donnelly *et al*.,
	↑ Fizz, Arg and Relm1	2005)
	↑ IL-10, PGE2	
	Promoted Th2 responses while Inhibited Th1 immunity	
	Induction of M2 macrophages independent of IL-4 and IL-13	(Donnelly *et al*.,
	Not dependent on enzyme activity	2008)

peptides by antigen-presenting cells (Table 9.1). In the case of cytokines, *F. hepatica*-derived molecules in general suppress IFN-γ, IL-17, TNF, NO and IL-12 that are associated with Th1- and Th17-mediated diseases such as sepsis (Donnelly *et al.*, 2010; Ramos-Benitez *et al.*, 2017; Aguayo *et al.*, 2019), EAE (Finlay *et al.*, 2017), arthritis (Khan *et al.*, 2020) and TID (Lund *et al.*, 2014), while in the mouse model of allergy Th2-specific cytokine responses were supressed (Finlay *et al.*, 2017). In some studies, there was a switch from Th1/Th17 cytokine profile to regulatory cytokines such as IL-10, TGFβ, IL-13, IL-4 and IL-5. In the mouse model of EAE, protection in mice was dependent upon IL-33 and IL-5 while independent of IL-4, IL-10 and Tregs (Walsh *et al.*, 2009). The C-terminal sequence of *Fasciola* helminth defence molecule-1 (C-FhHDM-1) suppressed levels of CIA-induced expression of TNF, IL-17, and IFN-γ in joints but not serum, suggesting a local rather than systemic suppressive effect (Khan *et al.*, 2020). These treatments also inhibited the migration of cells into diseased tissue, such as decreased infiltration of polymorphonuclear cells into the synovial fluid in a mouse model of arthritis (Khan *et al.*, 2015, 2020) and reduced infiltration of neutrophils, inflammatory monocytes, γδ T-cells and Th17 cells in brain tissue of EAE mice (Walsh *et al.*, 2009). Similarly in a mouse model of house dust mite-induced asthma, reduced cellular infiltration of lymphocytes, eosinophils and neutrophils was observed (Tanaka *et al.*, 2018), while C-FhHDM-1 suppressed ICAM-1 on macrophages, which could explain the reduced infiltration of these cells (Khan *et al.*, 2020).

M2 macrophages that are associated with *F. hepatica* infection are important in wound healing, tissue repair and regulatory mechanisms critical for parasite survival within the host (Orecchioni *et al.*, 2019; de Ruiter *et al.*, 2020). A number of stimuli, including Th2 cytokines, IL-4 and IL-13, adenosine released from epithelial cells and tissue-released collectins, can activate M2 macrophages (Inclan-Rico and Siracusa, 2018). The regulatory properties of these cells likely suppress the Th1/Th17 responses that mediate many inflammatory diseases. Peroxiredoxin is the first purified *F. hepatica* molecule to induce M2 macrophages as characterized by the expression of M2 genetic markers, namely Fizz1, Ym1 and Arg1 and high levels of IL-10 and prostaglandin E2. Peroxiredoxin-activated macrophages enhanced the secretion of IL-4, IL-5 and IL-13 from naive $CD4^+$ T cells (Donnelly *et al.*, 2005) and therefore promoted Th2 immune responses associated with *F. hepatica* infection. Administration of peroxiredoxin to $IL\text{-}4^{-/-}$ and $IL\text{-}13^{-/-}$ mice induced M2 macrophages independently of IL-4/IL-13 signalling, as confirmed *in vitro* as peroxiredoxin stimulated the expression of M2 markers in macrophages in the absence of these cytokines. Mutagenesis studies showed that the immunomodulatory property of peroxiredoxin is not due to its antioxidant activity, as an inactive recombinant could also induce M2 macrophages and Th2 responses (Donnelly *et al.*, 2008).

Fh12 and FhTLM are other *F. hepatica* molecules that induce M2-like phenotypes in human macrophages or monocytes. When human macrophages were stimulated with Fh12 a significant increase in arginase and chitinase-3-like protein expression was observed as well as an overexpression of IL-10 (Figueroa-Santiago and Espino, 2014; Sulaiman *et al.*, 2016). FhTLM, a growth factor belonging to the TGF superfamily bound to TGF-β RII, initiating the Smad2/3 pathway and activating a regulatory phenotype in human monocytes, as measured by elevated IL-10 and arginase-1, while expressing low levels of IL-12 and nitric oxide. The inhibitory receptor PD-L1 and mannose receptor were also elevated and the expression was lost following TGF-β RII mRNA knockdown (Sulaiman *et al.*, 2016).

Cathepsin L1, a protease secreted by the adult stages of infection that is important for nutrient acquisition and immune evasion, protected mice in a model of sepsis but not experimental autoimmune encephalomyelitis (EAE) or type-1 diabetes (TID) (Donnelly *et al.*, 2010; Lund *et al.*, 2014). Interestingly, cathepsin L does not induce M2 macrophages, which could explain its lack of therapeutic effect in mouse models of TID and EAE and highlights the importance of understanding the molecules' immune modulatory mechanisms (Lund *et al.*, 2014). Instead, cathepsin L1 prevents the release of NO, IL-6, TNF and IL-12 from M1 classically activated macrophages by specifically blocking the TLR4 and TLR3, MyD88-independent, TRIF-dependent signalling pathway through the degradation of TLR3 within the endosome

(Donnelly *et al*., 2010). In general, M1 macrophages infiltrate disease tissue associated with autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease and type-1 diabetes (Ma *et al*., 2019). M1 macrophages are activated by IFNγ and TLR ligands such as lipopolysaccharides (LPS). These pro-inflammatory macrophages secrete cytokines such as TNF, IL-1β, NO and IL-12 and express the full range of co-stimulatory molecules (CD80, CD86, MHCII and CD40) that promote Th1 cells (Orecchioni *et al*., 2019). In addition to cathepsin L, studies have shown that *F. hepatica* molecules such as Fh12 inhibit the production of nitric oxide (NO) and nitric oxide synthase expression as well as inhibiting TNF, IL-12 and IL-1β, in macrophages stimulated with LPS (Figueroa-Santiago and Espino, 2014; Martin *et al*., 2015). Similar properties were displayed by GST, FhTLM, FhHDM and Fh15, pointing to an important immune evasion mechanism employed by the parasite which would explain why, as infection progresses, Th1 immune responses are suppressed (Robinson *et al*., 2012; Sulaiman *et al*., 2016; Ramos-Benitez *et al*., 2018; Aguayo *et al*., 2019).

In macrophages, the processing and presentation of host peptides in conjunction with MHCII is important during immune-mediated diseases to activate adaptive immune responses and thereby produce autoreactive responses (Ma *et al*., 2019). FhHDM inhibits the activation of cathepsin-like lysosomal proteases – enzymes that act to process internalized antigen to smaller peptides for presentation to $CD4^+$ T cells. FhHDM binds to lipid rafts in the cell membrane through selective interaction with phospholipids and following internalization, and is cleaved by lysosomal proteases to release a short C-terminal peptide that subsequently inhibits the proton pump, vacuolar ATPase activity. This inhibition raises the pH of the lysosome, which prevents the activation of the lysosomal proteases, such as cathepsin B, from their inactive zymogens to active mature enzymes (Alvarado *et al*., 2017). Inactivation of lysosomal cathepsin B prevents formation of the NLRP3 inflammasome in response to inflammatory signals, which results in reduced IL-1β secretion by the FhHDM-treated macrophages (Alvarado *et al*., 2017). This suppression of IL-1β production has been hypothesized to inhibit the development of Th1 immune responses, which ensures *F. hepatica* survival within the host (Alvarado *et al*., 2017).

Some studies have examined the immune properties of *F. hepatica* antigens on dendritic cells given that they are important components of many immune-mediated diseases (Motran *et al*., 2017). Cathepsin L3 is released by NEJs and is important in their migration through the gut in the early stage of infection. This molecule promotes the NLRP3 inflammasome independently of the classical mechanism involving caspase activation (Motran *et al*., 2017). However, this process was dependent on protease activity of FhCL3 and the presence of the NLRP3 receptor. The production of NLRP3-dependent IL-1β and IL-18 in dendritic cells prompted the secretion of IFN-γ and IL-13 when these cells were co-cultured with $CD4^+$ cells, supporting a role for FhCL3 in the induction of the early mixed Th1/Th2 immune responses observed in the initial stages of *F. hepatica* infection (Brady *et al*., 1999; O'Neill *et al*., 2000). The mucin-like peptide promotes pro-inflammatory cytokines and Th1 immune responses in dendritic cells (Noya *et al*., 2017) while, in contrast, the serine/cysteine protease inhibitor Kunitz suppresses the release of pro-inflammatory cytokines from dendritic cells. Similarly, cathepsin L and GST induce partially activated dendritic cells that cannot produce IL-23 in responses to LPS. When cells were activated with these molecules and then adoptively transferred into D11.10 mice, a reduction in IL-17 was observed in $CD4^+$ cells in response to OVA peptide (Dowling *et al*., 2010). Th17 cells are implicated in the pathogenesis of many immune-mediated diseases and the targeting of effector cytokines such as IL-17 by *F. hepatica*-released products supports these molecules as potential treatment for immune-mediated diseases (Yasuda *et al*., 2019).

F. hepatica-secreted molecules can also have non-immune function that is important in managing pathogenesis associated with diseases such as arthritis. Osteoclasts have a critical role in the formation of arthritic joints, since disease is associated with alterations in osteoblast growth, differentiation and activity (Corrado *et al*., 2017). One study has shown that C-FhHDM-1 suppressed osteoblasts RANKL production, which inhibits osteoclast formation and lysosomal acidification. It also upregulates sequestome1/p62, a negative regulator of NF-κB expression.

C-FhHDM-1 improves clinical scores in a murine model of collagen II-induced arthritis, protecting against cartilage destruction and maintaining bone mass and bone architecture (Khan *et al.*, 2020). It is also important that, when examining these molecules, we should look for therapeutic benefits that are outside the realm of the immune response.

9.6 Conclusions and Future Directions

Within the definitive host, *F. hepatica* migrates through different anatomical locations, encountering a range of tissue types and distinct immunological compartments (Mas-Coma *et al.*, 2019). The pathology associated with infection is caused by the parasites migrating from the gut mucosa through the peritoneal cavity and liver tissue before taking up residence for many years in the bile duct and gallbladder (Cwiklinski *et al.*, 2016). The mature egg-producing flukes feed on host cells accessed through the bile duct wall, causing the pathology associated with the chronic stages of infection. The tissue damage caused by migrating flukes, or flukes feeding on host cells, in addition to parasite-released modulatory molecules, triggers the local inflammatory responses (Molina-Hernández *et al.*, 2015). When examining the immune response to the parasite, much of the analysis in the natural host has focused upon blood PBMCs, which are distal from the infection site, as well as local responses in the liver tissue, hepatic lymphocytes and lymph nodes (McCole *et al.*, 1998; Molina and Skerratt, 2005; Zafra *et al.*, 2010). However, in experimental animals it is also possible to examine immune responses in the peritoneal cavity as flukes migrate from the gut to the liver (Donnelly *et al.*, 2005, 2008).

Unfortunately, there are few studies in humans, leaving a major gap in the literature; however, evidence from the limited studies available suggests that infected individual humans, like ruminants, exhibit Th2/T-regulatory immune responses (Cwiklinski *et al.*, 2016). Cattle, sheep, goats and experimental rodent models exhibit similar immune responses characterized by initially mixed Th1/Th2 cells in the early stages of infection followed by biased Th2 response that ultimately leads to tolergenic/anergenic and regulatory T-cell responses associated with chronic stages of infection (Fig. 9.1). Studies support a role for IL-10 and TGFβ cytokines in initiating these regulatory adaptive immune responses during infection (Flynn and Mulcahy, 2008b; Japa *et al.*, 2015). There are many similarities in immune responses between species that point to the importance of Th1 immune responses, in particular IFNγ and IgG1, for immune protection (Clery *et al.*, 1996; O'Neill *et al.*, 2000) (Table 9.3). Mouse models are critical for examining immune responses in the peritoneal cavity, because this is difficult to achieve in the natural host. Studies have demonstrated enhanced cellular infiltration of M2a- and M2c-like macrophages as well as regulatory DCs that can induce biased Th2 and Treg cells associated with *F. hepatica* infection (Donnelly *et al.*, 2005; Walsh *et al.*, 2009).

Since the immune responses between ruminants and experimental rodent models are very similar, these models provide invaluable knowledge on the immune responses associated with *F. hepatica* infection, particularly the use of genetically modified animals when examining the phenotype of individual cell populations during infection as well as the interaction of *F. hepatica* molecules with immune cells (Fig 9. 1, Table 9.2). The continued improvement of these models with the introduction of genetically modified species will likely broaden their usefulness (Fig. 9.1). For example, the development of genetically modified bovinized or ovinized mice would facilitate the more complex studies required to understand the genetic and immunological components that are important to host immunity. Humanized mouse models have already enhanced our knowledge of human parasites; this would be an excellent starting point in the absence of large-scale studies in human populations. These models are also useful tools for assessing anti-parasitic interventions (Tao and Reese, 2017). An alternative option is the genetic manipulation of cattle leucocytes to examine the role of immune cells during infection. Kennedy *et al.* (2002) examined the modulation of immune responses to *Mycobacterium bovis* in cattle depleted of WC1(+) gamma delta T cells and in the future the use of genetically modified ruminants is also a viable option.

While there are some differences in immune responses between experimental and natural

infections, the result is the same, i.e. the establishment of a chronic infection and a potentially non-responsive immune state (Fig. 9.1). This may explain the general lack of success with vaccine programmes, as the tolerant state induced by exposure to *F. hepatica* would essentially render a vaccine ineffective by preventing the induction of protective immune responses. Without complete elimination of the parasite, any worms that reach the liver stage in vaccinated animals would effectively switch off the ability of memory T cells to respond to parasite antigen, thus disabling the vaccine-induced immune response. As antigen-specific responses in T cells from infected cattle were restored by the addition of IL-2 and neutralization of IL-10 and TGFβ (Sachdev *et al*., 2017), perhaps a similar approach should be considered in the formulation of adjuvants in future trials. In addition, vaccines that target the gut mucosa could prevent infection at the stage associated with extensive tissue damage.

The dearth of knowledge on the immune response in the gut and peritoneal cavity in the natural hosts infected with *F. hepatica* is a barrier to the development of a mucosal vaccine. These studies are critical to the development of an effective vaccine strategy that ideally blocks fluke migration at the level of the gut. Only one study has examined immune responses within the gut tissues during infection in rodents, where at 4 weeks pi cellular immune responses included IgE positive cells, granulocytes (mainly eosinophils), erythropoietin (EPO) positive cells and mucosal mast cells (Van Milligen *et al*., 1998). Few studies examined the role of mast cells during infection and a study in mice has shown increased numbers in the peritoneal cavity and liver but not the gut of *F. hepatica*-infected mice; the lack of knowledge warrants further studies examining this cell population (Vukman *et al*., 2013). Other studies have reported the release of IgA from liver plasma cells into the bile duct to the gastrointestinal tract (Hughes *et al*., 1981; Ferre *et al*., 1997), which may be protective and requires further studies.

Studies examining the effect of different parasite isolates, animal breed and gender on disease severity and outcome are warranted, in particular examining the host immune responses associated with host susceptibility and resistance when considering these factors. It is known

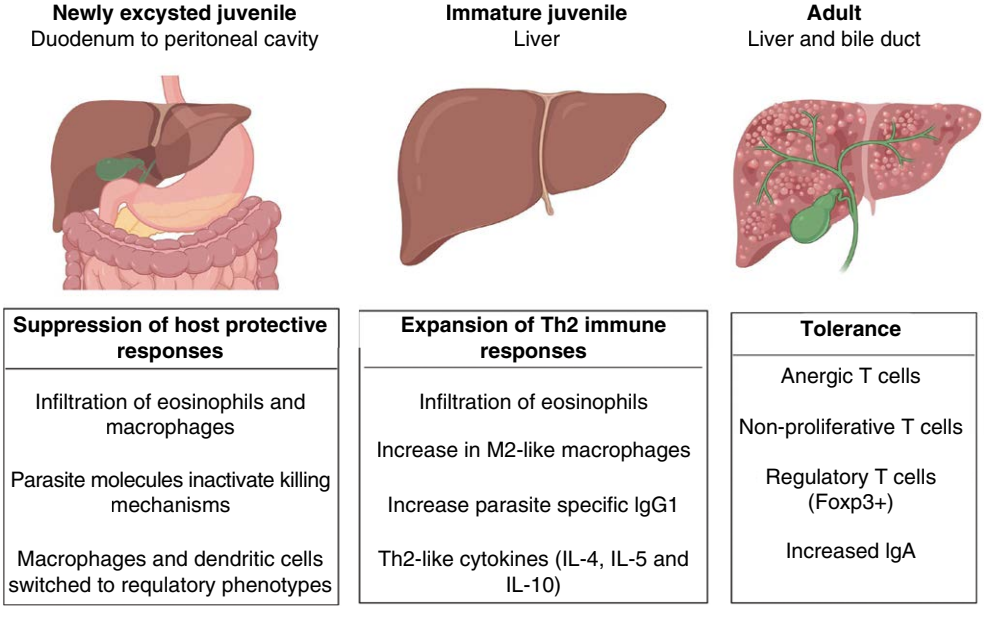

Fig. 9.1. Illustration to summarize the key immune responses observed in ruminants and rodents infected with *F. hepatica* in different anatomical locations as the parasite migrates through various tissues. (Figure created using Biorender.)

Table 9.3. Summary of immune responses measured in different species during *F. hepatica* infection (↑ increased; ↓ proliferation)

	Cattle	Sheep	Goats	Rats	Mice
Gut	No data	No data	No data	↑ Eosinophils, EPO positive cells ↑ Granulocytes ↑ IgE positive cells ↑ Mucosal masts cells	No data
Peritoneal cavity	No data	↑ Eosinophils ↑ Granulocytes ↑ Macrophages ↑ Neutrophils ↑ NO, H_2O_2	No data	↑ Eosinophils ↑ IL-10 ↑ Macrophages, ↑ Neutrophils ↑ NO & H_2O_2 in peritoneal cells	↑ M2a cells, ↑ Regulatory DCs ↑ Eosinophils, ↑ Macrophages, ↑ Neutrophils ↑ Treg cells ($CD25^+$, TGF^+, $IL\text{-}10^+$, $IL\text{-}10r^+$, $CTLA4^+$, $CCR5^+$, T1/TS2+)
Serum	↑Eosinophils, ↑TGF, IL-10, IL-13 ↑IgM, IgG1 CD4 cells ($CTLA4^+$/$PD\text{-}1^+$)	↑ IgG1, IgM	No data	↑IL-1β, IL-5, IL-13, IFNγ, IL-4 ↑Eosinophils	↑IgG1, ↑TGF, IL-4.
PBMCs	↓ Proliferation ↓ IL-4, ↓IFNγ	↑ Eosinophils ↑ TGF, IL-10, IFNγ ↓ NOS2, IL-18, IL-12 ↓ Proliferation	↑ proliferation ↓ oxidative responses	No data	No data
Spleen	No data	No data	No data	↑IL-10, IL-4 IFNγ ↓IL-2, ↓proliferation	Th2, Treg cells ↑ IL-10, IL-4, IL-5, IL-13
Lymph nodes	Th1 and Th2 clones ↑ IL-10, ↓IL-2r ↑ CTLA4/PD-1	↑ Proliferative Plasma B-cells ↑ CD3, $CD4^{+Foxp3+}$ ↑ IL-4, IFNγ	↑ CD3, $CD4^{+Foxp3+}$ ↑ γ/δ T-cells ↑ B-cells (IgM^+, lambda-IgG^+ or $CD79^+$)	↑IL-5, IL-10, IL-4, IFNγ.	↑ Th2, Treg cells ↑ IL-10, IL-4, IL-5, IL-13. ↑$CD4^+CD25^+$Foxp3+ cells
Liver	↑Eosinophils ↑Mast cells, ↑Granulocytes, ↑$CD68^+$ macrophages, ↑$CD163^+$ macrophages, ↑IgA producing cells	↑ Macrophages ↑ Eosinophils ↑ CD3, $CD4^{+Foxp3+}$	↑ Macrophages ↑ Eosinophils ↑ CD3, $CD4^{+Foxp3+}$ ↑ Plasma cells ↑ γ/δ T-cells ↑ B-cells (IgM^+, lambda-IgG^+ or $CD79^+$)	No data	↑Eosinophils ↑Neutrophils ↑Macrophages ↑Lymphocytes ↑Plasma Cells ↑IL-5, IL-10, IL-4, IFNγ

that even within a single host species, breed differences in responsiveness and susceptibility can exist, as there are also indications of differences at a transcriptomic level between breeds of cattle (Garcia-Campos *et al*., 2019). This must be more extensively investigated in order to establish if some breeds are more resistant to infection.

The dose and strain of metacercariae can affect the immune response observed during *F. hepatica* infection (Martínez-Moreno *et al*., 1997; Brady *et al*., 1999), but while the strain of metacercariae influenced infection outcome in rats, these differences were not linked to host immune responses (Hanna *et al*., 2011). The development of well-characterized *F. hepatica* isolates with in-depth genomic background should allow for *in vivo* comparisons of between- and within-host virulence (Alvarez Rojas *et al*., 2015).

Wesolowska *et al*. (2018) compared the impact of gender on *F. hepatica* infection by examining $CD4^+$ and $CD8^+$ cells in peritoneal fluid, MLN, and HLN of male versus female Sprague Dawley rats. While in general there was no change in the number of $CD8^+$ cells, there was a significant decrease in $CD4^+$ cells in the peritoneal cavity and HLN for both males and females, with significant differences observed between the two groups. This study measured metabolic activity as an indication of cell growth in cells isolated from blood, peritoneal fluid and HLN and demonstrated a decrease in metabolic activity in infected male rats only at all sites tested, while no changes were observed in infected female rats (Wesołowska *et al*., 2018). Metabolic activity can be linked to specific immune responses; in particular, different classes of cell phenotype can be associated with specific metabolic signatures (Viola *et al*., 2019). The existence of sexually dimorphic innate responses has already been identified in cattle, which underlies the importance of these comparisons in fluke infection (Wesołowska *et al*., 2018).

There have been significant advances in the development of *F. hepatica* vaccines, with many potential targets identified (Molina-Hernández *et al*., 2015). Given the focus to develop an affordable commercial vaccine, many of these target molecules were produced in recombinant systems to facilitate potential upscaling for large-scale vaccine production. We now understand the immune modulatory properties for many of these vaccine targets and, therefore, a promising derivative of these studies is the potential to develop this panel of molecules as therapeutics for a broad range of inflammatory diseases (Walsh *et al*., 2009; Robinson *et al*., 2012; Lund *et al*., 2014; Finlay *et al*., 2017; Aguayo *et al*., 2019). Studies examining *F. hepatica* bio-therapeutic molecules to date have focused upon preclinical murine models, which have shown promise; therefore, it is only a matter of time before these molecules progress to human clinical trials. Many of the molecules are proteins derived from adult parasite stages; however, there are many NEJ molecules that have yet to be characterized as well as other compounds like *F. hepatica*-derived miRNAs and glycans. The tegumental coat that surrounds the fluke is a rich source of oligosaccharides that were recently characterized (Garcia-Campos *et al*., 2016; Ravidà *et al*., 2016). While they show good immune modulatory properties (Rodríguez *et al*., 2015; Ravidà *et al*., 2016), the precise glycan structures that could be exploited as vaccine or therapeutics have yet to be identified.

We have made significant advances in the study of *Fasciola* immune responses, but what is lacking is a comprehensive characterization of cellular phenotypes during the course of infection. To date, studies have produced data that used a limited antibody panel specific to particular cell phenotypes. In the ruminant host this is due to the lack of available antibodies, a scenario that will change in the near future as more antibodies are brought to the market annually. However, many of the cell markers used are expressed by more than one cell type, so the data obtained to date does not provide a full picture of the complexity of the immune responses associated with infection. With the recent development of multi-parameter immune profiling by single-cell mass flow cytometry, in the future cells can be evaluated using a unique repertoire of cell markers using several antibodies together, each coupled with different fluorochromes. This will shed light on the full complexity of the cell populations associated with infection and will facilitate the monitoring of cell plasticity over time as infection progresses (Böttcher *et al*., 2019; Winkler and Bengsch, 2019). These advances will ensure that in the next 10 years, we will have a more comprehensive understanding of host immune response associated with *F. hepatica* and its

secreted molecules, which is critical if we are to advance our understanding of host–parasite interactions and to develop effective vaccines and diagnostics to prevent and manage infected populations. Furthermore, understanding these mechanisms will accelerate these molecules along the drug development pipeline in order to treat a range of inflammatory disorders.

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10 Diagnostics for Animal and Human Fasciolosis

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10.1 Introduction

Sensitive and specific methods for the diagnosis of fasciolosis are required not only to determine the presence or absence of the disease in an individual (or in a collective) but also to evaluate the efficacy of treatments in humans and animal species. Ideally, diagnostic methods should detect the infection early to avoid severe damage of the liver tissue (Tantrawatpan *et al.,* 2005) and the spreading of eggs to the environment, particularly those harbouring drug-resistant *Fasciola* strains (Hotez *et al.,* 2007; Fairweather *et al.,* 2020). It is also advisable that diagnostic methods can detect fasciolosis at all stages of infection.

Over the past decades, many investigations led to the development of increasingly fast and more sensitive methods for the diagnosis of animal and human infections, although no method covers all possible scenarios. This is mainly due to limitations imposed by (i) the complexity of the *Fasciola* biological cycle in definitive hosts; (ii) the intensity of infection; (iii) the existence of two species (*F. hepatica* and *F. gigantica*) with different geographical distribution but also with some overlapping areas; (iv) history of reinfections and previous flukicide treatments; (v) access limitations to commercial kits in some countries; and (vi) cultural/ancestral human traditions, which make the collection of some samples difficult.

Regarding human infections, four clinical phases, stages or periods (incubation, acute, latent and chronic) were historically reported (Chen and Mott, 1990; Espino and Dumenigo, 2003). Ideally, diagnosis of fasciolosis during the incubation phase or at the early acute stage is preferred, to minimize liver damage, but this task is difficult for the following reasons:

- The duration of these phases varies considerably among patients.
- About half of acute infections are asymptomatic (Ashrafi *et al.*, 2014; Cwiklinski *et al.*, 2015).
- Even in symptomatic patients, findings are unspecific and many patients are not diagnosed until they are in the chronic phase (Cabán-Hernández *et al.*, 2014).

For these reasons, a careful anamnesis is essential to guide clinicians to suspect fasciolosis, particularly in non-endemic developed countries where the diagnosis of sporadic cases of human fasciolosis is challenging (Cabada and White,

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2012; Patel *et al.*, 2016). In contrast to humans, the absence of symptomatology is the rule in livestock fasciolosis, although acute infections with sudden death can be seen in very sensitive host species, such as sheep, after ingestion of a massive dose of metacercariae. On the other hand, it is not possible to eliminate fasciolosis in most farms; thus, the aim is to maintain low infection levels that do not compromise animal welfare, health and productivity. Therefore, diagnostic tests are carried out under a schedule determined by farm-specific factors such as the animal species, type of production, physiological status and herd grazing history.

Due to the diversity of diagnostic scenarios and possible protocols, in this chapter we focus on the basis and usefulness of the reported methods for diagnosis of fasciolosis, correlating them with the pathological events that occur along the time-course of the infection.

10.2 Methods for Diagnosis of Fasciolosis at the Early Stage of Infection

This section deals with diagnostic methods capable of detecting *Fasciola* infections early, i.e. within the first 4–5 weeks post-infection (pi). This period encompasses the time required by the newly excysted juveniles (NEJs) to reach the peritoneal cavity (24–72 h, depending on the animal species) (Espino and Dumenigo, 2003), the peritoneal migration of young flukes to the liver (48–90 h) and the first 3–4 weeks of hepatic migration, which corresponds to the early stage of acute infection (Marcos *et al.*, 2008). From the pathogenesis perspective of the disease, this period normally falls within the incubation phase in humans, which was estimated to be 6 weeks on average (Chen and Mott, 1990; Espino *et al.*, 1998), although a broad range of 0.5–22 weeks was also reported (Marcos *et al.*, 2008). As clinical symptoms are infrequent at this stage, early acute primary *Fasciola* infections will frequently be unnoticed. However, either if disease is suspected in humans, or when it is expected in livestock management, early diagnosis of fasciolosis is feasible using mainly immunological methods.

10.2.1 Detection of circulating antigens

Several ELISA methods were developed to detect circulating *Fasciola* antigens released by juvenile flukes of either *F. hepatica* (Espino *et al.*, 1997; Dumenigo *et al.*, 2000) or *F. gigantica* (Anuracpreeda *et al.*, 2013; Kueakhai *et al.*, 2015, 2019). These methods gained early interest for the following reasons:

- They are able, *a priori*, to detect the infection very early (from the first or second week pi).
- They are highly specific, mainly when monoclonal antibodies (mAbs) are used for the design of capture ELISAs (cELISAs).
- They detect active infections.

However, they also showed important inconveniences:

- Most of the reported methods gave poor signal-to-noise ratios even when animals were infected with a high number of metacercariae (Langley and Hillyer, 1989; Rodriguez-Perez and Hillyer, 1995; Dumenigo *et al.*, 2000).
- At least for *F. hepatica* infections in sheep, the ELISA optical density (OD) signal becomes negative after approximately 4–6 weeks pi (Dumenigo *et al.*, 2000).
- Most of them are in-house methods only available for use in the laboratory or country where they were developed.
- They were not evaluated by independent researchers.

Among these limitations, the recording of negative OD signals at 4–6 weeks after infection is the most serious drawback, since such tests may give false negative results. Low OD signals can be attributed to old unsuitable ELISA designs in some studies; for example: (i) the use of the same unlabelled rabbit anti-*Fasciola* polyclonal antibodies for capture and detection of circulating antigens (Sanchez-Andrade *et al.*, 2001); or (ii) direct coupling of the serum sample containing the target circulating antigens to the wells of ELISA plates (Guobadia and Fagbemi, 1996). Importantly, it should be noted that the amount of antigens released into circulation by young flukes is expected to be very low, since trickle infections are the rule in field conditions and young flukes only reach 3–4 mm in length by week 4 pi (Behm and Sangster, 1999). A low

antigen release, together with a dilution factor imposed by the volume of the host-animal blood, may explain why the detection of early circulating antigens by sandwich ELISA gives higher OD signals in rats (Espino *et al*., 1997) compared with sheep (Dumenigo *et al*., 2000). Nevertheless, low OD signals and a tendency to become negative over time were also observed in mice targeting cathepsin B3, which is only expressed in metacercariae, NEJs and juvenile flukes (Anuracpreeda *et al*., 2013).

A drastic fall in the concentration of circulating antigens in serum from infected animals is expected to occur when the flukes migrate from the liver parenchyma to the bile ducts, since at this stage only a small amount of regurgitated/tegumental antigens has the opportunity to re-enter into the circulation (for example, through lesions induced at the fluke feeding site or by absorption through the biliary epithelium). However, this fact cannot explain why ELISA methods fail to detect *F. hepatica* circulating antigens during the late parenchymal migration of flukes (i.e. between weeks 4 and 8 pi). Therefore, other causes should be considered, such as: (i) the formation of circulating immune complexes and their continuous removal from circulation by phagocytic cells (Lu *et al*., 2018); and (ii) antibody competition between circulating antibodies and the capture/detection antibody pairs used in cELISAs.

ELISA methods were also reported to detect circulating antigens in mice, livestock and humans infected by *F. gigantica* (Guobadia and Fagbemi, 1997; Velusamy *et al*., 2004; Demerdash *et al*., 2011; Attallah *et al*., 2013; Anuracpreeda *et al*., 2016; Kueakhai *et al*., 2019). From these studies, it seems that circulating antigens released by *F. gigantica* during primary experimental infections remain detectable in sera for more time than for *F. hepatica*, which could be related to the higher pathogenicity of this species (Valero *et al*., 2016) and the longer duration of its parenchymal phase (Valero *et al*., 2009). In this regard, methods with high specificity and sensitivity were reported for testing blood samples from naturally or experimentally infected cattle (Anuracpreeda *et al*., 2013; Anuracpreeda *et al*., 2016) and humans (Demerdash *et al*., 2011; Attallah *et al*., 2013). Unfortunately, as for *F. hepatica*, most of the reported ELISAs have poor signal-to-noise ratios and have relatively high backgrounds, which can compromise their performance, especially under field conditions. Moreover, regardless of the technical limitations, immunological methods for detection of circulating antigens in cases of human and animal fasciolosis (either by *F. hepatica* or by *F. gigantica*) are of limited usefulness, due to the absence of clinical symptoms at early stages, which makes the illness difficult to suspect during the first weeks pi. Also, it has not been demonstrated that such methods are able to detect early low-burden primary infections in livestock (e.g. one to five flukes), as experienced with available methods for detection of coproantigens (see below).

10.2.2 Detection of anti-*Fasciola* antibodies in serum or plasma

As for circulating antigens, antibodies to *Fasciola* antigens can be detected early, starting by week 2–3 post-primary infections in most susceptible hosts (Fig. 10.1), which contrasts with the 8–12 weeks required to find parasite eggs in faeces (see below). Methods for detection of antibodies in serum are very attractive for the following reasons:

- They are not fully dependent on the infection burden, as antibodies are produced even in cases of low infections (Mezo *et al*., 2010a).
- The samples (normally serum or plasma) are easy to obtain, handle and store in the majority of laboratories.
- They are suitable for high-throughput formats.
- They can be used to detect early primary infections.
- Antibodies are present during the entire time-course of fasciolosis, for a long time after infection, and antibody titres increase during reinfections.
- Due to antigenic cross-reactivity, some methods can be used to detect antibodies induced by *F. hepatica* and *F. gigantica*.
- Excellent signal-to-noise ratios with clear backgrounds can be obtained for many target antigens.
- In general, they are fast, inexpensive, simple and clean, which favours their use in routine diagnosis and research.

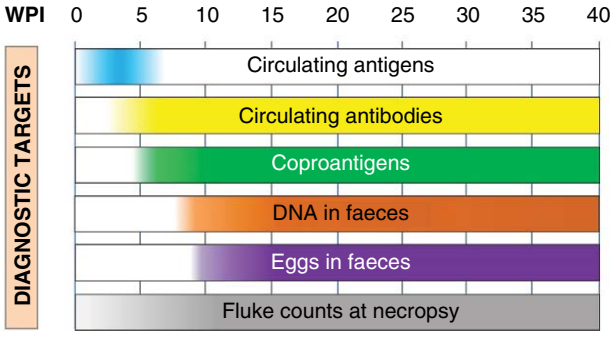

Fig. 10.1. Graphic representation showing the applicability of different diagnostic methods throughout the time-course of a primary *F. hepatica* infection in the sheep model. Coloured areas represent weeks post-infection (WPI) when each method is positive. The represented periods change depending on the infecting species (*F. hepatica* or *F. gigantica*), the host species, the intensity of the infection, or the existence of reinfections. Other laboratory findings of major interest in human infections such as eosinophil counts and hepatic enzymes levels were not represented, because eosinophilia is too common in grazing animals, usually parasitized by other helminths, and hepatic enzymes are not routinely measured in livestock.

However, methods to detect antibodies also have some important drawbacks:

- Even in primary infections, antibody titres do not correlate with parasite burden.
- They are unsuitable to follow up the efficacy of flukicide treatments, as antibodies take a long time to return to basal levels.
- They are not adequate to reveal human or animal reinfections after a flukicide treatment, as frequently occurs in endemic areas.
- In some designs, it is necessary to use different secondary antibodies to detect infections according to the host species and antibody isotypes.
- They may be influenced by the administration of previous vaccines sharing epitopes with target antigens.
- Some of them can be affected by cross-reactive natural antibodies present in sera from non-infected hosts, which may react with the target antigen, with proteins included in blocking solutions, or with any other antibody used in the diagnostic procedure (e.g. anti-species antibodies).
- They are not appropriate for diagnosing infection in young animals being fed with colostrum from infected dams, since colostrum antibodies persist for at least 3 months in blood.
- If required, no methods have yet been reported that can differentiate infections by *F. hepatica* and *F. gigantica*.

Early methods for detection of anti-*Fasciola* antibodies included complement fixation tests, precipitation reactions (e.g. double immunodiffusion, immunoelectrophoresis), immunofluorescence and indirect haemagglutination (reviewed by Chen and Mott, 1990; Mas-Coma *et al.*, 2014a). However, these methods were progressively relegated after the introduction of the Western blot (WB) technique and, more particularly, by modern ELISAs. Initially, WB was proposed as a confirmation method to be used in combination with ELISA (Hillyer, 1999). However, since WB is more laborious, more expensive and less amenable than ELISA for testing multiple samples in parallel, this technique is mainly restricted to laboratory-based research. Still, there is an available commercial WB (*Fasciola* ES WB IgG for serodiagnosis of human infections; LDBIO Diagnostics, Lyon, France). Consequently, during the past three decades, most improvements on the serodiagnosis of human and animal fasciolosis were made in the development and refinement of ELISA techniques. In this sense, researchers have focused on the evaluation of new native and recombinant antigens to improve specificity of ELISAs, as well as on the development of other serological methods such as

lateral flow immune assay (LFIA) rapid tests for point-of-care use.

Most of the proposed serological methods to detect human and animal infections by *F. hepatica* and *F. gigantica* are based on the detection of specific antibodies of the IgG isotypes, as they predominate in human and animal infections; for example, IgG1 and IgG4 isotypes in humans (O'Neill *et al*., 1998) and IgG1 in other species (Clery *et al*., 1996; Mulcahy *et al*., 1999; Phiri *et al*., 2006). Additionally, a great number of target antigens were reported to achieve combined values of specificity and sensitivity higher than 95% in ELISA (reviewed by Álvarez-Rojas *et al*., 2014). Below, we review some of the target antigens with the highest potential for serodiagnosis of human and animal fasciolosis, emphasizing those that have been evaluated in more than one laboratory or that are included in commercial diagnostic kits.

10.2.2.1 Methods using excretory/secretory (ES) antigens

Some serological diagnostic tests use ES antigens from *F. hepatica* or *F. gigantica* as targets, since they exhibit better specificity than whole-parasite extracts. Although with variations between laboratories, the bulk of *Fasciola* ES antigens are normally produced using cleaned adult flukes (obtained from livers of infected sheep or cattle) placed in a cell culture medium supplemented with antibiotics (with or without addition of protease inhibitors) and incubated at 37°C for 6–24h in a CO_2 incubator (Espino and Dumenigo, 2003; Mezo *et al*., 2007). The *Fasciola* ES fraction comprises a complex mix of antigens regurgitated by the parasite through the oral sucker, but may also contain some tegumental antigens – antigens that are released through the tegument surface (e.g. MF6p/FhHDM-1 protein) (Martinez-Sernandez *et al*., 2011, 2017) and some host proteins (Morphew *et al*., 2007). Two-dimensional gel electrophoresis isolation and further identification of *F. hepatica* ES products by peptide mass fingerprinting revealed about 60 proteins, of which cathepsin Ls (CLs) are the majority (Jefferies *et al*., 2001).

Numerous in-house indirect ELISAs (iELISA) targeting *Fasciola* ES antigens for serodiagnosis of fasciolosis were developed over several decades with reported values of sensitivity and specificity ranging between 95–100% and 97–100% for humans (Sampaio-Silva *et al*., 1996; Carnevale *et al*., 2001a; Rokni *et al*., 2002) and between 72–100% and 70–99.3% for cattle and other ruminants (Ferre *et al*., 1995; Anderson *et al*., 1999; Cornelissen *et al*., 1999; Bossaert *et al*., 2000; Salimi-Bejestani *et al*., 2005a; Charlier *et al*., 2008; Kuerpick *et al*., 2013; Mazeri *et al*., 2016). Regarding specificity, some false positives were reported using sera from animals or individuals infected with other helminths (Hillyer *et al*., 1992; Cornelissen *et al*., 1999; Bossaert *et al*., 2000; Mezo *et al*., 2007; Figueroa-Santiago *et al*., 2011). Independent evaluations of these in-house methods reported similar values of sensitivity and specificity (Valero *et al*., 2012a; Munita *et al*., 2019). Despite these potential specificity issues, some ELISA tests targeting *F. hepatica* whole ES antigens are commercially available, for example the DRG *Fasciola* IgG ELISA kit (DRG Instruments GmbH, Marburg, Germany), intended for serodiagnosis of human infections by *F. hepatica* and *F. gigantica*, and SVANOVIR® *F. hepatica*-Ab (Boehringer Ingelheim Svanova, Uppsala, Sweden), for detection of antibodies in cows using serum, meat juice or milk.

10.2.2.2 Methods using purified native antigens

Besides their usefulness as target antigens in iELISAs, ES antigens from *Fasciola* are also frequently used as the starting material for antigen purification. Indeed, early WB studies carried out with ES antigens already recognized bands of 12, 17, ~23–30 and 63 kDa as specific targets for serodiagnosis of fasciolosis (Santiago and Hillyer, 1988; Hillyer *et al*., 1992; Sampaio-Silva *et al*., 1996; Bossaert *et al*., 2000; Escalante *et al*., 2011).

Among the great variety of proteins present in ES antigens, cysteine proteases are of outstanding interest as target for immunodiagnosis since they contain immunodominant epitopes, they are expressed during all developmental stages in definitive hosts (Dalton *et al.,* 2003) and they are recognized both by humans (Cordova *et al*., 1997; O'Neill *et al*., 1998; Cordova *et al*., 1999; Rokni *et al*., 2002) and by animals (Cornelissen *et al*., 1999; Cornelissen *et al*., 2001). Considering ES antigens from adult

flukes, the family of CLs, which constitute about 80% of the total secreted protein (Morphew *et al.*, 2007; Robinson *et al.*, 2009), are among the most used target antigens in ELISA. In this sense, Cordova *et al.* (1997) isolated two cysteine protease fractions from ES antigens released by adults of *F. hepatica*, Fas1 (26 kDa) and Fas2 (25 kDa), which were obtained by ethanol precipitation and subsequent fractionation of ES antigens by ion exchange chromatography. Although both fractions are antigenic, Fas2 seemed to be more suitable for diagnosis of human infections in indirect ELISA, and provided sensitivity and specificity values between 92.4–95.5% and 83.6–100%, respectively (Cordova *et al.*, 1997, 1999; Espinoza *et al.*, 2005, 2007). Two commercial iELISA kits to detect antibodies in serum or plasma targeting Fas2 antigens (Fas2-ELISA® and IgM Fas2-ELISA®) are currently commercialized by Bionoma (Lima, Peru). Likewise, Smith *et al.* (1993) purified a FhCL1 (27 kDa) using two chromatographic methods (molecular exclusion and ion exchange). This cathepsin was evaluated in iELISA in two studies conducted in humans from endemic areas of Bolivia and Iran, respectively, obtaining high sensitivity and specificity values (O'Neill *et al.*, 1998; Rokni *et al.*, 2002).

Another partially purified *F. hepatica* antigenic fraction called 'f2 antigen' is currently being used in a commercial iELISA for determination of anti-*Fasciola* antibodies in serum samples of sheep and cattle, as well as in bovine milk (IDEXX Laboratories, Westbrook, Maine, USA). This antigen was initially obtained from *F. hepatica* ES antigens and purified in three steps, including a precipitation with dextran sulfate to eliminate lipoproteins, a chromatographic isolation on Sephadex G-75 and a precipitation with 3.5 M potassium phosphate buffer pH 6.5 (Tailliez and Korach, 1970); this antigen was further used to develop an indirect haemagglutination test (Levieux *et al.*, 1992a,b; Levieux and Levieux, 1994). Although the exact nature of the f2 antigen was never revealed, we know it contains a mosaic of antigens among which there are *Fasciola* CLs, given its strong reactivity with mAbs recognizing such molecules. The f2 IDEXX ELISA test has been evaluated in several studies testing sera from ruminants, and the sensitivity and specificity values obtained ranged between 84% and 100% (Reichel, 2002; Molloy *et al.*, 2005; Rapsch *et al.*, 2006; Charlier *et al.*, 2008; Kuerpick *et al.*, 2013; Munita et *al.*, 2019).

An antigenic fraction rich in *Fasciola* CL1 (peak IV) obtained by size-exclusion chromatography of *F. hepatica* ES antigens (Mezo *et al.*, 2003) was also used as immunogen to obtain the IgG1/κ mouse MM3 mAb (Mezo *et al.*, 2004). This mAb, which recognizes a specific conformational epitope on native and folded recombinant *Fasciola* cathepsins L1, L2 and L5 (Martinez-Sernandez *et al.*, 2018), was used to develop a cELISA (MM3-SERO) for the serodiagnosis of fasciolosis in ruminants (Mezo *et al.*, 2007, 2010b; Perez-Creo *et al.*, 2016). In this method, native CLs present in *F. hepatica* ES are captured in-plate with immobilized mAb MM3 in a single step, while the remaining antigens present in the ES fraction are eliminated in the washing step. Due to the capture format of the test, all wells in the ELISA plate contain immobilized MM3 mAb, but only half of the wells contain antigen (odd positive wells). This special design allows having an individual control to each serum to be tested (even negative wells), which contrasts with most iELISA tests that use a single blank value for all determinations made with each ELISA plate. The main advantages of this strategy over iELISAs are as follows:

- There is great sensitivity, as all clades of CLs (L1, L2 and L5) produced by adult flukes are available as target antigens.
- The antigens are oriented in space after being captured by MM3.
- False positive results can be minimized by having an individual control for each tested serum.

In contrast, the conformational epitope recognized by mAb MM3, and perhaps some residues in the boundary of such epitope, will not be available for serum antibodies once the corresponding MM3-antigen complexes are formed. In practice, however, it seems not to be a problem, since excellent values of sensitivity and specificity, and high signal-to-ratio values, are obtained when testing sera from different ruminants, including sheep infected with *F. gigantica* (Mezo *et al.*, 2007; Valero *et al.*, 2009). The test MM3-SERO was also used to detect serum antibodies in human infections by *F. hepatica* (Martinez-Sernandez *et al.*, 2011). Like for other *Fasciola* native antigens, a commercial version of

MM3-SERO has been marketed under the name of BIO K 211-Monoscreen AbELISA *Fasciola hepatica* (Bio-X Diagnostics, Rochefort, Belgium) intended for the detection of anti-*Fasciola* antibodies in sera, plasma and milk of sheep and cattle. A recent independent field study comparing several commercial ELISA methods to detect circulating anti-*Fasciola* antibodies in experimentally infected cattle showed that BIO K 211 gave results with maximal sensitivity and specificity without loss of OD signal along a 10-week observation study (Munita *et al*., 2019).

Although some *F. hepatica* antigens (e.g. CLs) can be used as targets to detect circulating antibodies induced by *F. gigantica* infections (Valero *et al*., 2009), some authors have developed in-house iELISA methods targeting antigens obtained from *F. gigantica*. For example, a 28 kDa fraction containing CLs, obtained by ethanol fractionation of ES antigens (followed, or not, by ion exchange chromatography), was used to test serum samples of sheep (Dixit *et al*., 2002), cows (Sriveny *et al*., 2006) and buffalo (Raina *et al*., 2006) experimentally or naturally infected by *F. gigantica*. Similar to the *F. hepatica* methods, specific antibodies were detected early (by weeks 2–4 pi), and the sensitivity and specificity were high (97–100%), but no commercial tests are yet available.

10.2.2.3 Methods using synthetic and recombinant antigens

To avoid the requirement of live flukes to obtain native antigens for use in ELISA methods, synthetic peptides and recombinant proteins and peptides have been evaluated as target antigens for serodiagnosis of human and animal infections. This strategy allows the production of unlimited quantities of defined antigens, including some that are difficult to obtain in native form, such as those that are primarily expressed in juvenile stages (Law *et al*., 2003; Cwiklinski *et al*., 2015).

Some lineal peptide sequences were proposed as target antigens in iELISA to minimize potential cross-reactions between common epitopes present in *Fasciola* antigens and proteins from other organisms (Cornelissen *et al*., 1999). However, since linear immunodominant epitopes are normally scarce within antigens (Haste Andersen *et al*., 2006) and peptides are randomly oriented when coupled directly to ELISA plates, these targets are normally not as sensitive as whole antigens to detect mild infections. An alternative to the use of short lineal sequences is the construction of chimeric antigens belonging to different *Fasciola* antigens, a strategy that is also of interest in designing chimeric vaccines (Orbegozo-Medina *et al*., 2019a). In this line of research, it was reported that a 25 kDa recombinant chimera composed of two parental sequences from an *F. hepatica* leucine aminopeptidase (gb|AAV59016, residues 192–281) and a CL1 (gb|CAC12806, residues 173–309), was recognized in WB by sera from cows naturally infected by *F. hepatica* (Hernández-Guzmán *et al*., 2016), but these results have not yet been validated in field studies. As a result, the bulk of the research on recombinant antigens has focused on the use of whole *Fasciola* recombinant proteins, among which CLs are the most investigated.

Regarding recombinant CLs (rCLs), a review of the literature revealed important differences in gene constructs (e.g. presence/absence of the propeptide), expression system (prokaryotic versus eukaryotic) and purification strategies, which may affect the diagnostic performance. Most of the reported diagnostic methods use FhCL sequences expressed in *Escherichia coli*, since it allows the obtention of high amounts of protein (up to 10 mg/l of culture) at relatively low cost (Carnevale *et al*., 2001b). Nevertheless, these proteins are expressed as insoluble inclusion bodies, which is due to the high expression rate and to the inability to form disulfide bridges under the reducing conditions of the bacterial cytosol (Lilie *et al*., 1998; Huang *et al*., 2012). Consequently, these inclusion bodies need to be solubilized under denaturing conditions and subsequently submitted to refolding methods in order to make proteins functional (Carnevale *et al*., 2001b; Ling *et al*., 2015; Martinez-Sernandez *et al*., 2018), which is a cumbersome task and usually results in poor recoveries (Hoffmann *et al*., 2018). Despite these inconveniences, several studies reported good sensitivity and specificity in iELISA using recombinant proCL1 (rpCL1) from *F. hepatica* (rFhpCL1) and *F. gigantica* (rFgpCL1) expressed in *E. coli* to detect circulating anti-*Fasciola* antibodies either in humans or in livestock (Carnevale *et al*., 2001b; Raina *et al*., 2006; Gottstein *et al*., 2014; Martinez-Sernandez *et al*., 2018).

Another refolded rFhpCL1 expressed in *E. coli* was successfully used to develop the first one-step LFIA (ICT-*SeroFluke*®) for serodiagnosis of human fasciolosis. The ICT *SeroFluke*® can be performed with serum or whole-blood samples taken by fingertip puncture and, contrary to ELISA, it requires only minimal training, making it especially suitable for point-of-care testing in developing countries where fasciolosis is endemic (Martinez-Sernandez *et al*., 2011; Muino *et al*., 2011). This test was constructed using colloidal gold-rFhpCL1 antigen in the conjugated pad, recombinant protein A/G in the test line and the mAb MM3 in the control line. In the current format, the test is for exclusive use with human samples (serum or blood) and the results can be obtained in about 10 min when testing serum samples and about 20 min (10 min of running plus a 10 min washing step to remove coloration by haemoglobin) when testing blood samples. In the laboratory, the ICT *SeroFluke*® test was reported to be as sensitive as the MM3-SERO, but confirmation of these results has not yet been performed under field conditions in fasciolosis-endemic countries. More recently, a new LFIA intended for serodiagnosis of sheep fasciolosis was developed using gold-staphylococcal protein G in the conjugated pad, rFhCL1D in the test line and sheep IgG in the control line. Using post-mortem liver inspection as gold standard of infection, the authors reported a sensitivity and specificity of 100% and 97%, respectively (Xifeng *et al*., 2019).

In contrast to *E. coli*, the expression in yeasts allows the direct production of functionally active CL. Roche *et al*. (1997) first cloned a pre-pCL1 of *F. hepatica* (gb|U62288) in *Saccharomyces cerevisiae*, which was secreted in its active form (mature rFhCL1). This rFhCL1 was evaluated in iELISA with human sera from an endemic region of Bolivia and provided similar results to those obtained with the native FhCL1 (O'Neill *et al*., 1999). Cornelissen *et al*. (2001) also cloned and expressed an rFhCL1 (gb|CAC12806) in *S. cerevisiae* that was recognized in iELISA by sera from experimentally infected cows and sheep with sensitivities and specificities near to 100%, although under field conditions these figures dropped to 90% and 75%, respectively. In these experiments, antibodies appeared between weeks 5 (cattle) and 7 (sheep) and remained detectable for more than 20 weeks post-primary infection, which entails a diagnostic delay of about 3 weeks compared with methods targeting native cathepsins (Valero *et al*., 2009; Mezo *et al*., 2010a).

Since the expression of rFhCL1 in *S. cerevisiae* is low and, therefore, less convenient for large-scale production, a more efficient system for cysteine protease expression such as *Pichia pastoris* (Brömme and Schmidt, 1999) has been used in other studies. In this sense, Kuerpick *et al*. (2013) evaluated the serodiagnostic value of a mature rFhCL1 (gb|U62288) expressed in *P. pastoris*. By testing sera from experimentally infected calves in iELISA at week 7 pi, the authors obtained values of sensitivity and specificity of 85% and 89%, respectively, which were clearly lower than those obtained (both 100%) using a commercial test with native f2 antigen. In order to prevent an autocatalytic degradation during the purification procedure and a possible hydrolysis of immunoglobulins in iELISA, Collins *et al*. (2004) generated a single C26/Gly26 mutation affecting the active centre of rFhpCL1 (gb|U62288). This rFhpCL1Gly26 mutant, also expressed in *P. pastoris*, has been used to develop an iELISA commercialized by Ildana Biotech (Dublin, Ireland), with a reported sensitivity and specificity of 98%. This test was used in a comparative study to detect anti-*Fasciola* antibodies in serum of cattle (Munita *et al*., 2019), humans (Gonzales Santana *et al*., 2013) and horses (Quigley *et al*., 2017). In these studies, values of sensitivity and specificity close to 100% were obtained for cattle and humans, but the sensitivity dropped to 42% when evaluating horse serum samples. The mutated rFhpCL1Gly26 was also incorporated in a multiplex assay based on Luminex xMAP® technology that allows simultaneous detection of antibodies against *F. hepatica*, *Cooperia oncophora* and *Dictyocaulus viviparus* in bovine serum samples with values of sensitivity and specificity close to 100% (Karanikola *et al*., 2015). In addition to CLs, cathepsins B have been investigated as diagnostic markers of pre-patent fasciolosis. However, as these proteases are only temporally expressed during the *Fasciola* life cycle, specific antibodies peaked at week 5 pi but fell during subsequent weeks (Law *et al*., 2003), which discourages the use of these antigens for serodiagnosis of *Fasciola* infections.

Although CLs are the most frequently used antigens for the diagnosis of fasciolosis, other antigens such as saposin-like proteins (SAPs), ferritin and leucine aminopeptidase have also been evaluated (Grams *et al*., 2006; Orbegozo-Medina *et al*., 2019b). Besides CLs, only SAPs were reported to give consistently high values of sensitivity and specificity in different laboratories (Figueroa-Santiago *et al*., 2011; Gottstein et *al*., 2014; Kueakhai *et al*., 2015; Shin *et al*., 2016). SAPs are a family of lipid-interacting proteins of relatively low MW (about 11 kDa) which are present in *F. hepatica* (FhSAP-1 and FhSAP-2) and *F. gigantica* (FgSAP-1, FgSAP-2, FgSAP-3) (Grams *et al*., 2006; Kueakhai *et al*., 2015). However, only SAP-2 variants were extensively evaluated as targets for immunodiagnosis of fasciolosis (Kueakhai *et al*., 2015; Shin *et al*., 2016; Mirzadeh *et al*., 2018). Like CLs, *Fasciola* SAPs also have three disulfide bridges (Espino and Hillyer, 2003; Mirzadeh *et al*., 2017) and require a correct folding to make them functional, although they seem easier to fold than CLs, which is probably due to their smaller size (Naganathan and Muñoz, 2005). Several authors reported the use of rSAP-2 as target antigen for the serodiagnosis of human fasciolosis (Figueroa-Santiago *et al*., 2011; Shin *et al*., 2016). Specifically, two immunological assays (a WB and a fluorescent bead-based Luminex® assay) using a GST-FhrSAP-2 antigen were approved for the diagnosis of *Fasciola* infections by the US Centers for Disease Control and Prevention (CDC); these tests have sensitivities and specificities ≥ 94% and ≥ 98% to detect humans with chronic *Fasciola* infections, but they have not yet been validated for acute infections.

10.3 Diagnosis During the Early Biliary and Chronic Phases of Infection

Once the flukes migrate to the bile ducts, the antigenaemia typically decreases (Espino *et al*., 1998; Pelayo *et al*., 1998), but the diagnosis can be performed by several methods, including: (i) detection of specific antibodies, as indicated above; (ii) detection of faecal antigens (coproantigens); (iii) detection of ova in faeces by microscopical examination; and (iv) detection of fluke DNA by molecular techniques.

10.3.1 Detection of circulating antibodies

The same serological methods reported above for early diagnosis, including LFIA tests, are valid to detect circulating antibodies during the entire parenchymal migration of flukes, as well as at chronic phases of infection when the parasites are in the bile ducts (Table 10.1a). In fact, some kinetic studies reported consistently high OD values for observation periods of up to 53 weeks pi in sheep and cattle using the MM3-SERO ELISA (Mezo *et al*., 2007, 2010a; Valero *et al*., 2009). These results indicate that adult flukes continue to stimulate the production of antibodies during the chronic phase of infection while residing in the bile ducts. However, it was also observed in some cows that the level of serum antibodies induced by a primary infection with a very low infecting dose (10 metacercariae) fell below the cut-off limit of the assay by week 40 pi (Mezo *et al*., 2010a). Consequently, in the absence of long-term studies (i.e. for several years), it is unknown whether serum antibodies in livestock remain high throughout the entire life of adult flukes during a primary infection. This aspect may be of interest to detect primary human infections in asymptomatic patients from non-endemic areas, since, if negative, the infection may remain unsuspected. In contrast, detection of circulating antibodies in livestock and in humans from endemic areas is common, since in these areas humans and animals are frequently reinfected, which causes antibody levels to remain indefinitely high. Nevertheless, it should be noted that about 5% of low-burden (one to three flukes) naturally infected cattle having chronic active *Fasciola* infections may lack detectable antibodies to some target antigens used in ELISA (Mezo *et al*., 2010b).

10.3.2 Detection of coproantigens

Some *Fasciola* antigens produced by adult flukes during the early biliary and chronic phases are released into the bile as relatively stable waste products and can be detected in faeces by immunological methods. Unlike antibodies, the presence of such antigens (coproantigens) is indicative of an active/current infection (with

Table 10.1. Selected immunological methods able to detect circulating antibodies and coproantigens in *Fasciola* infections.
(a) Antibody detection methods

Technique	Target antigen	Product	Company/Centre[a]	Tested species	Samples	References
iELISA	FhES	In house		Human, cattle, sheep	S/M/MJ	Charlier *et al*., 2009; Kuerpick *et al*., 2013; Mirzadeh *et al*., 2018
		Fasciola IgG ELISA	DRG Instruments	Human	S	Valero *et al*., 2012a
		SVANOVIR *F. hepatica*-Ab	Boehringer Ing. Svanova	Cattle	S/M/MJ	Munita *et al*., 2019
	Fas2	Fas2-ELISA	Bionoma	Human	S	Espinoza *et al.,* 2005, 2007; Kazantseva *et al*., 2017
	f2	In house		Cattle, sheep	S/M	Reichel, 2002; Molloy *et al*., 2005
		IDEXX Fasciolosis Verification	IDEXX Lab.	Cattle, sheep	S/M	Charlier *et al*., 2008; Munita *et al*., 2019
	FhCL	In house		Human	S	O'Neill *et al*., 1998 ; Rokni *et al*., 2002
	FgCL	In house		Cattle, sheep, buffalo	S	Dixit *et al*., 2002; Sriveny *et al*., 2006; Raina *et al*., 2006
	rFhpCL1	In house		Human, cattle, sheep	S	Carnevale *et al*., 2001b; Martínez-Sernández et al., 2018
	rFhpCL1Gly26	*Fasciola ELISA*	Ildana Biotech	Human, cattle	S/M	Gonzales-Santana *et al*., 2013; Munita *et al*., 2019
	rFhSAP2	In house		Human	S	Figueroa-Santiago *et al*., 2011; Shin *et al*., 2016
icELISA	FhCL1/FhCL2/ FhCL5	In house (MM3-SERO)	USC/ IAQBUS	Human, cattle, sheep	S/M	Mezo *et al*., 2007, 2010a; Martínez-Sernández *et al*., 2011
		BIO K 211	BIO-X Diagnostics	Cattle	S/M	Munita *et al*., 2019; Brockwell *et al*., 2013
LFIA	rFhpCL1	ICT-SeroFluke®[b]	USC/ IAQBUS	Human	S/B	Martínez-Sernández *et al*., 2011
	rFhCL1D	In house		Sheep	S	Xifeng *et al*., 2019
Luminex xMAP®	rFhpCL1Gly26	In house		Cattle	S	Karanikola *et al*., 2015
WB	Fh ES	*Fasciola* ES WB IgG	LDBIO-Diagnostics	Human	S	Agnamey *et al*., 2012

Continued

Table 10.1. Continued.

(b) Coproantigen detection methods

Technique	Capture/ detection Ab	Target antigen	Product	Company/Centre	Tested species	References
cELISA	pAb/mAb (MM3)	CL1/CL2/CL5	In house (MM3-COPRO)	USC/ IAQBUS	Human, cattle, sheep	Mezo *et al*., 2004, 2008; Ubeira *et al*., 2009
			BIO K 201/2	BIO-X Diagnostics	Cattle, sheep, red deer	Martínez-Sernández *et al*., 2016; French *et al*., 2019
	mAb (ES78)/pAb	Glycoproteins	In house (FasciDIG®)[c]	IPK (Cuba)	Human, cattle, sheep	Espino *et al*., 1990; Dumenigo *et al*., 2000; Marcet *et al*., 2012
	mAb/mAb	CL1	In house		Cattle	Estuningsih *et al*., 2009
	mAb/mAb	Glycoproteins	In house[c]		Human	Demerdash *et al*., 2011

Note:

Preferred techniques were those with commercial versions, known target antigens, robustness and continuity of use for the past two decades. As indicated in the text, authors and companies reported values of sensitivity and specificity higher than 95% for most methods. Nevertheless, these values were not included in this table, since they were obtained under non-comparable experimental conditions.

[a]Commercial methods not reporting the nature of target antigen were excluded from the Table. [b]SeroFluke® is a trademark of Inmunogal SL (Santiago de Compostela, Spain).

[c]Used to detect coproantigens and circulating antigens;

B, blood; CL, cathepsin L; cELISA, capture ELISA; iELISA, indirect ELISA; icELISA, indirect ELISA with prior in plate antigen capture; ES, excretory/secretory antigens; Fg, *Fasciola gigantica*; Fh, *Fasciola hepatica*; Gly26, inactive Cys26Gly mutated form; LFIA, lateral flow immune assays; M, milk; mAb, monoclonal Ab; MJ, meat juice; pAb, polyclonal Ab; pCL, proCL; S, serum; SAP, saposin-like protein; WB, Western blotting.

the exception of a brief period of 1–2 weeks after parasites die, for example during an effective flukicide treatment). Although not as early as the detection of circulating antigens, methods for detection of coproantigens have many advantages:

- They can detect coproantigens during the late pre-patent phase of the infection.
- Normally, they are not influenced by the formation of immunocomplexes.
- Sampling is not invasive.
- High specificities and sensitivities as well as high signal-to-noise ratios are normally obtained.
- Frequently, at least for livestock, OD signals have a good correlation with parasite burden (Mezo *et al.*, 2004; Brockwell *et al.*, 2013).
- Faecal samples can be stored frozen or in specific preservatives.
- They are not overly time-consuming.
- They allow high-throughput testing.
- Highly trained personnel (as needed for microscopy) are not required.

By contrast, some limitations should also be taken into account:

- The stability of some coproantigens may be compromised in some hosts/individuals while passing through the digestive tract (e.g. in horses).
- Adapted sample buffers may be required for an efficient extraction of coproantigens in faeces from different hosts.
- Although rare, they are not suitable for diagnosis of fasciolosis when flukes are only located in ectopic locations (Mas-Coma *et al.*, 2014a,b).
- The sensitivity of some methods may be compromised with samples obtained from hosts with very low fluke burden and large faecal volume (e.g. cattle).

The first methods developed for coproantigen detection in stools of humans and cattle included counter-immunoelectrophoresis (CIE) (Youssef *et al.*, 1991) and WB (el-Bahi *et al.*, 1992), respectively. However, these methods soon revealed some disadvantages, mainly as follows:

- There is a high expenditure of undiluted hyperimmune serum (CIE).
- They are time consuming (WB).
- There is the need for a previous step for concentration of faecal extracts (WB).
- They are not adequate for quantitative analysis (CIE, WB).

Consequently, they were soon displaced by cELISA techniques (Table 10.1b). The first cELISA broadly used for detection of coproantigens in human faecal samples was developed by Espino and Finlay (1994). In the original design, this method, registered as FasciDIG® test (Pedro Kouri Institute of Tropical Medicine, Havana, Cuba), used the mAb ES78 (mouse IgG2a) to capture the antigen and a rabbit peroxidase-conjugated anti-*F. hepatica* ES antigens polyclonal antibody for detection. The exact nature of the antigens recognized by mAb ES78 is still unknown, but it was reported to react with antigenic fractions of 14–20, 25–29 and 36–45 kDa (Marcet *et al.*, 2002). The FasciDIG® test was able specifically to detect *F. hepatica* coproantigens by weeks 4–6 in sheep (Dumenigo *et al.*, 2000) and by week 9 in humans (Espino and Dumenigo, 2003). In order to improve the antigen detectability of the original test (15 ng/ml), the FasciDIG® test was later modified by changing the single-step detection system (peroxidase-conjugated rabbit serum) to a two-step procedure (Marcet-Sánchez *et al.*, 2012) in which biotin was conjugated to anti-*Fasciola* rabbit polyclonal antibodies and bound antibodies were revealed using peroxidase-conjugated ExtrAvidin® (Sigma-Aldrich, St Louis, Missouri, USA). With this modification, the detection limit (ES antigens diluted in dH_2O plus Tween-20) improved to about 2 ng/ml. In a similar design, Abdel-Rahman *et al.* (1998) developed a cELISA combining the mAb (M2D5/D5F10) and a rabbit polyclonal antibody, both reacting with a 26–28 kDa glycoprotein from *F. hepatica*. In this cELISA the mAb is coupled to the wells of ELISA plates to capture the antigen, which is then revealed in two steps using a rabbit polyclonal antibody followed by a peroxidase-conjugated goat anti-rabbit polyclonal antibody. This method could detect cattle infections by week 6 pi and the results correlated positively with fluke burden. In the laboratory, this cELISA was able to detect as little as 300 pg of purified antigen/ml, but under field conditions it only detected all parasitized animals with ≥ 10 flukes. Two other in-house cELISA methods

were reported to detect infections by *F. gigantica* in cattle (Estuningsih *et al*., 2009) and humans (Demerdash *et al*., 2011). Both methods combined a pair of mAbs for capture and detection of coproantigens and were able to detect antigen concentrations of 200 ng/ml and 3 ng/ml, respectively. Regardless of some technical limitations and differences in sensitivity and specificity, a major inconvenience of all these methods is their availability, as FasciDIG® seems to be only available in Cuba and the remaining methods were never commercialized.

Besides its application to capture native *Fasciola* CLs in the MM3-SERO test, the mAb MM3 was also used to design a cELISA to detect *Fasciola* coproantigens (MM3-COPRO). In its original design (Mezo *et al*., 2004) the MM3-COPRO test used anti-*F. hepatica* ES antigens and polyclonal antibodies produced in rabbits to capture coproantigens, the mAb MM3 to specifically detect bound FhCLs, and a peroxidase-conjugated goat anti-mouse IgG to amplify the signal. Another characteristic of this assay was the use of double wells (test and control), where the test wells are coupled with the polyclonal anti-*Fasciola* antibody while the control wells contain IgG from normal rabbits. As for MM3-SERO, this design has an individual blank control for each sample to be tested, which may be relevant for specificity considering the heterogeneity of faecal samples and hosts susceptible to be tested. The sensitivity of MM3-COPRO was tested in sheep and cattle, reaching values of 100% for animals harbouring one and two flukes, respectively (Mezo *et al*., 2004). The test also proved to be highly specific, as no cross-reactions were observed with other helminths, including *Dicrocoelium dendriticum* and intestinal nematodes (Mezo *et al*., 2004). The MM3-COPRO test was able to detect coproantigens by weeks 5 and 7 pi in sheep infected with *F. hepatica* or *F. gigantica*, respectively (Mezo *et al*., 2004; Valero *et al*., 2009), and by weeks 8–10 pi in cattle infected with *F. hepatica* (unpublished data). These values precede egg detection by 4–7 weeks and 3–6 weeks for *F. hepatica* and *F. gigantica*, respectively. By contrast, MM3-COPRO does not react with antigens from the giant liver fluke *Fascioloides magna* (unpublished results).

To avoid possible batch-to-batch variations in the peroxidase-conjugated anti-mouse antibodies, the test was later modified using biotinylated MM3 for antigen detection and NeutrAvidin®-POase as amplification reagent. This in-house MM3-COPRO version was initially validated with human faeces processed in CoproGuard® (Inmunogal SL, Santiago de Compostela, Spain), with reported values of sensitivity and specificity of 100% and a detection limit of 1.1 ng/ml (Ubeira *et al*., 2009). Subsequently, the test was evaluated in a community study conducted on children from two endemic regions of Bolivia and Peru and the sensitivity was superior to the parasitological stool examination, although an unexpected small proportion of false-negative children were observed in Huacullani and Cajamarca (Valero *et al*., 2012b). This version of MM3-COPRO ELISA has been marketed for veterinary use under the name of BIO K 201 – Monoscreen AgELISA *Fasciola hepatica* (Bio-X Diagnostics, Rochefort, Belgium), although both tests have some differences related to extraction buffer, incubation conditions and cut-off. As observed for MM3-COPRO, several authors confirmed the specificity of BIO K 201 in several field studies reporting no cross-reactions with other frequent parasites in cattle such as *Paramphistomum cervi*, *Calicophoron daubneyi*, gastrointestinal nematodes and coccidia (Kajugu *et al*., 2012, 2015; Gordon *et al*., 2013). Regarding the sensitivity of BIO K 201, several studies confirmed that the detection of coproantigens using the commercial test preceded the identification of eggs in faeces (McConville *et al*., 2009; Flanagan *et al*., 2011a; Brockwell *et al*., 2013) and showed greater sensitivity than coprological examination in natural infections (Charlier *et al*., 2008; Hanna *et al*., 2015). Moreover, a coproantigen reduction test (CRT) based on the use of the Bio K 201 test was proposed as an alternative to the classical faecal egg count (FEC) reduction test (FECRT) to measure the efficacy of flukicide treatments in different hosts (Flanagan *et al*., 2011b; Novobilský *et al*., 2012; Brockwell *et al*., 2014; Elliott *et al*., 2015; Hanna *et al*., 2015; Novobilský and Hoglund, 2015; French *et al*., 2016; McMahon *et al*., 2016; George *et al*., 2017). While a flukicide treatment is considered effective when the release of coproantigens ceases after 14 days post-treatment (Flanagan *et al*., 2011a,b), the FECRT test requires at least 28 days, due to the release of eggs accumulated in gallbladder. Nevertheless, since only antigens released by late immature/adult flukes are detected, assessment of the efficacy against

immature flukes requires a coproantigen retesting 4 weeks later, i.e. 6 weeks after treatment (Kelley *et al.*, 2020).

Despite the good performance observed in most studies for Bio K 201, a significant proportion of false negative results in animals that were in the patent phase of the infection was also reported (Gordon *et al.*, 2012; Novobilský *et al.*, 2012; Palmer *et al.*, 2014; Kajugu *et al.*, 2015). A lesser sensitivity of Bio K 201 to detect low-burden *Fasciola* infections compared with the original MM3-COPRO can be attributed to differences in secondary reagents, incubation protocol, extraction buffer, and/or cut-off optimization (Brockwell *et al.*, 2014). However, when comparisons are made between different field studies using the same protocol, such differences are more probably related to sample characteristics, as well as the procedure used for sampling and conservation before analysis. In this sense, it should be noted that detection of coproantigens by a cELISA such as MM3-COPRO is only possible when both epitopes (those recognized by the polyclonal antibody and MM3 mAb, respectively) remain intact and freely available after CLs pass into the faeces. Consequently, any biochemical reaction or blocking molecule capable of degrading or blocking these key epitopes on *Fasciola* CLs may give a false negative result.

To avoid, to a certain extent, the limitations inherent in the sample characteristics and to increase the sensitivity of former MM3-COPRO and the commercial Bio K 201 version, the amplification system and the recommended incubation procedure were modified (Martinez-Sernandez *et al.*, 2016). With the modifications introduced, the new MM3-COPRO cELISA, now commercialized by Bio-X as Bio K 201/2 – Monoscreen Ag ELISA, improved its detectability to 150 pg/ml of *Fasciola* ES antigens and the assay can be performed in only 100 min (see Fig. 10.2 for a comparison of the performance of the in-house new version of MM3-COPRO and the commercial Bio K 201/2 kit). This improvement is of high relevance when composite faecal samples are analysed (Graham-Brown *et al.*, 2019; Kamaludeen *et al.*, 2019) or in any circumstance where only small amounts of intact cathepsins are available for detection in faecal samples. Current preliminary unpublished field studies testing hundreds of CoproGuard®-preserved samples from positive and negative animals revealed that the new MM3-COPRO test is 100% sensitive and specific for detection of sheep infected by *F. hepatica* and similar figures were obtained testing infected cattle. Moreover, the new MM3-COPRO was able to detect one-third more true positive samples than FEC using a sedimentation method with a sensitivity of 1 egg/g. Nevertheless, it was also observed that, although testing positive, faecal samples from some cattle herds tended to produce lower cELISA OD signals than expected with respect to egg burden. This suggests that the antigenicity of some CLs may be altered during intestinal transit in discrete individuals or in some host species. The antigen destruction during intestinal transit, for example due to hindgut fermentation, was proposed to explain the low sensitivity of Bio K 201 to detect *Fasciola* infections in horses (Palmer *et al.*, 2014).

The new Bio K 201/2 has been commercially available since 2016, and while extensive field studies on its performance are still awaited, the comparative data in Fig. 10.2 show that its performance is similar to that of the new MM3-COPRO. However, recent field studies of faecal samples from sheep and cattle indicate that the cut-off of Bio K 201/2 needs to be lowered from a percentage of positivity (PP) of 8% to a PP value of 2% to equalize the sensitivity of both methods. Alternatively, the use of a fixed conservative cut-off value (OD = 0.03) has also been found suitable in our laboratory.

10.4 Detection of Ova in Stools by Microscopy

After a prepatent period of about 9–10 weeks and 12 weeks pi for sheep and cattle, respectively, and 12–16 weeks for humans, flukes in bile ducts reach sexual maturity. At this location, they start the production of eggs, which are easily identifiable by microscopical examination at 40× or higher magnification (Mezo *et al.*, 2004, 2007; Mas-Coma *et al.*, 2014a). *Fasciola* spp. eggs are large, operculated, ellipsoidal, non-embryonated and light yellowish-brown (Esteban *et al.*, 2019). Typically, *Fasciola* spp. eggs measure 130–150 μm in length × 63–90 μm in width (Chen and Mott, 1990), but these features depend on hosts. In this sense, it was reported that the size of *Fasciola* spp. eggs in humans (101–191 μm in length × 64–120 μm in width) is different

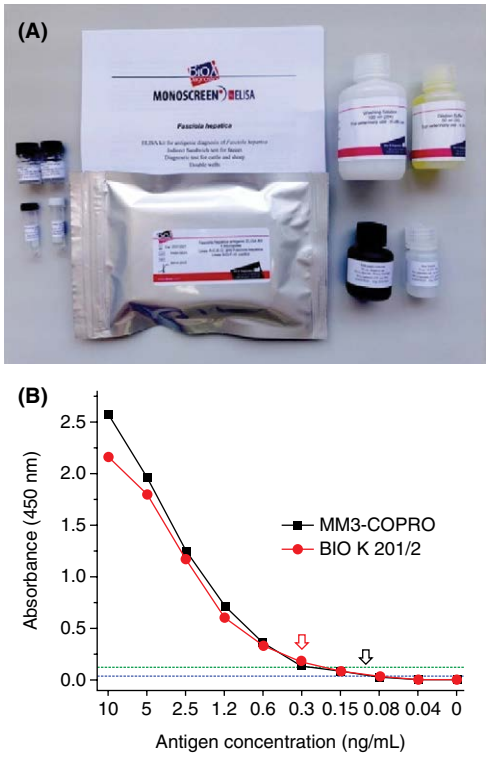

Fig. 10.2. Comparison of the performance of the tests MM3-COPRO and Bio K 201/2. The performance of the in-house MM3-COPRO test (USC/ IAQBUS, Santiago de Compostela, Spain) and its commercial version (*Fasciola hepatica* Monoscreen AgELISA, Bio K 201/2, Bio-X Diagnostics SA, Rochefort, Belgium **(A)** was tested in parallel. A pool of faeces from fluke-free sheep were resuspended 1:3 v/v in CoproGuardR or in the Bio K 201/2 dilution buffer and spiked with *F. hepatica* ES antigens at a concentration of 10 ng/ml. Then the samples were thoroughly mixed, centrifuged at 2000 ×g for 15 min and the supernatants were used as the starting material for comparisons **(B)**. Pure and one-half dilutions were tested in parallel following, respectively, the instructions reported for MM3-COPRO (Martinez- Sernandez *et al*., 2016) and those included with the kit Bio K 201/2, except that 30 min incubations at RT with shaking at 750 rpm were used with both methods. A combined cut-off value for MM3-COPRO (OD = 0.03; horizontaldashed blue line in **(B)**) was determined as being 3 standard deviations (SD) above the highest OD value observed on testing sheep and cattle negative samples (171 samples from nine seronegative sheep farms and 140 samples from four seronegative cattle farms). The current cut-off recommended by the manufacturer for Bio K 201/2 (PP = 8%) was indicated by a horizontal dashed green line **(B)**. Accordingly, vertical arrows show the detection limit for both methods according to each cut-off (300 pg/ml for Bio K 201/2 and 100 pg/ml for MM3-COPRO; **(B)**).

than that reported for livestock (Mas-Coma *et al*., 2014a).

Faecal egg count is still the foremost method used for diagnosis of fasciolosis in humans and animal species for several reasons:

- Like coproantigen determinations, it is non-invasive.
- The presence of ova is indicative of an active infection, except in rare cases of spurious human infections, for example consuming raw infected liver (Poovorawan and Wattanagoon, 2020), or when they are retained in the gallbladder after an effective flukicide treatment (Mezo *et al*., 2004).
- Some methods for FEC allow the diagnosis of co-infections with other helminths (Verocai *et al*., 2020).
- When carried out by trained personnel, FEC is 100% specific.

- It is low-cost and does not require sophisticated equipment.
- In addition to stool samples, other abattoir samples such as duodenal or bile aspirates and even liver biopsies can be used to detect the presence of *Fasciola* eggs.
- It is the only recommended technique to decide the treatment schedule in people with massive infections (Mas-Coma *et al.*, 2014a).

In contrast, FEC has also important drawbacks:

- Egg detection in faecal samples is not possible before patency (i.e. before 9–12 weeks pi), or in the cases where egg elimination in faeces does not occur (for example in ectopic infections, or when flukes or eggs encapsulate in liver abscesses or granulomas).
- As egg sizes vary depending on *Fasciola* species and hosts (Mas-Coma *et al.*, 2014a), technical training is required to identify *Fasciola* eggs and to differentiate them from those of other co-existing trematodes with similar morphology (Fig. 10.3). In this regard, eggs of *Fasciolopsis buski*, *Paragonimus* spp., *Gastrodiscoides hominis* and *Echinostoma* spp. can be found in human stools, mainly in Asian regions (Sripa *et al.*, 2010; Bless *et al.*, 2015), while eggs of *Fascioloides magna* in cervids (Nagy *et al.*, 2018) and a large number of trematode eggs from species belonging to the family Paramphistomidae are frequently found in faeces from many ruminants (Verocai *et al.*, 2020).
- Sensitivity is poor in general, dependent on the intensity and age of infection, the host source of faecal samples (Keiser and Utzinger, 2009; Charlier *et al.*, 2014) and because egg shedding is irregular.
- FEC is time-consuming and not adequate for high-throughput analysis as, for example, to carry out epidemiological studies (Cabada and White, 2012).
- FECRT alone is not recommended to assess flukicide efficacy (McMahon et *al.*, 2016).

From a technical point of view, there are numerous methods for FEC, which differ in terms of sensitivity and complexity. Although in heavy infections the eggs can sometimes be revealed using a simple direct smear, the use of concentration methods, where eggs are separated from the stool by sedimentation and/or flotation techniques, are normally required (Álvarez-Rojas *et al.*, 2014; Mas-Coma *et al.*, 2014a; Verocai *et al.*, 2020). The preferential use of each method depends on the particularities of each case, as well as their availability in each country when it comes to commercial devices.

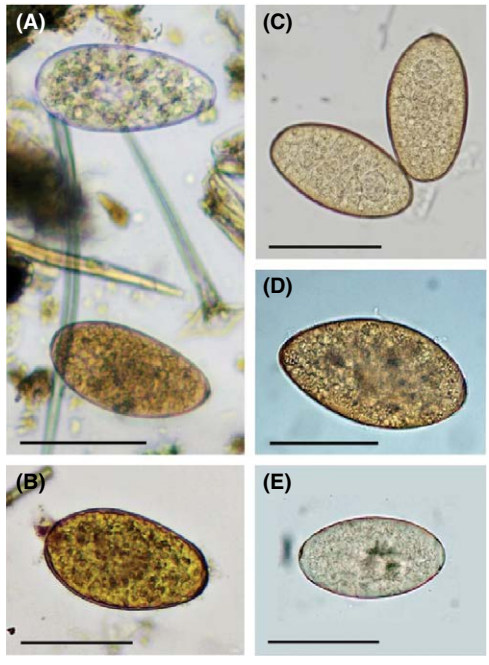

Fig. 10.3. Fasciolid eggs in animal and human biological samples. **(A)** *F. hepatica* egg in a faecal sample from sheep showing the characteristic yellowish-brown appearance (see below), which contrasts with the absence of colour of a *C. daubneyi* egg (see above), both in the same microscopical field (sample from Galicia, Spain). **(B)** *Fascioloides magna* egg in a faecal sample from a red deer from Hungary (courtesy of Dr G. Nagy, Kaposvar University, Hungary). **(C)** *F. hepatica* eggs collected from gallbladders of infected cattle (sample from Galicia, Spain). **(D)** *F. gigantica* egg obtained from an adult fluke collected in Vietnam (courtesy of Drs M.A. Valero and S. Mas-Coma, Departamento de Parasitología, Universidad de Valencia, Spain). **(E)** *Isthmiophora hortensis* (formerly *Echinostoma hortense*) egg in a faecal sample from a human patient from Korea (courtesy of Dr J.Y. Chai, Institute of Parasitic Diseases, Korea Association of Health Promotion, Seoul, Korea). Scale bars = 100 µm.

10.4.1 The Kato–Katz method

The Kato–Katz thick smear is frequently used to determine the prevalence and intensity of infection of human parasitosis caused by *Schistosoma mansoni* and soil-transmitted helminths in endemic regions (Levecke *et al*., 2011; Kure *et al*., 2015; Periago *et al*., 2015). This method has also been used for surveys to quantify egg burdens in human infections by *F. hepatica* in populations of several continents (WHO, 2007; Zumaquero-Rios *et al*., 2013; Mas-Coma *et al*., 2014a; Rodriguez-Ulloa *et al*., 2018). Basically, in the Kato–Katz method (WHO, 1994) a small amount of sieved fresh faeces is used to fill the hole (6 mm diameter, 1.5 mm height) of a plastic template placed on a microscope slide. Then the template is removed and the faecal material is covered with a cellophane strip pre-soaked in a glycerol–malachite green solution and extended on the microscope slide by pressing on a flat surface. After clarification of the faecal material, the sample is examined by microscopy at 100× total magnification. Although this method is simple and cheap, and there are commercial kits (e.g. Kato–Katz kit, Sterlitech, Kent, Washington, USA), it also has important drawbacks:

- It is time-consuming to carry out epidemiological surveys.
- It is not useful for egg detection in livestock.
- It has poor sensitivity (30–65%), mainly for detection of infections with low intensity (Zumaquero-Rios *et al*., 2013), as only a mean of 41.7 mg of faecal material is examined (Leuenberger *et al*., 2016; Zarate-Rendon *et al*., 2019).
- Since human stools can harbour pathogens, including dangerous viruses (Alidjinou *et al*., 2018; Xu *et al*., 2020), its use under a non-controlled environment may present a biohazard risk to examiners.

10.4.2 Sedimentation and flotation techniques

Sedimentation is commonly used in routine analysis to detect trematode infections in domestic and wild ruminants, and less frequently in humans. The faecal samples can be tested either fresh or preserved (typically resuspended 1:3 in 10% formalin v/v), and the eggs are concentrated using a sedimentation technique such as faecal sieving–staining (FSS) methods (Graham-Brown *et al*., 2019), or a combined sedimentation–flotation procedure (Becker *et al*., 2016; Zarate-Rendon *et al*., 2019). The amount of faeces recommended for diagnosis of fasciolosis by an FSS method is 3–4 g for sheep and 10 g for humans and big ruminants such as cattle, horses and buffalo. Typically, in an FSS method, fresh or preserved faecal samples are suspended in tap water alone, or water containing 1% Tween-20 (Roberts and Copeman, 2006), and homogenized. Then the faecal material is passed through a combination of at least two stacked sieves with apertures of, for example, 210 μm (= 80 mesh) and 44 μm (= 325 mesh) with the aid of a powerful jet of tap water. The material retained by the 44 μm sieve is tipped out into a sedimentation flask, filled with tap water, left to sediment for about 6 min and the supernatant is aspirated; this process is repeated until the supernatant is clear. Finally, the sediment is collected in a small volume of liquid (about 10 ml) and the remnant debris is stained with methylene blue before transferring it to a Petri dish for microscopic observation (for example, using an inverted microscope at 20–40× magnification). Compared with liver inspection at abattoirs, the sensitivity of sedimentation techniques oscillates between 60% and 80% (Anderson *et al*., 1999; Mezo *et al*., 2004; Rapsch *et al*., 2006; Mazeri *et al*., 2016). A commercial device called Flukefinder® (Soda Springs, Idaho, USA) containing two sieves of unknown mesh size was used in several studies, with promising results, to detect *F. hepatica* and *F. gigantica* eggs in humans and cattle infections (Nzalawahe *et al*., 2018; Zarate-Rendon *et al*., 2019).

Compared with FSS methods, sedimentation–flotation techniques are less sensitive and can distort trematode eggs by the effect of hypertonic solutions (Chen and Mott, 1990; Esteban *et al*., 2019). Nevertheless, since these techniques produce clearer backgrounds than FSS and are less time-consuming, either in-house techniques or closed commercial devices (FLOTAC and Mini-FLOTAC) were used by some authors to detect *Fasciola* eggs in faeces in comparative studies (Charlier *et al*., 2008; Cringoli *et al*., 2010; Barda *et al*., 2013; Zarate-Rendon *et al*., 2019).

10.5 Diagnosis by Molecular Techniques

In recent years, some molecular methods (MMs) were reported to detect and quantify DNA from *F. hepatica* infections in faecal samples from definitive hosts and in intermediate snails (Cabada *et al.,* 2017; Alda *et al.,* 2018; Rathinasamy *et al.,* 2018), as well as to differentiate infections caused by *F. hepatica* and *F. gigantica* and 'intermediate forms' (Alasaad *et al.,* 2011a, b; Le *et al.,* 2012). Most reported polymerase chain reaction (PCR)-based, loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) methods for diagnosis of fasciolosis targeted rDNA spacer sequences (ITS-1 or ITS-2) or the mtDNA cytochrome C oxidase 1 (*Cox*1) gene (Arifin *et al.*, 2016; Martinez-Valladares and Rojo-Vazquez, 2016; Cabada *et al.*, 2017; Rathinasamy *et al.*, 2018; Calvani *et al.*, 2018a,b). Nevertheless, complete sequencing of ITS-1 and ITS-2 rDNA spacers together with mtDNA *Cox*1 and *Cox*2 genes, or alternatively the *nad*1 gene, was recommended for precise detection of hybrid forms (Amer *et al.*, 2016; Mas-Coma *et al.*, 2019).

While MMs are specific and are of interest in epidemiological studies or in other situations where differentiation between *Fasciola* species is required, their application for routine diagnosis of human and animal fasciolosis is not extended. This is probably due to some misinterpretations and contradictions between results reported by different authors with respect to the sensitivity, source of fluke DNA and optimization of methods for DNA extraction (Calvani *et al.*, 2018a; Fairweather *et al.*, 2020), as well as to some intrinsic limitations, including the following:

- Assuming that *Fasciola* eggs present in the faeces are the main source of DNA to be amplified, the ability of MMs (e.g. PCR-based, LAMP and RPA) to detect Fasciola infections during the prepatent period (i.e. before weeks 9–10 pi in sheep and before week 12 pi in cattle and humans) is limited (Arifin *et al.*, 2016; Calvani *et al.*, 2018b).
- As *Fasciola* eggs accumulate in gallbladder, MMs can still test positive up to a month after death of flukes (Robles-Pérez *et al.*, 2013), which makes them less adequate than coprological cELISAs for monitoring treatment efficacy (Fairweather *et al.*, 2020).
- MMs cannot differentiate active versus spurious infections.
- Even using LAMP or RPA, the time required for sample preparation and DNA extraction is lengthy and the reagents are more expensive than those used in conventional FSS and cELISA methods.
- There are no standardized methods for DNA extraction in faecal samples from different host species.
- A single set of primers is not enough to detect all *Fasciola* species and hybrids, which limits its use as a general-purpose technique.
- Some MMs can be inhibited by substances present in faecal samples (Schrader *et al.*, 2012).

In view of these limitations, new improvements and simplifications are required to make MMs more reliable and robust before they can be included in routine analysis.

10.6 Adaptations and Complementary Diagnostic Methods

10.6.1 Methods for detection of anti-*Fasciola* antibodies in milk and other biological fluids

Most methods designed for antibody detection in serum can be adapted with minor changes to measure antibodies in milk and other biological fluids (Salimi-Bejestani *et al.*, 2005b; Charlier *et al.*, 2009; Mezo *et al.*, 2010a,b). As for serum, antibodies can be detected at any time starting at the beginning of lactation (Mezo *et al.*, 2010a). Nevertheless, it should be considered that, except in colostrum, the total concentration of IgG in cow's milk is 20–35 times lower than in the serum, and therefore the tests used must be highly sensitive (Mezo *et al.*, 2010b). This is particularly relevant when testing anti-*Fasciola* antibodies in milk tanks, as they frequently contain milk from infected and non-infected animals. Although dependent on the sensitivity and specificity of each ELISA method (Munita *et al.*, 2019), it was reported that a proportion of 12–25% of infected dairy cows in a herd may be necessary to detect anti-*Fasciola* IgG antibodies in bulk

tanks (Mezo *et al*., 2010b; Duscher *et al*., 2011; Sekiya *et al*., 2013). Consequently, a negative result does not mean that a given herd is free of infection. As for serum, the presence of antibodies in individual or bulk samples does not indicate active infection. However, when anti-*Fasciola* antibodies are monitored regularly, valuable information can be obtained about the parasite exposure status (Sekiya *et al*., 2013) and on the degree in which infection can affect production parameters. In this sense, it was reported that cut-off ODs ranges ('economic thresholds') can be established for bulk tanks and for individual milk samples revealing milk production losses (Mezo *et al*., 2011; Kostenberger *et al*., 2017; May *et al*., 2020).

As for faecal samples, collection of milk is not invasive and the samples are easy to handle and store. Moreover, the surplus of milk samples routinely collected to monitor animal productivity and milk quality can be used for analysis. Nevertheless, the high fat content of these samples may cause interferences in ELISA and should be removed. Typically, fresh individual and bulk milk samples can be stored overnight at 4°C without adding preservatives, and, after removal of fat by centrifugation, stored frozen at −20°C for further analysis. As an alternative, a biocide such as bronopol (2-bromo-2–nitro1,3 propanediol), a combination of bronopol and the antifungal natamycin, or azidiol (a combination of sodium azide and chloramphenicol) can be added to the milk samples before or after centrifugation to prevent the growth of microorganisms (Upadhyay *et al*., 2014). While serum samples are tested diluted 1/100–1/800 in a suitable buffer, individual and bulk tank samples are tested undiluted. Under these conditions, excellent correlations are normally obtained comparing the levels of antibodies in serum and milk (Salimi-Bejestani *et al*., 2005b; Mezo *et al*., 2011).

Regarding the use of other biological fluids, the measurement of anti-*Fasciola* antibodies in meat juice obtained from carcasses of beef cattle has been reported (Charlier *et al*., 2009). In this case, meat samples are collected from the diaphragmatic muscle and stored at −20°C for 3 days. Afterwards, the samples are thawed, the transudate is collected and then centrifuged, and the supernatant is stored frozen prior to ELISA analysis. Antibody determinations in meat juice may be a simple, non-invasive, cost-effective, method to carry out epidemiological studies on *Fasciola* infections in beef cattle and this method was reported to be more sensitive than liver examination at abattoir (Charlier *et al*., 2009) but, as occurs with antibodies in serum, a positive result does not indicate an active infection. Nevertheless, storage of faecal samples collected in the field, or at the time of sending animals to abattoirs, for further coproantigen determinations is probably a convenient alternative for better evaluation of the prevalence of liver fluke in beef cattle.

10.6.2 Fluke and egg counts at necropsy

Fluke counts can be performed as a method to confirm infections and to quantify parasite burden in animals. For example, the detection of young flukes during the first days of infection in the peritoneum of euthanized animals was reported by several authors, mainly in rats and mice, in the course of experimental infections (Harness *et al.,* 1973, 1977; Burden *et al*., 1983). However, due to the difficulty of performing peritoneal washes in ruminants and the lack of sensitivity and repeatability of this diagnostic technique, early fluke counts are not of practical use in these animals. Instead, counting juvenile flukes in the hepatic parenchyma of ruminants from the second week of infection is more feasible. Typically, young and mature flukes can be detected in liver following a protocol basically consisting of slicing the organ into 1–2 cm segments, incubation in warm saline media at 37°C for 2 h, squeezing each fragment, and examining the media under a dissecting microscope (Mazeri *et al*., 2016; George *et al*., 2017). This method is often used as the gold standard to estimate the sensitivity and specificity of other methods and to monitor the efficacy of flukicide treatments and of experimental vaccines (Geurden *et al*., 2012; Orbegozo-Medina *et al*., 2018). However, it should be considered that the sensitivity may be less than 70% if a detailed examination of the liver fragments is not performed, and that infections with few parasites may be overlooked (Rapsch *et al*., 2006; Charlier *et al*., 2008). Post-mortem confirmation of *Fasciola* chronic infections in animals, with more than 90% sensitivity, can also be done at the slaughterhouse by searching for the presence of eggs in

bile from the gallbladder by direct microscopic examination (Rapsch *et al.*, 2006).

10.6.3 Complementary laboratory findings and non-invasive imaging techniques

The diagnosis of human fasciolosis in non-endemic countries is difficult but can be suspected in a patient with fever, abdominal pain (more frequently located in right hypochondrium), gastrointestinal disturbances and eosinophilia, which are frequent findings during the invasive or acute phase (Chen and Mott, 1990). This clinical picture may be accompanied by a discrete increase of liver enzymes and perhaps a history of consumption of raw vegetables (e.g. wild watercress). Due to abdominal pain, most patients undergo abdominal ultrasonography (US), computed tomography (CT), and/or magnetic resonance imaging (MRI). In the parenchymal phase of fasciolosis, US normally reveals non-specific liver alterations, but CT and MRI may reveal subcapsular and parenchymal liver lesions compatible with fasciolosis (Kabaalioglu *et al.*, 2007; Dusak *et al.*, 2012). The parenchymal lesions regress at the beginning of the biliary phase, but ductal dilatations and signal-filling defects due to the presence of flukes can be observed (Dusak *et al.*, 2012). However, in the absence of a clear guiding symptom, eosinophilia, in combination with gastrointestinal symptoms, is what make a helminth infection a primary concern (Micic *et al.*, 2020).

10.7 Concluding Remarks

The diagnosis of fasciolosis poses different challenges in humans and in animal species. In early primary human infections in non-endemic regions, as well as in cases of ectopic infections, serological methods such as ELISAs or LFIAs are recommended; however, clinical suspicion is challenging and requires a detailed anamnesis and the finding of compatible image scans and complementary laboratory data (e.g. eosinophilia, abnormal liver function tests). When flukes start migration to the bile ducts, or during the chronic stage of infection, detection of coproantigens by a sensitive cELISA, if available, remains the simplest quantitative technique in the routine diagnosis of fasciolosis, since it can detect infections before MMs and FEC. Coproantigen determinations are also the preferred methods to evaluate the efficacy of flukicide treatments in humans and animal species. Moreover, combined with serological methods, coproantigen-based tests may also be useful to detect old chronic infections that test negative by FEC and/or MMs, as well as to differentiate real from spurious infections. Although *Fasciola* coproantigens in faecal samples are more stable than ES antigens in solution, on a few occasions their antigenicity may be altered during the intestinal transit, or due to inadequate conservation. In such cases, testing a new sample several days later can probably solve the problem, although FEC and/or MMs may be alternatively used to confirm the diagnosis. In endemic regions where humans are continuously reinfected and have other helminth infections, eosinophilia is not of value as a guide sign, but since fasciolosis is a frequent pathology, diagnosis can be confirmed by serological methods combined with coproantigen tests, FEC, or MMs. Finally, MMs are required to differentiate infections produced by *F. hepatica*, *F. gigantica* and their hybrids, which is of underlying interest for epidemiologists but not so important for physicians and veterinarians, since human and animal infections by *F. hepatica* and *F. gigantica* are managed in the same way.

In the case of livestock, both the diagnostic methods to use and the schedule for being carried out are mainly determined by the production type and the grazing management procedures. Serological methods are very useful tools to test milk and serum samples collected in animal health and/or production control programmes to locate herds with prevalence levels indicative of production losses. These methods are also of choice to detect early primary infections in first-grazing season animals that can be useful sentinels to detect high-risk periods for *Fasciola* infections. Ideal moments for such analysis are the entry to the feedlot, in the case of meat herds, and before first lactation, in the case of dairy herds. However, in animals with previous grazing experience, it is recommended that active infections are confirmed by a coproantigen-based cELISA test capable of detecting infections before the patency, to avoid pasture contamination. Conveniently, the analysis should be done

when animals are individually managed, as occurs around parturition and at drying off in dairy animals. Where these tests are not available, or when faecal samples are not suitable for coproantigen determinations, FEC and/or MMs may be an alternative, although they are not useful for detecting infections before patency. Moreover, due to its poor sensitivity and being time-consuming, FEC is not a recommended method to use in large herds.

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11 Applying 'Omics' Technologies to Understand *Fasciola* spp. Biology

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11.1 Introduction

The liver flukes *Fasciola hepatica* and *F. gigantica* are digenean trematodes that exhibit a similar complex life cycle involving a snail intermediate host vector and an invertebrate definitive host. Within the snail, the parasites undergo asexual clonal expansion, passing through sporocyst and redial stages that ultimately give rise to many cercariae that encyst as metacercariae on vegetation or float in water. *F. hepatica* and *F. gigantica* are hermaphroditic and have the ability to self- or cross-fertilize. In addition, in regions where they coexist, for example in Asia and Africa, hybridization can occur, giving rise to intermediate or hybrid forms as determined by analysis of mitochondrial genes and intergenic genome sequences (reviewed by Calvani and Šlapeta, 2021).

Infection of the mammalian host occurs following the ingestion of the infective metacercariae. Within the intestine, parasites excyst as newly excysted juveniles (NEJ) that migrate across the intestinal wall into the peritoneal cavity before penetrating the Glisson capsule of the liver and entering the parenchyma tissue. During this migration through various tissues, they actively feed, develop and grow rapidly, encountering new environments while defending attack from the host's innate and adaptive immune responses until they enter the bile ducts. Here they become sexually mature adults and remain for many years within this immunologically safe environment and feed on blood to support the production of 20,000–24,000 eggs per fluke per day (Boray, 1969) (see Chapter 1, this volume). *F. hepatica* is known to infect a wide range of mammalian hosts, including rodents, ruminants, ungulates and primates (Robinson and Dalton, 2009), some of which they have encountered only within recent times (coypu, camelids, kangaroos), implying that the parasite has evolved a one-fits-all mechanism of infection and immune defence.

The extensive collection of 'big data' sets now available for *F. hepatica* includes draft genomes, stage-specific transcriptomes and proteomic datasets for the somatic proteome, secretome, extracellular vesicles and glycoproteome of the outer tegumental surface (Cwiklinski and Dalton, 2018). The information for *F. gigantica* is not yet as extensive but includes draft genomes and stage-specific transcriptomes, with proteomic studies focusing on the host responses to infection (Zhang *et al.*, 2017; Zhang *et al.*, 2019b; Choi *et al.*, 2020; Hu *et al.*, 2020; Pandey *et al.*, 2020). These datasets are used to investigate the

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complex features of the *Fasciola* spp. life cycle, particularly their effects on life history traits that directly influence gene flow within liver fluke populations, influencing the spread of drug resistance, host selection and virulence/pathogenicity traits. They are advancing our understanding of liver fluke migration, growth, development and cellular biology, and elucidating the mechanisms central to virulence, infection and long-term survival within both the snail vector and mammalian definitive host.

The methods used to collect these datasets are referred to as 'omics' technologies and include technologies to:

- sequence and annotate genes and analyse their structure and evolution (genomics);
- establish which and when genes are transcribed during parasite development (transcriptomics);
- profile and characterize the proteins that are translated from transcribed mRNA, especially those involved in host–parasite interactions (proteomics); and
- characterize post-translational events such as glycosylation (glycomics) and phosphorylation.

These technologies are not mutually exclusive; rather, they lead seamlessly from one to the next and, therefore, they can be integrated and interrogated together to attain a global view of parasite developmental biology (Fig. 11.1). Furthermore, they can be compared with studies of

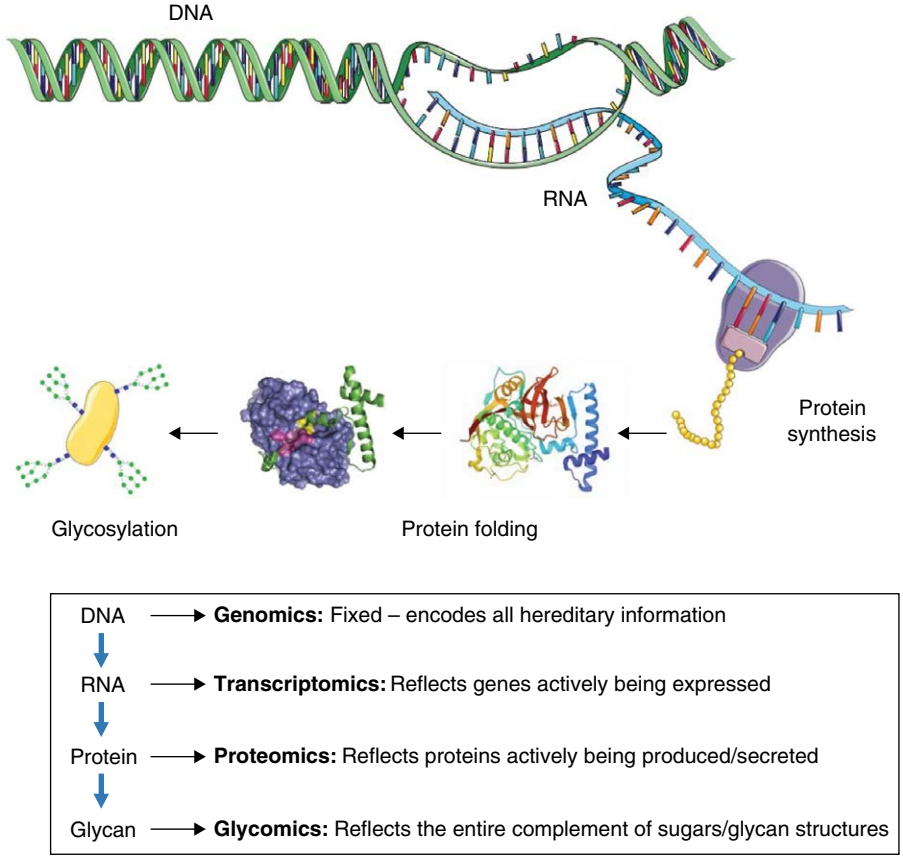

Fig. 11.1. The processes involved in the synthesis of DNA, RNA and proteins, the addition of glycans during post-translational modification processes, and the terminology used for their study. Protein structures provided by PDB (line protein) and reprinted from *Advances in Parasitology*, Cwiklinski *et al*. (2019) (globular protein). Medical art is provided by Les Laboratories Servier (https://smart.servier.com).

host physiology and immunology to gain an interactive picture of the host–parasite relationship. The ultimate goal, however, is to exploit this data in the search for new anti-parasite interventions such as drugs and vaccines.

11.2 The Mitochondrial and Nuclear Genomes

DNA sequencing technologies have evolved rapidly from the traditional Sanger methodology used to map the first human genome (Lander *et al*., 2001; Venter *et al*., 2001) to the recent high-throughput sequencing technologies used today, including next-generation sequencing tools such as Roche 454 and Illumina (Reuter *et al*., 2015; Goodwin *et al*., 2016; McCombie *et al*., 2019) and long-read sequencing technologies developed by Pacific Biosciences (PacBio) and Oxford Nanopore Technologies (Amarasinghe *et al*., 2020; Kraft and Kurth, 2020). Combined with better analytical techniques for nucleic acids extraction and library preparation (Price *et al*., 2009; Mason *et al*., 2017), sequencing projects can be performed on even the most challenging parasites to propagate in the laboratory, as the necessary quantities of nucleic acids required nowadays are in the nano- to microgram levels. Accordingly, sequencing of nucleic acids from the microscopic *Fasciola* NEJ and various intra-molluscan stages of the life cycle is now attainable.

Information on helminth genomes from human and veterinary parasites of global importance are housed on WormBase ParaSite (https://parasite.wormbase.org) which in its current version (WBPS15) comprises > 190 genomes, representing > 160 species. WormBase ParaSite also integrates genomic and transcriptomic data to provide functional annotation and expression information for each species, and thus facilitates comparisons between genomes (comparative genomics). The genomic sequence data available for *Fasciola* species at WormBase ParaSite currently includes two *F. hepatica* datasets (PRJEB25283 and PRJNA179522) and one *F. gigantica* dataset (PRJNA230515). Another *F. gigantica* assembly is also available at the European Nucleotide Archive (ENA) under accession MKHB03000000 (Table 11.1).

11.2.1 *Fasciola* spp. mitochondrial genomes

F. hepatica was the first trematode to have its mitochondrial genome completely sequenced (Le *et al*., 2001). More recently, the complete mitochondrial genome of *F. gigantica* has been reported (Liu *et al*., 2014), which now provides species-specific references that can be used in characterization studies. The identification of *Fasciola* spp. and differentiation of *F. hepatica* and *F. gigantica* using solely morphological features of adult parasites, in particular, is often unreliable. By contrast, molecular identification based on nuclear ribosomal and mitochondrial genes is a more robust method of species classification and can be employed for any stage of the parasite.

These molecular tools have been employed to provide markers for population genetic studies and epidemiological analysis of *Fasciola* spp. (Ai *et al*., 2011; Hodgkinson *et al*., 2013). These markers have facilitated the analysis of the global geographical dispersal of *Fasciola* spp. isolates, which has highlighted high levels of genetic diversity within all populations. In particular, seminal work by Madoka Ichikawa-Seki and Tadashi Itagaki has characterized *Fasciola* spp. isolate populations throughout the world, identifying most recently the following.

- Nigerian *F. gigantica* isolates reflect the extensive transport of livestock throughout Nigeria, and Africa in general, resulting in a genetically diverse population (Ichikawa-Seki *et al*., 2017c).
- *F. hepatica* isolates in China may have originated in Afghanistan (Thang *et al*., 2019).
- Cambodian *F. gigantica* isolates may be an ancestor of Southeast Asian isolates (Loeurng *et al*., 2019).
- *F. hepatica* isolates on the high plateau and steppe areas of Algeria bear most resemblance to Spanish isolates (Laatamna *et al*., 2021).
- *F. gigantica* isolates found in buffalo and goats in Pakistan likely originated from India and the Middle East (Rehman *et al*., 2021).

Mitochondrial markers have also been used to assess genetic diversity within triclabendazole-resistant *F. hepatica* populations. The study by Elliott *et al*. (2014) evaluated adult flukes from three triclabendazole-susceptible and resistant

Table 11.1. Summary of the *Fasciola* spp. genome assembly statistics.

	F. hepatica UK v1	*F. hepatica* UK v2	*F. hepatica* USA v1	*F. hepatica* USA v2	*F. gigantica* India	*F. gigantica* Uganda
Assembly accession	PRJEB6687	PRJEB25283	PRJNA179522	PRJNA179522	MKHB03000000	PRJNA230515
Genome length	1.3 Gb	1.3 Gb	1.14 Gb	1.14 Gb	1.04 Gb	1.13 Gb
N50 scaffold length	204 kb	1.9 Mb	161.1 kb	160.9 kb	129 kb	181.8 kb
Protein coding genes	22676	9732	14642	15739	20858	12647
% BUSCO (complete)	90 (CEGMA)	68.6	43.9	74	51.3	77.2
Repetitive content (%)	32	Not reported	55.3	65.8	46.85	58.2
Mean exons per mRNA	5.3	Not reported	3.3	7.5	3	5.9
Mean exon length (bp)	303	Not reported	259.8	217.7	302.5	233.2
Mean intron length (bp)	3700	Not reported	2902	4170	2612	3982

References: *F. hepatica* UK v1: Cwiklinski *et al*., 2015a; *F. hepatica* USA v1: McNulty *et al*., 2017; *F. hepatica* USA v2: Choi *et al*., 2020; *F. gigantica* India: Pandey *et al*., 2020; *F. gigantica* Uganda: Choi *et al*., 2020.

populations in Australia. Similarly, Martínez-Valladares and Rojo-Vázquez (2014) analysed eggs from sheep from three strains in Spain that displayed different susceptibilities to three anti-*Fasciola* anthelmintic drugs, namely albendazole, triclabendazole and clorsulon. Both of these studies concluded that the susceptibility/resistance profile of the liver fluke isolates did not influence genetic diversity. Indeed, all the *F. hepatica* isolates analysed displayed high levels of genetic diversity, in keeping with other *F. hepatica* population genetic studies.

The availability of mitochondrial genomes and the corresponding mitochondrial markers has also been particularly useful in identifying *F. hepatica* and *F. gigantica* and their hybrids where the two parasites coexist (see Chapter 13, this volume). PCR-based tools such as those described by Castilla Gómez de Agüero *et al.* (2020) can differentiate between the *Fasciol*a spp. and have shown that both species are present within the cattle and sheep populations in Nigeria. In 2014, Liu and colleagues sequenced the complete mitochondrial genome of an intermediate/hybrid form of *F. hepatica* and *F. gigantica* found in Heilongjiang province, China, which was classified as a hybrid based on the intergenic spacer regions (ITS-1 and ITS-2). Comparative analyses between *Fasciola* spp. mitochondrial genomes revealed that the parasite was more closely related to *F. gigantica* than to *F. hepatica* (Peng *et al*., 2009). This study revealed that hybridization is not uniform across the genome and sequence variations occur at different sites, in this case within the nuclear ribosomal genes and the maternally inherited mitochondrial genes. Importantly, the study highlights the complexity associated with hybridization of *Fasciola* spp. and the challenges that their subsequent characterization presents.

In addition to the mitochondrial markers, population genetic studies of *Fasciola* spp. are increasingly exploiting other markers, including the intergenic spacer regions (ITS), ribosomal markers (Ai *et al*., 2011) and nuclear markers (pepck and pold) (Shoriki *et al*., 2016; Hayashi *et al*., 2018), to allow for accurate species differentiation, though discordant sequences can still be identified. Several studies have identified co-endemic areas where there is a potential for hybridization to occur, including Egypt (Amer *et al*., 2016) and Armenia, where *F. gigantica* isolates are more genetically diverse than the *F. hepatica* isolates (Aghayan *et al*., 2019), and South Africa, where *F. hepatica* predominates (Mucheka *et al*., 2015; Chikowore *et al*., 2019; Haridwal *et al*., 2021).

To date, molecular classification of hybrid *Fasciola* parasites has been carried out on samples from across Southeast Asia (Hayashi *et al*., 2018), including Thailand (Wannasan *et al*., 2014), Nepal (Shoriki *et al*., 2014), China (Ichikawa-Seki *et al*., 2017a), Vietnam (Nguyen *et al*., 2018) and Japan (Ichikawa-Seki *et al*., 2017b; Saijuntha *et al*., 2018; Ohari *et al*., 2021). Hybrids have also been identified in Ecuador (Saijuntha *et al*., 2018) and Chad (Evack *et al*., 2020) (see Chapter 13 of this volume for more on hybridization).

11.2.2 Nuclear genomes of *F. hepatica* and *F. gigantica*

11.2.2.1 F. hepatica *genome assemblies*

The first *F. hepatica* draft genome assembly representing a UK isolate, published by Cwiklinski *et al*. (2015a) (PRJEB6687), was a defining moment for liver fluke research as it provided the foundation that is necessary for the annotation, characterization and analysis of all other omics technologies. Surprisingly, *F. hepatica* has the largest trematode genome sequenced to date (1.3 Gb), the reason for which is currently unclear, although the large genome size appears to be associated with the divergence of the *Fasciola* species line (Choi *et al*., 2020). While a similar number of genes are found across the trematode genomes, comparative analyses revealed that increases in genome size are reflective of greater average exon and intron lengths, although this alone does not fully explain the increased size of the *F. hepatica* genome. Since *F. hepatica* ensures its survival by producing an enormous number of eggs each day, the evolution of a large genome is puzzling, as it imposes a high cost on egg production (Cwiklinski *et al*., 2015a).

A second draft genome of *F. hepatica* isolates from the Americas uncovered the presence of a *Neorickettsia* endobacterium genome, indicating the potential interaction between *Fasciola* and endosymbionts/endobacteria. The bacteria were found by immunochemistry within the eggs, reproductive system and the oral suckers of adult flukes (McNulty *et al*., 2017). However, since no other geographical isolates of *F. hepatica* have been reported with *Neorickettsia* spp., this endobacterium may have occurred since the introduction of *F. hepatica* to the Americas.

In 2018, a revised assembly of the genomic data of the UK isolate was made available within the 11th release of WormBase ParaSite (PRJEB25283), which comprises 2816 scaffolds and 78,522 contigs, representing an improved genome assembly (scaffold N50: 1.9 Mb). Since the publication by McNulty *et al*. (2017), this assembly has also undergone improvements relating to the gene annotation, as reported by Choi *et al*. (2020).

11.2.2.2 F. gigantica *genome assemblies*

Recently, two genome assemblies have been made available for *F. gigantica*, representing an isolate from Uganda (Choi *et al*., 2020) and an isolate from India (Pandey *et al*., 2020). Consistent with the *F. hepatica* genome assemblies, *F. gigantica* has a genome size of > 1 Gb (Uganda isolate, 1.13 Gb; India isolate, 1.04 Gb), and therefore a large genome size is a trait associated with the genus *Fasciola*. Moreover, the *F. gigantica* genomes contain a similar number of protein-coding genes, which, as highlighted by Choi *et al*. (2020), implies that the large genome size is not a consequence of whole-genome duplication. However, the *Fasciola* spp. genomes contain longer repetitive elements compared with other trematode species (Choi *et al*., 2020).

11.2.2.3 Application of the liver fluke genomes

The availability of the *Fasciola* spp. genome assemblies allows in-depth analysis of fluke biology, providing novel insights into how these parasites have evolved and adapted to infect a range of hosts and survive within very different environmental niches. Whole-genome comparative analysis has revised the proposed time when *F. hepatica* and *F. gigantica* diverged from about 19 million years ago, which was based on evolution of the cathepsin cysteine peptidases (Irving *et al*., 2003), to about 5 million years ago at the Miocene–Pliocene boundary (Choi *et al*., 2020).

Comparative genomics across the parasitic helminths housed at WormBase ParaSite reveals the expansion of key gene families that are associated with parasitism (International Helminth Genomes Consortium, 2019). Consistent with

the genome studies by Cwiklinski *et al.* (2015a) and McNulty *et al.* (2017), several gene families have expanded within the *F. hepatica* genome, highlighting the functionally important role these molecules play during the parasite life cycle (International Helminth Genomes Consortium, 2019). Key families of interest are the papain-like cysteine peptidases, cysteine peptidase inhibitors and Kunitz-like inhibitors that are major components of the excreted/secreted products. Similarly, large gene families for G protein-coupled receptors (GPCRs), important for neuromuscular function, and a range of kinases that are regulators of cellular phosphorylation were identified, consistent with the studies by McVeigh *et al.* (2018b) and by Haeberlein and colleagues (Houhou *et al.*, 2019; Morawietz *et al.*, 2020), respectively. A large number of glycosyltransferase genes were also observed, emphasizing the important role that glycans play for *F. hepatica* biology (see Glycomics, section 11.7 below).

Although the data from the *F. gigantica* genomes were not available for the comparative analysis above, analysis of *F. gigantica* gene families demonstrates similar gene expansions for those molecules associated with the excretory/secretory (ES) products such as the cathepsin peptidases, legumains, glutathione *S*-transferases (GSTs) and fatty acid-binding proteins (FABPs) (Choi *et al.*, 2020; Pandey *et al.*, 2020). This indicates that the expansion event likely occurred prior to the divergence of the two species (Choi *et al.*, 2020). In addition, as highlighted by the *F. hepatica* genome analysis, GPCRs were significantly enriched within the *F. gigantica* genome (Choi *et al.*, 2020; Das *et al.*, 2020).

One of the major applications of the *F. hepatica* genome is the elucidation of the underlying causes of drug resistance. The emergence and global spread of parasites resistant to the frontline drug triclabendazole (TCBZ) has intensified the pursuit to understand the genetics behind drug resistance and to seek new avenues for future drug discovery (Fairweather *et al.*, 2020, Castro-Hermida *et al.*, 2021) (see Chapter 7, this volume). This information is also critical for future surveillance programmes monitoring the emergence of TCBZ in *F. gigantica* and the *Fasciola* hybrids. Triclabendazole belongs to the benzimidazole class of anthelminthics that target the β-tubulin molecules of cytoskeletal microtubules (Devine *et al.*, 2011; Fuchs *et al.*, 2013). *F. hepatica* expresses a cohort of tubulins, comprising five α-tubulin and six β-tubulin molecules (Ryan *et al.*, 2008) that display differential expression across the life cycle (Fuchs *et al.*, 2013; Cwiklinski *et al.*, 2015a). The transcription of the diverse range of β-tubulin isotypes is temporally regulated, which may explain the stage-specific efficacy of benzimidazole anthelminthics (Sanabria *et al.*, 2013). However, although the β-tubulin molecules likely play a role in resistance to the benzimidazole derivative albendazole (ABZ), ABZ-resistant *F. hepatica* isolates remain susceptible to TCBZ, indicating different modes of action (Sanabria *et al.*, 2013). A genome-wide approach to determine the molecules involved in the mode of action and resistance of TCBZ can now be carried out following the availability of the *F. hepatica* genome (Hodgkinson *et al.*, 2013).

In keeping with the genetic diversity observed within the ribosomal and mitochondrial markers, a genome-wide analysis using microsatellite markers reveals that high genetic diversity in *F. hepatica* is spread throughout the genome and may contribute to gene flow within UK field isolates (Beesley *et al.*, 2017). High levels of genetic diversity and gene flow may be important to counter the decline of allelic diversity due to self-fertilization in the mammalian host (Noel *et al.*, 2017) and clonal expansion within the intermediate snail host (Hodgkinson *et al.*, 2018). Moreover, genetic diversity may also play a critical role in the ability of *Fasciola* spp. parasites to infect a wide range of mammalian hosts. As evidenced by studies in Argentina, Cuba and the UK, *F. hepatica* has a propensity for cross-fertilization; as such, self-fertilization may not impact greatly on genetic diversity (Vazquez *et al.*, 2016; Beesley *et al.*, 2017, 2021).

Analysis of susceptible and resistant *F. hepatica* UK isolates (Hodgkinson *et al.*, 2013) revealed high levels of single nucleotide polymorphisms (SNPs) throughout the genome (Cwiklinski *et al.*, 2015a). At least one non-synonymous change was observed in 48% of the 22,676 gene models of the original assembly of the UK isolate. Comparative analysis focused on orthologous genes in other platyhelminths revealed that *F. hepatica* displays higher levels of polymorphism in those genes it shares with *Clonorchis sinensis*, *Schistosoma mansoni* and *Echinococcus multilocularis* relative to the free-living planarian, *Schmidtea mediterranea* (Cwiklinski

et al., 2015a). This gene-set likely reflects those important to a parasitic lifestyle and further highlights the adaptability of *F. hepatica*. Functional analysis revealed that there is a marked over-representation of genes with high levels of non-synonymous polymorphism associated with axonogenesis, chemotaxis and responses to external stimuli, reflecting the changing environments the parasite encounters during its migration in the snail and mammalian host (Cwiklinski *et al*., 2015a).

11.3 Transcriptomics and Stage-specific Gene Expression

The deep understanding of parasite biology and development in the context of host–parasite interaction is essential for the discovery of innovative control strategies, vaccines and diagnostic tools directed at specific life cycle stages. The emergence of large-scale RNA sequencing (RNAseq) methods has facilitated the high-throughput analysis of all gene transcripts within each parasite developmental stage as well as their specific transcriptional abundance. A comparative analysis between parasite stages allows the profiling of the temporal changes in gene expression as the parasite develops and moves from one host niche to the next. Data detailing temporal and developmental changes are commonly presented in the form of heat-maps using hierarchical clustering (Fig. 11.2).

11.3.1 *F. hepatica* developmental stage transcriptomes

The first extensive analyses of *F. hepatica* gene transcription using next-generation sequencing technologies were carried out by Young *et al*. (2010), who employed Roche 454 sequencing technology to generate 590,927 high-quality reads from mRNA of adult *F. hepatica*. Functional analysis of the assembled genes identified molecules consistent with the adult ES products, including all 160 proteins identified earlier by Robinson *et al*. (2009), in addition to a number of FABPs and redox enzymes (peroxiredoxin, FhPrx, and thioredoxin, FhTrx) (Young *et al*., 2010). This study was followed by the 454-sequencing project by Wilson *et al*. (2011), which focused on the molecules expressed in the adult tegument and identified a range of structural proteins, such as annexins and tetraspanins, transporters and membrane transport-associated ATPases (Wilson *et al*., 2011).

Gene transcription analysis of *Fasciola* spp. was greatly advanced with the development of short-read Illumina sequencing that increased sequence depth and coverage (Reuter *et al*., 2015; Goodwin *et al*., 2016; McCombie *et al*., 2019) and allowed the probing of life cycle stages that had been previously difficult to analyse. Thus, Cwiklinski *et al*. (2015a) reported the transcriptomes of several early life cycle stages, including the infective metacercarial stage, the NEJ (at 1, 3 and 24 h post-excystment (pe)), immature parasites taken from the liver parenchyma at 21 days post-infection (pi) and mature bile-duct dwelling adult parasites. Similarly, McNulty *et al*. (2017) reported comparable results in their transcriptome analysis of *F. hepatica* eggs, metacercariae and adult stages. The comprehensive transcriptional profile for *F. hepatica* revealed that, as the life cycle progresses, more genes are 'switched on' and that the genes display different levels of transcription as the parasite grows and develops, suggesting that specific genes are important for specific parasite stages (Fig. 11.2A). This was further demonstrated by clustering the genes based on their pattern of transcription. Functional analysis of these clusters identified over-representation of distinct biological processes associated with specific parasite stages (Cwiklinski *et al*., 2015a). Genes associated with the Wnt signalling pathway, which regulates critical processes involved in cell determination, migration and proliferation, and GPCR and neuropeptide signalling pathways were expressed throughout development from metacercariae stage to the immature parasites; however, these processes were downregulated within the adult parasites (Fig. 11.2B). Energy catabolism and metabolism and nucleic acid metabolism are key processes highly transcribed by the immature and adult parasites (Fig. 11.2B).

11.3.1.1 Transcription of multi-copy gene families

Consistent with the important functions that peptidases play throughout the life cycle, the

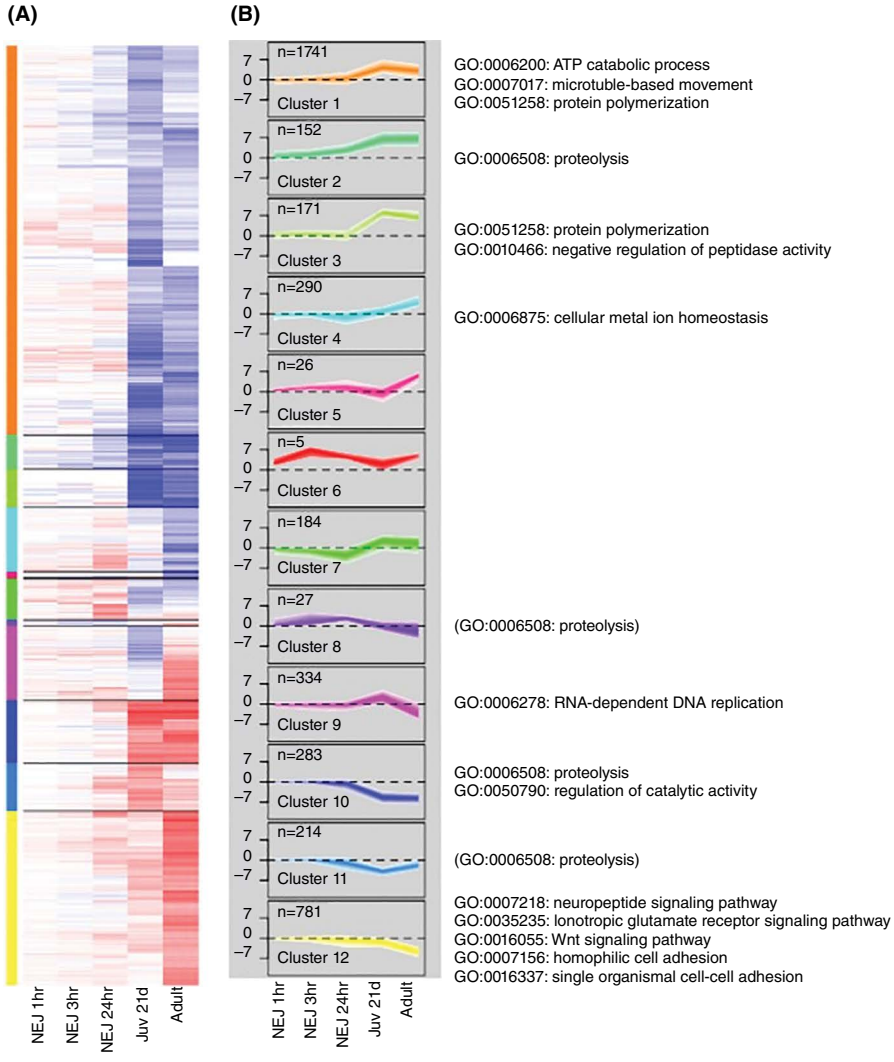

Fig. 11.2. Graphical representation of gene transcription across the *F. hepatica* life cycle. **(A)** Heatmap showing the profile of gene expression with genes highly transcribed in blue and genes with lower levels of transcription in red. Genes are clustered by the pattern of their transcription. **(B)** Graphs highlighting the transcription profile of the clusters and the types of biological processes that are associated with each cluster, represented as gene ontology (GO) terms. (Figure was originally printed in Genome Biology, BioMed Central (BMC); Cwiklinski *et al*., 2015a.)

genes associated with expression and control of cathepsin-like peptidases were the most significantly differentially regulated genes by the *F. hepatica* parasite stages (Fig. 11.2) (Cwiklinski *et al.*, 2015a). The cathepsin peptidases belong to a large gene family that arose following gene duplication and functional divergence (Robinson *et al.*, 2008; Turk *et al.*, 2012; Cwiklinski *et al.*, 2019). Both *F. hepatica* genome assemblies show that these families comprise at least 23 cathepsin L sequences and 11 cathepsin B sequences, data that were corroborated by transcriptome analysis (Cwiklinski *et al.*, 2019). Phylogenetic analysis of the cathepsin L gene sequences separates the family into five well-supported clades, FhCL1–FhCL5, that are functionally diverse and

differentially expressed by the three major life cycle stages: NEJ, immature flukes and adult flukes (Robinson *et al*., 2008; Cwiklinski *et al*., 2019). Similarly, the 11 cathepsin B sequences separate into two groups that display specific expression within the NEJ and adult stages, respectively (Cwiklinski *et al*., 2019).

Gene family expansion is a key feature of *F. hepatica* adaptation and survival. Comparable to the expanded cathepsin peptidase family, the proteins that regulate cathepsin peptidase activity, namely cystatins and Kunitz-type (KT) inhibitors, are also encoded by multi-membered gene families (Cancela *et al*., 2017; Smith *et al*., 2020). The cystatin gene family comprises three single-domain cystatins, termed FhStf-1, FhStf-2 and FhStf-3 (Cancela *et al*., 2017). All three cystatin proteins are potent inhibitors of both *F. hepatica* and host cysteine peptidases and their genes are transcribed at high levels throughout the life cycle, with particularly high levels within the adult stages, although FhStf-2 is the most abundant (Cwiklinski and Dalton, unpublished).

The KT inhibitors are typical inhibitors of serine peptidases (Ranasinghe and McManus, 2013). Unusually, the *F. hepatica*-specific KT inhibitors have evolved to inhibit predominantly cathepsin peptidases, adding to the repertoire of inhibitors that *F. hepatica* has in its arsenal to regulate its own cathepsin peptidases (Smith *et al*., 2016). The KT gene family consists of seven members, of which the most highly expressed belong to the FhKT1 group (FhKT1.1, FhKT1.2 and FhKT1.3), which display a marked upregulation in transcription by the NEJ 24 h post-excystment and adult parasites, compared with the metacercariae and immature fluke stages (Smith *et al*., 2020). The other FhKT genes display distinct patterns of transcription; FhKT2 and FhKT5 are predominantly transcribed by the adult parasites, whereas FhKT4 is transcribed solely by the metacercariae and NEJ stages (Smith *et al*., 2020). In contrast to the majority of the FhKT family, FhKT4 is predicted to be an inhibitor of trypsin-like serine proteases. Interestingly, as the FhKT4 homologue in *F. gigantica* is abundantly transcribed by the cercarial stage, it is suggested to play a role in anti-trypsin defence within the snail intermediate host (Smith *et al*., 2020).

The serine protease inhibitors (serpins) are also encoded by a multi-membered gene family that displays differential transcription across the life cycle (De Marco Verissimo *et al*., 2020; Sánchez Di Maggio *et al*., 2020). The lack of serine proteases within the *F. hepatica* secretomes implies that the secreted serpins play a role in the regulation of host rather than parasite serine proteases (De Marco Verissimo *et al*., 2020). Seven serpin genes were identified in the *F. hepatica* genome, which separate into four clusters based on phylogenetic analysis of the sequence containing the reactive centre loop (1. FhSrp1 and FhSrp3; 2. FhSrp2 and FhSrp4; 3. FhSrp5 and FhSrp6; 4. FhSrp7). The transcriptional profile mirrors the identification of specific serpins within the ES products, with the highest level of transcription observed in the metacercariae and NEJ stages (De Marco Verissimo *et al*., 2020). Only FhSrp1 and FhSrp3 are transcribed in high abundance by the adult parasite. Interestingly, FhSrp1 and FhSrp2, as representatives of clusters 1 and 2, have distinct inhibitory profiles that suggest they have specific roles; FhSrp1 inhibits kallikrein whereas FhSrp2 is a potent inhibitor of chymotrypsin (De Marco Verissimo *et al*., 2020; Sánchez Di Maggio *et al*., 2020).

11.3.1.2 Key metabolic pathways associated with the F. hepatica *life cycle*

Interrogation of the transcriptome data showed that the metacercariae, a stage previously thought to be dormant awaiting activation within the mammalian host, are metabolically active (Cwiklinski *et al*., 2018). Specifically, the metacercariae upregulate 1607 genes relative to the other NEJ stages within the 24 h period post-excystment. These genes are associated with biological processes such as superoxide metabolism, protein catabolism, regulation of Wnt signalling and cellular glucose homeostasis (Cwiklinski *et al*., 2018). Metacercariae are non-feeding, relying on endogenous glycogen stores, which are not replaced until 24 h post-excystment. The glycogen stores are broken down via the glycolysis/gluconeogenesis pathway. Transcriptome analysis revealed that the components of the pathways related to aerobic energy metabolism, including the glycolysis/gluconeogenesis pathway, display comparable transcription to the active NEJ at 3 h post-excystment. Similar levels of transcription were observed for all the metabolic pathways analysed and further emphasized that

this stage is not dormant. To ensure their survival, the parasites must rapidly trigger the excystment process, infect the mammalian host and begin feeding while they have sufficient energy stores to do so (Cwiklinski *et al.*, 2018).

Comparative analysis of the early juvenile stages by Cwiklinski *et al.* (2018) demonstrated that the transcription of the metabolic pathways related to aerobic energy metabolism are tightly regulated, facilitating the transition from glycogen metabolism by the metacercariae and the NEJ 1 h post-excystment to glycogen catabolism and synthesis by the NEJ 24 h post-excystment (NEJ 24h). In particular, the components of the glycolysis/gluconeogenesis pathway are highly transcribed by the NEJ 24h relative to the earlier NEJ stages. Similarly, the NEJ 24h display increased transcription of several metabolic pathways, including pathways associated with the synthesis of genes, proteins and glycans, reflecting a developing parasite primed for migration through the mammalian host.

Once *F. hepatica* has reached the host liver, the parasite regulates the transcription of many of its genes, with progressively more genes being highly transcribed in preparation for the migration through the host liver parenchyma (> 8000; Cwiklinski *et al.*, 2015a, 2021). Functional analysis of these genes revealed an enrichment for processes associated with transcription and translation, highlighting the intensive production of proteins by the immature flukes (Cwiklinski *et al.*, 2021). Increased transcription of the signal transduction pathways that drive cellular differentiation and proliferation mediating growth and development was also observed. These pathways play a critical role in regulating the metabolic pathways and energy consumption of the rapidly growing immature flukes. Specifically, the PI3K-Akt signalling pathway regulates neoblast/pluripotent stem cells that drive growth and development in *F. hepatica* (McCusker *et al.*, 2016; Cwiklinski *et al.*, 2018).

As the immature flukes grow and develop into sexually mature adults, oxygen is no longer able to diffuse throughout the parasite, resulting in the processes used for energy metabolism switching to anaerobic dismutation via aerobic acetate production (Tielens *et al.*, 1982) (see Chapter 8, this volume). This change can be observed at the gene and protein levels within the immature flukes by the identification of components of both the TCA/Krebs cycle, which requires oxygen, and the glyoxylate and dicarboxylate metabolism pathway, which occurs under anaerobic conditions (Cwiklinski *et al.*, 2021).

11.3.1.3 Applications of F. hepatica *transcriptome data*

Transcriptome sequencing is being used to assess the effects of drug treatment on *F. hepatica* and the potential changes in gene expression in drug-resistant isolates (Radio *et al.*, 2018; Miranda-Miranda *et al.*, 2021). The study by Radio *et al.* (2018) analysed the transcriptional profile of three isolates that displayed different drug susceptibilities, namely: resistance to both TCBZ and ABZ; resistance to ABZ but susceptibility to TCBZ; and susceptibility to both drugs. Their transcriptome analysis demonstrated that the isolate resistant to both TCBZ and ABZ displayed lower overall gene transcription compared with the other isolates. Specifically, lower levels of transcription were observed for the cytoskeletal associated genes, α-tubulin, β-tubulin, kinesins and dyneins. However, the values relating to β-tubulin transcription were not statistically different from the susceptible isolate. Moreover, the pattern of expression did not follow that of the ABZ-resistant isolate, which displayed higher levels of β-tubulin transcription compared with the other two strains, implying that the dual resistant isolate may display different levels of ABZ resistance compared with the ABZ-alone resistant isolate. The isolates used by Radio *et al.* (2018) have been experimentally maintained for several years, which may have affected the genetic diversity and gene transcription of these isolates, as well as their drug resistance profile. In addition, as the authors did not drug-treat the animals used in the study, they did not apply a selection pressure to their isolates, which may have resulted in a different transcriptional profile. A recent study by Miranda-Miranda *et al.* (2021) aimed to address this issue by exposing drug-sensitive and resistant *F. hepatica* isolates to TCBZ and ABZ in culture prior to transcriptional sequencing. Their *Data in Brief* article describes the identification of many novel genes and 5213 long non-coding RNA that may be playing a role in the differential expression between these drug-susceptible/resistant isolates.

The function of a large proportion of the genes identified within the *F. hepatica* genome remains unknown, because the current databases available for annotation represent mainly non-trematode organisms. To elucidate the function of these genes and specifically identify those genes involved in host immune response evasion, Haçarız *et al*. (2015) applied in-depth Illumina sequencing to study adult *F. hepatica* molecules that interact with the host immune system. They identified 64 immunomodulation-related transcripts, representing 62 previously uncharacterized genes and two genes associated with CD59, based on known virulence factors within publicly available databases, such as VIOLIN (He *et al*., 2014) and Victors (Sayers *et al*., 2019), and by assessing which genes are under positive selection due to their importance in immune evasion. Further investigation is required to determine how these unknown molecules interact with the host. Comparative analysis with *C. sinensis* and *Opisthorchis viverrini* reveals that the bile-dwelling trematodes may share common immune evasion strategies based on their virulence and immunomodulation genes, of which 36 are shared between the three species (Haçarız and Sayers, 2018).

11.3.2 *F. gigantica* developmental stage transcriptomes

Young *et al*. (2011) reported the first characterization of the *F. gigantica* adult transcriptome using Illumina sequencing, which allowed a comparative investigation between *F. hepatica* and *F. gigantica*, particularly those molecules that are important at the host–parasite interface. This study was further complemented by the comprehensive transcriptome analyses by Zhang and colleagues of eight *F. gigantica* life cycle stages, which profiled the complex and dynamic changes the parasite undergoes during its life cycle and represents the first *Fasciola* spp. transcriptomes of the snail-associated stages (Zhang *et al*., 2017, 2019b).

Interrogation of the data revealed that gene transcription is host-associated, with the snail-associated stages transcribing a different subset of genes to the mammalian-related counterparts, facilitating host-specific interactions (Zhang *et al*., 2019b). Consistent with the high levels of activity shown by the miracidia to seek the snail host, this stage is transcriptionally active, specifically expressing genes involved in location and invasion of the snail host. However, in comparison with the other stages, the aerobic metabolic pathways that are reliant on glycogen energy stores exhibit lower relative transcription. The rediae transcribe genes associated with transcription, translation and repair, reflective of the clonal expansion that this stage is undergoing within the snail. Comparable to the analysis by Cwiklinski *et al*. (2018) of the *F. hepatica* metacercariae transcriptome, the *F. gigantica* metacercariae are similarly transcriptionally active, expressing genes involved in regulating metabolic processes to maintain homeostasis while encysted on pasture.

A key feature associated with the *Fasciola* genus is the reliance on the cathepsin peptidases for a myriad of biological processes by all the life cycle stages (Cwiklinski *et al*., 2019). An abundance of both the cathepsin L peptidases (37 transcript clusters) and cathepsin B peptidases (24 transcript clusters) were observed within the *F. gigantica* transcriptomes, which display developmental regulation (Zhang *et al*., 2019b). Intriguingly, this study identified a novel subset of cathepsin L peptidases that are highly transcribed by the rediae. In addition, a distinct profile for the cathepsin B peptidases was observed, highlighting the importance of these peptidases for the mammalian host life cycle stages in contrast to the egg, miracidia and rediae stages (Zhang *et al*., 2019b).

Comparative analyses revealed high levels of homology between the *F. gigantica* and *F. hepatica* genes (about 71%) (Zhang *et al*., 2019b), consistent with the study by Young *et al*. (2011). However, these genes display different expression profiles within comparable life cycle stages, resulting in species-specific enrichment of key biological processes such as cathepsin peptidase regulation by the *F. gigantica* eggs, and mannose metabolism and iron binding by the adult *F. gigantica* parasites. Similarly, analyses of the most abundantly transcribed genes for both species did not display comparable levels of gene transcription, suggesting species-specific processes for gene regulation, which may be initiated by the interaction with the species-specific intermediate/definitive host.

11.4 Non-coding RNAs

Gene transcription and translation processes require a myriad of RNAs that do not code for protein (non-coding or ncRNAs), which can be broadly separated into housekeeping ncRNAs and regulatory ncRNAs (Hombach and Kretz, 2016; Zhang *et al.*, 2019a). The housekeeping ncRNAs, including ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs), are expressed in all cells and play critical roles in the removal of introns during RNA splicing, the chemical modification of RNA and protein synthesis. The regulatory ncRNAs control gene expression at the transcriptional and post-transcriptional levels and are further classified as: (i) small ncRNAs (sequences < 200 bp), which include microRNAs (miRNAs) and the RNA interference pathway associated small interfering RNAs (siRNAs); and (ii) long ncRNAs comprisimg sequences > 200 bp that do not code for proteins (Zhang *et al.*, 2019a). To date our knowledge of *Fasciola* spp. specific ncRNAs is limited to miRNAs (as described below) and a small subset of sequences relating to tRNA fragments (Hu *et al.*, 2021).

11.4.1 MicroRNAs

MicroRNAs (miRNAs) were first discovered in the free-living nematode *Caenorhabditis elegans* when it was found that the *lin-4* and *let-7* genes, both necessary for the regulation of developmental progression, did not produce mRNAs. Instead, they encoded very short RNAs of approximately 22 nucleotides (nt) in length (Lee *et al.*, 1993; Reinhart *et al.*, 2000). When it was also found that these short *lin-4* and *let-7* RNAs had imperfect complementarity to conserved sites within the 3′ untranslated region (UTR) of regulatory genes, it was proposed that small ncRNAs mediated the regulation of gene expression through antisense interactions (Lee *et al.*, 1993; Olsen and Ambros, 1999; Reinhart *et al.*, 2000). Subsequently, the sequence and temporal pattern of expression of *let-7* was shown to be conserved in higher organisms (Pasquinelli *et al.*, 2000). With the advent of genome sequencing, numerous different miRNAs were identified in the genomes of diverse organisms, including humans, plants, viruses and parasitic helminths. Since not all of these were expressed in a temporal manner, it became evident that miRNAs had a much broader biological role than merely regulating the timing of developmental transitions; indeed, it emerged that all mRNAs were likely regulated by one or more miRNAs (Friedman *et al.*, 2009).

The synthesis of mature miRNAs occurs through different processes; however, the canonical miRNA biogenesis pathway in all animal and plant species (Chong *et al.*, 2010) begins within the nucleus when non-coding miRNA genes are transcribed by RNA polymerase II (Pol-II) to create a stem-loop structure that is the primary miRNA (pri-miRNA) (Fig. 11.3) (Cai *et al.*, 2004; Lee *et al.*, 2004). Pri-miRNAs are similar to mRNAs, in that they are 5′ 7-methylguanosine (m7G)-capped and polyadenylated at their 3′ ends. But the pri-miRNA is processed by a microprocessor complex containing an RNase III, Drosha, to yield stem-loop structures of around 70 nt termed precursor-miRNAs (pre-miRNA) (Nguyen *et al.*, 2015). The pre-miRNAs are then exported to the cytoplasm by Exportin-5 (XPO5) where a second RNase III, Dicer, cleaves the stem loop to form an imperfect duplex of around 22 base pairs (bp) comprising a guide strand and a passenger strand (miRNA*) (Hutvagner *et al.*, 2001). The guide strand is incorporated into the RNA-induced silencing complex (RISC) and loaded on to one of the four Argonaute subfamily proteins (Ago1-4) which directs the complex to the target mRNA.

Target recognition occurs at the seed region (residues 2–8 of the 5′ end) of the miRNA guide strand and the 3′ UTR of the mRNA target (Bartel, 2009). Two outcomes can occur, depending on the complementarity of the binding: (i) a perfect complementarity in the seed sequence will result in the mRNA being degraded through the deadenylation of the PolyA tail or by direct cleavage of the mRNA itself, thus halting any further translation; or (ii) an imperfect complementarity will result in the miRNA remaining attached to the mRNA target, preventing the ribosome from elongating and effectively stopping translation (Chekulaeva and Filipowicz, 2009). For some miRNAs, the passenger strand of the duplex (miRNA*) can be functional, but it is more commonly degraded once the guide strand is incorporated into RISC.

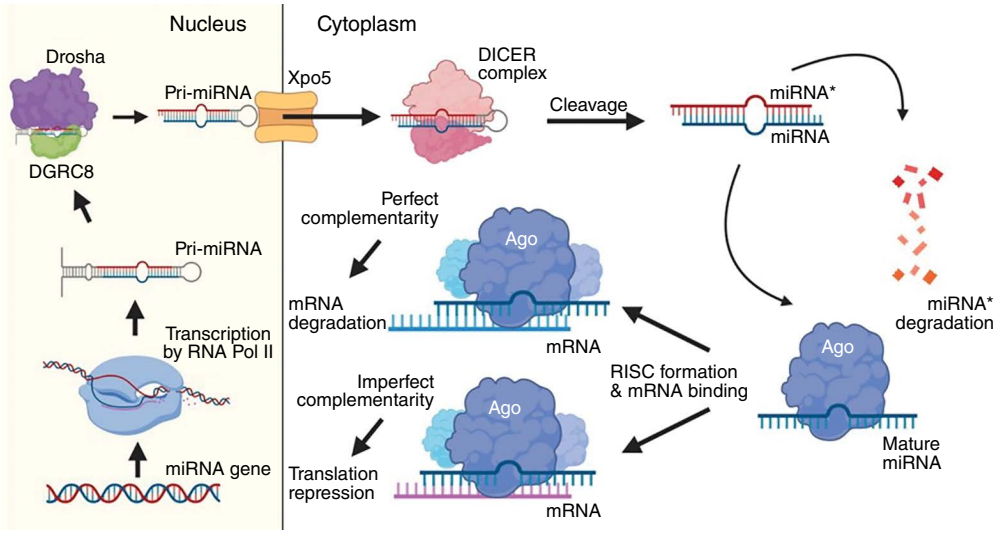

Fig. 11.3. The canonical pathway of the biogenesis of miRNAs and the mechanism of gene regulation. (Created with Biorender.)

Ambros *et al.* (2003) proposed a series of criteria to identify and annotate miRNAs and to distinguish them from other non-coding RNAs such as siRNAs, tRNAs, mirtrons and 5′ capped small transcripts. These criteria related to unique aspects of the miRNA canonical biogenesis pathway, in particular the processing by the RNAses Drosha and Dicer. Thus *bona fide* miRNAs were those that were expressed from endogenous transcripts that form hairpin structures, which are processed in a way that mature miRNAs from the two arms form a duplex, and that the 5′ end of each mature miRNA is processed with high consistency. Those miRNAs authenticated using this system were added to an online database, miRBase, which remains the largest repository of metazoan miRNA sequences.

However, more recently it was suggested that the hairpin structures of miRNAs may be more variable than originally thought and that the annotation of new miRNAs should require expression of the 20–26 nt sequence in both arms of the hairpin precursor with 2 nt offsets between the 5p and 3p arms (Fromm *et al.*, 2015). This proposal set the basis for a new set of criteria for the annotation of metazoan miRNAs and the establishment of the miRNA gene database MirGeneDB, which is curated manually. This database employs an internal nomenclature, which differs from the naming system utilized by miRBase that assigns the next number in succession (i.e. miR-3 was reported after miR-2, etc.) to new sequences, with paralogues indicated by a letter (if there is a difference of a single nucleotide) or a number (if the mature sequences are identical). The MirGeneDB nomenclature was developed to capture the phylogenetic relationship between miRNAs, where genes of common descent are assigned the same miRNA family name (Fromm *et al.*, 2020). Because MirGeneDB is a relatively new database, it contains fewer entries than miRBase.

11.4.1.1 *Identification of* Fasciola *miRNAs*

Identification of *de novo* miRNAs unique to one species is achieved using deep sequencing in tandem with tools that rank the probability of these sequences to be a genuine miRNA within a reference genome (Britton *et al.*, 2014). One of the software packages most commonly used is miRDeep2, which scores the likelihood of sequences representing genuine miRNA based on the folding energies of the genomic regions around the reads, the similarity of these regions to miRNA hairpins, evidence of processing by Drosha and Dicer, and similarity to sequences within the genome of species supplied to the tool (Friedländer

et al., 2012). In the absence of a complete genome, identification of miRNAs can be completed by reference to a comparative genome. This approach identifies homology in miRNA sequences by comparing sequencing data of a species to the genome of closely related species.

To date, *F. hepatica* RNA sequence data has been examined for the presence of miRNAs in several independent studies. The first of these was performed prior to the release of the parasite's genome and, therefore, putative miRNAs within the adult worm RNA sequencing data were mapped to the *Schistosoma japonicum* genome using the Short Oligonucleotide Alignment Programme (SOAP) (Xu *et al.*, 2012). This approach yielded the first repertoire of 16 *F. hepatica* miRNAs. Of these, eight were found to be homologous to *S. japonicum* miRNAs deposited in miRBase and were, therefore, deemed to be conserved sequences. The remaining eight miRNAs were classified as novel sequences, unique to *F. hepatica.* Subsequently, the data from this study and an additional sequencing dataset derived from the extracellular vesicles (EVs) of adult parasites were examined using a different bioinformatic pipeline (Fromm *et al.*, 2015). A modified version of MirDeep2 was used to predict miRNA loci within assembled contigs from *F. hepatica* genomic reads (from the 50 Helminth Genome Initiative). This workflow produced an expanded list of 55 miRNAs, consisting of 42 conserved miRNAs and 13 novel miRNA sequences. The eight conserved miRNAs described by Xu *et al.* (2012) were again identified within the adult miRNA sequences, validating their first identification. However, the putative eight novel miRNAs were not found, most likely due to the use of an *F. hepatica* rather than *S. japonicum* reference genome. The sequences for all 55 miRNAs were found in both the cellular miRNA of adult parasites and in EVs, but several miRNAs were enriched within the EVs, most notably miR-125b, miR-87, miR-2a-B, miR-2b-A and miR-1993.

Fontenla *et al.* (2015) expanded the identification of *F. hepatica* miRNAs to the NEJ. Initially, the cleaned small RNA reads from the NEJ sequences were matched with miRNAs listed on Sanger, Rfam, and functional RNA databases, and also to a local database which consisted of RNAs documented in previous publications but absent from the RNA databases. All reads identified by this process were regarded as conserved miRNAs. Sequences that produced no match with lengths of 20–23 nt were then mapped to an assembled genome comprising *F. hepatica* partial genomic reads generated by the Welcome Trust Sanger Center using miRDeep2. These resultant sequences, which were regarded as novel miRNA candidates, were then mapped to the two genome assemblies to confirm their location on the genome. This pipeline identified a total of 45 putative miRNAs, of which 34 shared sequence identity to miRNAs previously found within the adult miRNA and belonging to ancient miRNAs conserved in metazoans. The expression level of the miRNAs was quite varied with a range of single to hundreds of thousands of reads reported. Surprisingly, only five miRNAs represented 90% of total reads in the NEJ sequences; miR-125b was the most abundant and accounted for more than three-quarters of the total reads.

Of the 72 miRNAs identified from the above studies, only 38 fit the standard criteria for annotation and are currently featured in the miRBase database (F_hepaticav1), suggesting that these are the only sequences regarded as authentic miRNAs. However, the sequence data from these three studies was recently re-examined according to the MirGeneDB system of annotation (Fromm *et al.*, 2017; Ovchinnikov *et al.*, 2020). Compiling the findings from all of these studies suggests that, in addition to the 38 miRNAs listed on miRBase, *F. hepatica* expresses another 39 bona fide miRNAs. Of these 77 miRNAs, 36 were expressed in both NEJ and adult parasites, 15 were specific to NEJ and 26 were exclusive to adult parasites and their EVs (Ricafrente *et al.*, 2020).

The miRNome of *F. gigantica* was most recently characterized in a study that examined the small RNA sequences from eight stages of the parasite's life cycle: egg, miracidium, redia, cercaria, metacercaria, two juvenile stages and adult (Hu *et al.*, 2021). Similar to the bioinformatic pipeline applied to *F. hepatica*, sequencing reads were mapped against a draft genome assembly of *F. gigantica* and miRDeep2 was used to predict potential miRNAs. The likelihood of these being bona fide miRNAs was determined using the miRGeneDB criteria for annotation. This approach identified 56 conserved miRNAs and four that were specific to *Fasciola.* Despite the much broader range of life cycle stages that were analysed, the miRNA complement of *F. gigantica*

was identical to sequences that previously described for *F. hepatica*. Furthermore, alignment of the miRNA sequences revealed a high degree of conservation in the mature and star arms of most miRNAs for both species, except for two miRNAs (mir-1992_3p and Mir-Novel-3_3p) with nucleotide variations in the mature arms. Hu *et al.* (2021) suggested that these minor differences in miRNA sequences could be applied to taxonomic differentiation of the two fluke species.

11.4.1.2 Biological function of Fasciola *miRNAs*

The function of a given miRNA is determined by the biological role of the product of its gene target. A single miRNA, however, can have multiple targets and, conversely, the 3′ UTR of an mRNA can contain various binding sites for multiple miRNAs. Thus, the first step in the identification of putative miRNA targets is commonly achieved *in silico* using several computational programs, each with slightly different algorithms (reviewed in Thomas *et al.*, 2010). Typically, these tools score the interaction of a miRNA sequence with likely binding sites in the 3′ UTR of mRNAs. Confidence in the authenticity of a particular gene as a target is based on a number of features, including: (i) sequence complementarity between the miRNA seed sequence and the target sequence; (ii) evolutionary conservation of the predicted miRNA binding sites; (iii) location of binding sites; and (iv) the free energy of the miRNA–mRNA heteroduplex. To provide the most robust analysis, multiple different predictive tools are typically applied to identify a putative gene target for any given miRNA, with the premise that if a gene is predicted to be a target by a variety of tools, there is a greater likelihood that it is a genuine target.

As many *Fasciola* miRNAs are conserved, functionality can also be inferred by their biological role in other species. For example, *bantam*, one of the most ancient miRNAs that is highly represented in the NEJ, has been implicated in the regulation of cell proliferation and apoptosis (Brennecke *et al.*, 2003; Fontenla *et al.*, 2015). Similarly, *let-7*, the miRNA characterized in *C. elegans* as a central regulator of the transition to adulthood, was found expressed by both NEJ and adult *F. hepatica* and may perform a similar biological role in this worm. Of interest, a recent comparison of the *Fasciola let-7* miRNAs revealed that the NEJ miRNA sequence differs slightly to that of the adult fluke and exhibits a different pattern of conservation. While the NEJ *let-7* aligned with Planaria, the adult fluke *let-7* was more closely conserved with other parasitic trematodes and mammalian sequences (Ricafrente *et al.*, 2020). This discovery suggests that developmental difference in *let-7* reflects the evolution of variants of the same miRNAs that diverged to become specific to distinct life stages of the parasite to ensure the regulation of mRNAs that are pivotal to the maturation of the worm. An essential next step to expand our understanding of the role of the *Fasciola* miRNAs (novel and conserved) in the developmental biology of the parasite is the identification of specific mRNA targets within the transcriptome of the different parasite life stages.

The availability of the *F. gigantica* stage-specific transcriptomes (Zhang *et al.*, 2019b) has facilitated the analysis of miRNA:mRNA relationships during the development of liver fluke (Hu *et al.*, 2021). The 3′ UTR within gene transcripts that were differentially expressed across each life stage were first identified by homology mapping to the *F. hepatica* and *S. mansoni* genomes. Next the miRNA binding sites within these 3′ UTR sequences were predicted using three different programs: Targetscan, RNAHybrid and mirSVR. Any target that was commonly identified by all three programs was assumed to be the most authentic gene target for any miRNA. The exploration of the functional properties of these *F. gigantica* genes suggests that the miRNAs fine-tuned the expression of genes that contributed to a range of biological processes, but most notably metabolism and transport.

Analysis of the *Fasciola*-specific miRNAs reveals that several of the sequences are homologous to mammalian miRNAs that are known to have an immune regulatory function. This implies that parasite-derived miRNAs also contribute to the host–parasite relationship, specifically modulating the host immune response to prevent parasite expulsion. Molecular analysis showing that many of these miRNAs are abundant within the cargo of *F. hepatica* EVs and that glycans on their surface direct a preferential internalization by macrophages provides strong support for this idea (Liu *et al.*, 2019; de la Torre-Escudero *et al.*, 2019). Amongst the miRNAs delivered to host

cells by EVs, *fhe-miR-125b* is the most abundant. Interestingly, the sequence of the seed region of this miRNA is identical to the mammalian *miR-125b*, which is known to regulate the activation of pro-inflammatory M1 macrophages (Tili *et al.*, 2007). Transfection of murine macrophages with a synthetic mimic of *miR-125b* resulted in the significant inhibition of TRAF6 signalling and prevented the activation of pro-inflammatory M1 macrophages (Tran *et al.*, 2021). Moreover, during mice infection, the *F. hepatica fhe-miR-125b* was found to be loaded on to the Ago-2 protein of macrophages *in vivo*, suggesting that the parasite-derived miRNA mimics the activity of the host miRNA and essentially hijacks the miRNA machinery to control the host immune response.

The continued characterization of the sequences and function of *F. hepatica* miRNAs will inform an understanding of the molecular pathways that contribute to the development of the parasite and of the interactions between host and parasite that supports the parasite's survival. In addition, information regarding the molecular signals that are unique to parasitism will be of enormous benefit to the development of novel strategies for infection control.

11.5 Proteomics Identify Proteins in Parasite–Host Interactions

Alongside advances made over the past two decades in techniques to analyse nucleic acids, progress in basic extraction protocols for soluble and membrane-bound proteins, together with more sophisticated methods for increasing the sensitivity of protein detection and characterization, principally mass spectrometry (MS) (Scherp *et al.*, 2011; Nature Methods Editorial, 2013), has allowed the sensitive and high-throughput analysis of complex protein preparations (Yarmush and Jayaraman, 2002; Brewis and Brennan, 2010; Kelly, 2020; Yang and Sun, 2021). Proteomic data can now be readily integrated with fundamental genomic/transcriptomic data to gain qualitative and quantitative data relating the expression of genes to their protein products, and to provide information of protein abundance, turnover and stage-expression (Fig. 11.4). Using proteogenomic techniques, gene prediction and annotation can be enhanced and splice variants and post-translational modifications (such as glycosylation, phosphorylation) elucidated (Ruggles *et al.*, 2017; Sotillo *et al.*, 2017). Proteomic techniques are also being used for *Fasciola* spp. differentiation (Sy *et al.*, 2020).

The mature adult *F. hepatica* parasites are readily obtained; hence, these have been characterized in most detail, with a particular focus on the molecules that the parasite excretes/secretes (ES products) into the host tissues. Molecules in the ES products most likely originate from the parasite's gut and tegument, from released vesicles/particles or via classical and non-classical secretory pathways. Using two-dimensional (2D) gel electrophoresis and subsequent characterization of specific spots by MS, Jefferies *et al.* (2000, 2001) showed that adult *F. hepatica* release an array of different functional molecules, including cathepsin L peptidases (FhCL), superoxide dismutase (SOD), peroxiredoxin (Prx), glutathione *S*-transferases (GSTs) and fatty acid-binding proteins (FABPs). Similarly, the study by Robinson *et al.* (2009) identified a comparable protein profile of 22 proteins, with the cathepsin L peptidases predominating. Since then, researchers have used various proteomic techniques and phylogenetic tools to elucidate how these proteins have evolved, diverged and adapted to their role in host–parasite interaction (Chemale *et al.*, 2006; Marcilla *et al.*, 2008; Robinson *et al.*, 2008; Morphew *et al.*, 2011, 2012, 2013, 2016; Cwiklinski *et al.*, 2015b; Di Maggio *et al.*, 2016; De Marco Verissimo *et al.*, 2020; Smith *et al.*, 2020; Stuart *et al.*, 2021).

Following the sequencing of the *F. hepatica* genome and comparable protein annotation, the available adult *F. hepatica* secretomes generated using both gel-based (Cwiklinski *et al.*, 2015b) and gel-free (Di Maggio *et al.*, 2016; Murphy *et al.*, 2020) proteomic approaches can be directly compared. In comparison to the number of proteins identified by Jefferies *et al.* (2000, 2001) and Robinson *et al.* (2009), the more recent analyses of the adult secretome identified in the range of 69–227 proteins (69 proteins, Cwiklinski *et al.*, 2015b; 202 proteins, Di Maggio *et al.*, 2016; 159 proteins, Murphy *et al.*, 2020; 227 proteins, Cwiklinski and Dalton, unpublished). These differences are likely reflective of the increasing sensitivity of proteomic technologies and the different protocols used for parasite

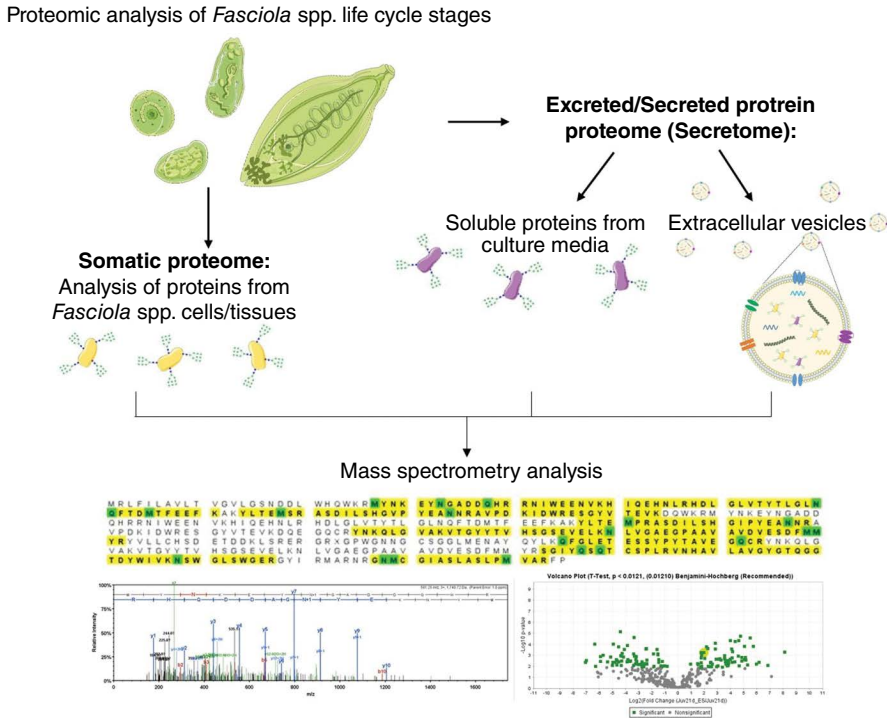

Fig. 11.4. Proteomic studies of *Fasciola* spp. proteins can be carried out on extracts from parasite cells and tissues, termed the somatic proteome, and the excreted/secreted parasite proteins, referred to as the secretome. During parasite culture, which simulates the host environment, the *Fasciola* spp. life cycle stages secrete proteins as soluble proteins and release proteins encapsulated in extracellular vesicles. The proteins are analysed using mass spectrometry, which results in peptide data that can be mapped back to a protein database for annotation and then used for quantitative analysis to discern variation in protein abundance between different samples, shown here as a volcano plot. Medical art provided by Les Laboratories Servier (https://smart.servier.com).

culture. Interestingly, these studies highlighted the turnover of the cathepsin L peptidases, whereby the concentration of these enzymes relative to the total protein secreted changes over time. The proportion ranges from 32.6% at 3 h (Di Maggio *et al*., 2016), to 10–22% at 5 h (which is predominantly found within the 15K microvesicles) (Murphy *et al*., 2020), to a final proportion of > 80% at 8 h (Robinson *et al*., 2009).

Consistent with these studies was the identification of *F. hepatica* cathepsin L peptidases within bile recovered from *F. hepatica*-infected sheep (Morphew *et al*., 2007). The adult liver fluke parasites reside within the immunologically safe environment of bile ducts, immersed in bile acids, phospholipids, cholesterol, bilirubin and inorganic salts (Farina *et al*., 2009). The abundance of the cathepsin L peptidases, which the parasites use for degrading host blood (Lowther *et al*., 2009), emphasizes that the main goal of the adult parasites is to secure nutrients for the production of eggs.

The parasite surface tegument has also been a focus for proteomic studies, as it represents the direct interface between parasite and host, and it has long been known that its rapid turnover is critical to prevent the attachment of immune effector cells (Hanna, 1980; Rogan and Threadgold, 1984; Hanna *et al*., 2019) (see Chapter 3, this volume). Not surprisingly, proteomic characterization of the adult tegument found it to be enriched in structural proteins, transporters, proteins involved in secretory pathways and antioxidant enzymes

(Wilson *et al.*, 2011; Haçarız *et al.*, 2012). Some of these molecules formed part of the 'tegumental immunoprecipitate' that formed when live adult *F. hepatica* were cultured in purified IgG from *F. gigantica*-infected Thin Tailed sheep (Cameron *et al.*, 2017). Therefore, antibodies elicited against *F. gigantica* during infection react with *F. hepatica* tegumental molecules, evidence of the immunogenic similarity in their structures.

Although the mechanism of action of the anthelminthic drug triclabendazole (TCBZ) is not fully understood, adult fluke secretome analysis suggests that the drug affects liver fluke metabolism (Chemale *et al.*, 2010). Morphew *et al.* (2014) showed that adult parasites that are susceptible or putatively resistant to TCBZ can be discerned following exposure to the active TCBZ metabolite triclabendazole sulfoxide (TCBZ-SO) by the proteomic signature of their ES products. Susceptibility to TCBZ was indicated by the presence of actin, gelsolin, DJ-1 and triose phosphate isomerase, whereas putative resistance was indicated by the presence of calreticulin, cathepsin L proteases and enolase, providing potential biomarkers for future TCBZ efficacy studies.

Proteomic analysis of the early developmental and migratory stages of *F. hepatica* is more challenging, given their small size and the difficulty in locating them in host tissues. However, an understanding of the proteomic profiles of these stages is crucial to develop vaccines or drugs that can prevent the migration of the parasite into the liver, when most of the clinical manifestations of fasciolosis are endured. Investigation of the early-stage NEJ has been facilitated by methods that stimulate excystment of parasites from the metacercarial cysts *in vitro* (see Chapter 1, this volume) and culturing conditions that enable the *in vitro* development of the parasites (McCusker *et al.*, 2016). Comparing the proteins secreted by the early infective stages, namely the NEJ, and the immature fluke 21 days pi with those secreted by the adult parasites has allowed stage-specific proteins to be determined (Robinson *et al.*, 2009; Di Maggio *et al.*, 2016; Cwiklinski *et al.*, 2018, 2021).

Initial analysis of the NEJ secretome by Robinson *et al.* (2009) identified 29 proteins. Consistent with the adult parasite secretome, the NEJ secretome comprised a range of proteins, including cathepsin and legumain peptidases, peptidase inhibitors, redox enzymes, metabolic enzymes and structural proteins. The NEJ secretome profile also confirmed the first characterization by *N*-terminal sequencing of the NEJ-secreted proteins carried out in the Meeusen laboratory (Tkalcevic *et al.*, 1995), which also described an abundance of cathepsin L proteases and legumains (asparaginyl endopeptidase cysteine proteases).

Facilitated by the sequencing of the *F. hepatica* genome and improved proteomic technologies, NEJ secretome analyses carried out by Di Maggio *et al.* (2016) and Cwiklinski *et al.* (2018) identified a wider range of proteins, which are complemented by the analyses of the corresponding somatic proteomes. Notably, analysis of the metacercariae somatic proteome revealed that the abundance of proteins involved in metabolism was at comparable levels to that observed in the active NEJ parasite stage, consistent with the transcriptome data (see Transcriptomics, section 11.3 above), highlighting that this stage is not dormant (Cwiklinski *et al.*, 2018). In addition, a range of cathepsin and legumain peptidases was identified, which suggests that these proteins are stored within the encysted stage, primed for activation once they reach the duodenum of the mammalian host.

Using a gel-based approach, Cwiklinski *et al.* (2018) identified 159 proteins across the secretomes of NEJ 1 h, 3 h and 24 h post-excystment, of which 95 proteins were further analysed. The protein composition of the NEJ secretomes was consistent with that reported by Robinson *et al.* (2009) and protein abundance analysis revealed that, despite secreting a large number of proteins, 70% of the total protein secreted comprised only ten proteins, namely FhCB3, FhCL3 members, FhStefin-1, thioredoxin and four uncharacterized proteins (Cwiklinski *et al.*, 2018). Comparing the secreted protein profile with the somatic proteomes of the metacercariae and NEJ (1671 proteins) revealed that 88 proteins are shared between the two datasets. This result was not surprising, given the fact that the majority of proteins in the somatic proteome are involved in metabolism (Cwiklinski *et al.*, 2018).

The study by Di Maggio *et al.* (2016) used a gel-free approach and focused on the NEJ 48 h post-excystment, identifying 90 proteins in the secretome and 575 proteins in the corresponding somatic proteome. Consistent with other NEJ

secretomes, this study identified a number of peptidases and peptidase inhibitors, representing > 70% and < 10% of the total protein secreted, respectively, highlighting the important role these proteins play during the early stages of infection (Di Maggio *et al*., 2016). Comparative analysis of the somatic proteomes generated in the studies by Di Maggio *et al*. (2016) and Cwiklinski *et al*. (2018) found that 513 proteins were shared between all datasets, highlighting the key molecules expressed by the NEJ that are important, particularly for a range of metabolic pathways.

To further understand the roles played by proteins during infection, recent proteomic analyses of the NEJ secretomes has focused on analysing the profile of secreted proteins following interactions with the snail and mammalian hosts. Di Maggio and colleagues (2019) investigated whether the snail intermediate host would affect *F. hepatica* and the subsequent proteins it secretes once it has infected a mammalian host. Their analysis of the NEJ ES proteins from parasites derived from two snail intermediate hosts, *Lymnaea viatrix* and *Pseudosuccinea columnella*, highlighted differences in the resulting proteomes based on protein identification and abundance. Most particularly, key *F. hepatica* molecules, including cathepsin peptidases, Kunitz-type inhibitors and peroxiredoxin, displayed higher expression in the *L. viatrix*-derived parasites. However, it should be noted that the different snail species were infected with different *F. hepatica* isolates, namely isolates from Uruguay and US Pacific North West, which may also influence the resulting secretome profile.

To investigate the role the mammalian host plays in determining which proteins the parasite secretes, González-Miguel *et al*. (2020) cultured NEJ in the presence of murine intestinal cells. Analysis of the tegumental and somatic proteomes revealed that the co-culture with intestinal cells resulted in a different NEJ secretome profile. Specifically, co-culture resulted in an upregulation of key molecules associated with phosphorylation and proteolysis in the tegument proteome, and metabolic processes specifically implicated in nitrogen compound processing and cytoskeleton organization within the somatic proteome. This study highlights that interaction with host molecules and microenvironments has a direct impact on parasite development and survival processes. This information is crucial for the future development of *in vitro* NEJ culture growth techniques such as those described by McCusker *et al*. (2016).

The first investigation of the 21-day immature fluke secretome was carried out by Robinson *et al*. (2009), using gel-based proteomic techniques, and it identified 45 proteins, including a range of cathepsin-like peptidase isotypes and antioxidant enzymes, which is consistent with the migratory and feeding traits of this stage (see Chapter 1, this volume). Complementing this study, Cwiklinski *et al*. (2021) characterized the protein profile within the somatic proteome and the secretome using a gel-free proteomic approach. Consistent with the NEJ somatic proteome, the immature somatic proteome is dominated by molecules associated with metabolism, specifically relating to carbohydrate metabolism and signal transduction, reflecting the rapid growth and development the parasite is undergoing (Cwiklinski *et al*., 2015a; Cwiklinski *et al*., 2021).

The secretome analysis identified 210 proteins, of which the most abundant comprised cathepsin peptidases, predominantly FhCL2 and FhCL3, and cathepsin peptidase inhibitors, predominantly FhKT1, representing 36% and 42% of the total protein, respectively. The abundance of these key cathepsin peptidases and cathepsin peptidase inhibitors reflects the major role these molecules play in tissue degradation, migration, feeding and immunomodulation (Cwiklinski *et al*., 2019). This study also identified several other immunomodulatory molecules that are abundantly expressed by the immature flukes, such as fatty acid-binding proteins (FABP; Fh2, Fh3, Fh15) and the helminth defence molecule (FhHDM) (Cwiklinski *et al*., 2021).

The comparative analysis of the major life cycle stages associated with the mammalian hosts, namely NEJ, immature flukes and adult flukes, has revealed that these stages secrete a distinct profile of proteins, reflecting their stage-specific microenvironments/niches and roles. Key molecules that display differential secretion include the large cathepsin L peptidase family and their inhibitors, reflecting the stage-specific functions these proteins display (Cwiklinski *et al*., 2021).

There is only a small number of proteomic studies focusing on other *F. hepatica* life cycle stages. Proteomic studies of liberated eggs have shown a substantially different protein profile from the

other developmental stages of *F. hepatica* and that protein complexity increases as the miracidial stage matures. This molecular investigation of egg embryonation characterized 28 proteins within the somatic proteome from 200,000 eggs (Moxon *et al.*, 2010), of which several were comparable to the *S. mansoni* egg protein profile, including actin and enolase. Ferritin and GST-omega class proteins were also particularly abundant within the egg proteome. Key molecules that were statistically upregulated in the embryonated eggs included phosphoenolpyruvate carboxykinase (PEPCK), peptidyl prolyl isomerase, yolk ferritin and alpha crystallin containing heat shock proteins (FhHSP35α), which may play an important role for miracidial development (Moxon *et al.*, 2010).

Investigations of the ES products of the intra-molluscan stages also present challenges but Gourbal *et al.* (2008) managed to transform mother sporocysts (388,000 parasites) *in vitro* to generate sufficient protein for analysis. In this preparation, they identified 17 abundant proteins, in particular Cu/Zn superoxide dismutase and thioredoxin. These two anti-oxidant enzymes have also been identified within the secretome of the stages associated with the mammalian host (Jefferies *et al.*, 2001; Robinson *et al.*, 2009; Cwiklinski *et al.*, 2018, 2021), suggesting a similar process of detoxification of reactive oxygen species by stages within the snail and mammalian host.

11.6 Extracellular Vesicles

While most proteins found in the *F. hepatica* ES products are secreted in soluble form, others can be released within membrane-bound vesicles of various sizes, collectively referred to as extracellular vesicles (EVs). EVs are classified by their size and biogenesis and can be broadly separated into two major subtypes: exosomes and microvesicles (as reviewed by van Niel *et al.*, 2018; Teng and Fussenegger, 2020). Exosomes are typically 30–150 nm stemming from the endosomal pathway, while microvesicles range between 100 and 1000 nm and bud from the plasma membrane (van Niel *et al.*, 2018; Teng and Fussenegger, 2020). Analysis by differential centrifugation shows that the *F. hepatica* EVs could be broadly separated into two major sub-populations; large EVs (50–200 nm) that were collected following centrifugation at 15,000 ×*g* (15K EVs), and smaller exosome-like vesicles (30–100 nm), collected by centrifuging the supernatant at 120,000 ×*g* (120K EVs) (Marcilla *et al.*, 2012; Cwiklinski *et al.*, 2015b).

11.6.1 EV biogenesis and release

Exosome biogenesis occurs via two main routes: (i) the endosomal sorting complex required for transport (ESCRT)-dependent pathway that utilizes the four ESCRT subcomplexes (0, I, II and III); and (ii) the ESCRT-independent pathway that utilizes tetraspanins, including CD63 and sphingomyelinase (van Niel *et al.*, 2018; Teng and Fussenegger, 2020). Microvesicle biogenesis is less well characterized but likely involves several components of the ESCRT-dependent and independent pathways (van Niel *et al.*, 2018; Teng and Fussenegger, 2020). Interrogation of the *F. hepatica* genome identified all the components of the ESCRT-dependent pathway, except the charged multivesicular body protein 6 (CHMP6) from the ESCRT-III subcomplex. In contrast, only a few components of the ESCRT-independent pathway were identified (Cwiklinski *et al.*, 2015b). This preference for the ESCRT-dependent pathway was also observed within the proteomic data, with significant enrichment of proteins associated with this pathway (Cwiklinski *et al.*, 2015b; de la Torre-Escudero *et al.*, 2019). Analysis of the intra-mammalian *F. hepatica* life cycle stage transcriptomes revealed that, in contrast to the putative parasite cargo proteins that are developmentally regulated reflecting the stage-specific niches in which *F. hepatica* resides within the mammalian host, the genes associated with the EV biogenesis pathways are constitutively transcribed at similar levels in all stages assessed (Cwiklinski *et al.*, 2015b).

The exact mechanism and location of the release of the various *F. hepatica* EV subtypes is currently unclear. Initial studies suggested that they may be released following blebbing and/or damage to the outer tegumental surface (de la Torre-Escudero *et al.*, 2016). More in-depth analysis by immunohistochemistry and transmission electron microscopy (TEM) suggested that the larger 15K EVs were released from the gastrodermal

epithelial cells that line the fluke gut, while the parasite tegument plays a more important role for the active release of the smaller 120K EVs (de la Torre-Escudero *et al*., 2019). Proteomic analysis supported this analysis, which showed that the gastrodermis-associated *F. hepatica* cathepsin L1 (FhCL1) is a marker of the 15K EVs (Collins *et al*., 2004; Cwiklinski *et al*., 2015b), whereas the 120K EV proteome shares proteins found within the parasite tegument (22 proteins; Wilson *et al*., 2011).

However, the recent immunohistochemistry study carried out by Bennett *et al*. (2020), using an extensive panel of known EV markers and *in vitro* ligation experiments, has shown that the picture is more complex. Key markers for exosome-like EVs, such as ALG-2-interacting protein X (ALIX), Ras-related protein Ral-A (RalA), CD63 receptor, DM9 domain-containing protein (DM9), T cell immunomodulatory protein (TIP) and acid sphingomyelinase-like phosphodiesterase 3b (SMPDL3) were localized throughout the body of the liver fluke, predominantly in parenchymal cells. In particular, immunolocalization of these markers was observed within the parenchymal cells under the gastrodermis and below the tegument, in the sub-protonephridial parenchymal cells, within the tegumental syncytium and the protonephridial duct epithelium (Bennett *et al*., 2020) (see Chapter 3, this volume). This study suggests that the gut plays the major role for EV release, with the tegument contributing to a lesser degree. In addition, the protonephridial system may play a role in the release of the exosome-like 120K EVs.

11.6.2 EV uptake and function

An increasing number of studies have shown that helminth parasites, including *F. hepatica,* may use EVs to interact and manipulate their hosts at a cellular level (Coakley *et al*., 2015; Wu *et al*., 2018; Sotillo *et al*., 2020). EV to host-cell communication is mediated via cellular docking via surface ligands and/or cellular fusion and vesicle internalization (endocytosis) (van Niel *et al*., 2018). Following uptake, the role the EVs play is dependent on the composition of the EV cargo, which can comprise a range of proteins, lipids and miRNAs (Wu *et al*., 2018; Sotillo *et al*., 2020). To date, studies have shown that there is a predominance for the uptake of parasite EVs by host cells via endocytosis (Chaiyadet *et al*., 2015; Coakley *et al*., 2017; Kifle *et al*., 2020). This is likely to be the case for *F. hepatica*, but the exact mechanisms used are currently unknown and may be dependent on the cellular source of EV and the resulting host cell that is being interacted with.

Insight into the possible mechanisms of uptake can be drawn from the analysis of the EV surface. Characterization of the *F. hepatica* EV surface proteome by de la Torre-Escudero *et al*. (2019) identified a number of proteins that may facilitate the fusion between cell membranes (including myoferlin, dynamin-like EHD1 protein and GAPDH), suggesting that this is the mechanisms by which the EV cargo is deposited into the host cellular cytoplasm.

The *F. hepatica* EVs also display oligosaccharides on their surface that are predominantly mannose-bearing glycoconjugates, which are distinct from the outer tegumental glycan profile (de la Torre-Escudero *et al*., 2019; Murphy *et al*., 2020). The presence of the glycan coat on the *F. hepatica* EVs supports the idea that these are actively synthesized and released, rather than being derived from blebbing of the outer tegumental surface (de la Torre-Escudero *et al*., 2016). Shaving of the surface oligosaccharides by the glycosydase PNGase F blocks the internalization of *F. hepatica* EVs by macrophages, suggesting that the surface glycans play a role in EV–host-cell recognition/communication (de la Torre-Escudero *et al*., 2019). Moreover, the uptake of EVs by macrophages was increased following the addition of anti-*F. hepatica* antibodies, or antibodies specific to EV surface proteins (DM9-containing protein, myoferlin, aSMase and CD63 receptor) (de la Torre-Escudero *et al*., 2019). Despite this process being more reflective of increased phagocytosis rather than directed uptake of the EVs, this is an example of how *F. hepatica* potentially manipulates a host defence strategy to its advantage, directing the EV cargo molecules to the endolysosomal system where they can modulate the immune cells.

Based on protein concentrations, the EVs represent only a small proportion of the *F. hepatica* adult secretome (15K EVs: 8.6%; 120K EVs: 3.6%) (Cwiklinski *et al*., 2015b). However, as many of the proteins identified within the *F. hepatica* EV proteomes lack a signal peptide for

classical secretion, the EVs offer an alternative route for parasite protein secretion/excretion (Cohen *et al.*, 2020). In addition, proteomic studies have shown that the EVs contain potent immunomodulators/virulence factors that can play an important role in the manipulation and control of the host immune response via direct interaction with immune cells. This role is also supported by the study by Roig *et al.* (2018), which showed that 120K EVs modulate the inflammatory responses in dextran sulfate sodium-induced colitis that is independent of B and T cells. Similarly, Murphy *et al.* (2020) demonstrated that co-culture with *F. hepatica* EVs activates dendritic cells, although the resulting FhEV-stimulated dendritic cells do not play a role in activating Th2 and T regulatory immune responses.

The first report and proteomic analysis of adult *F. hepatica* exosome-like EVs was made by Marcilla *et al.* (2012) using differential centrifugation, which identified 79 *F. hepatica* proteins consistent with those previously identified in the adult fluke ES products, and 19 host proteins, including a range of metabolic enzymes and proteins typically associated with exosomes. The *F. hepatica* EV proteomic studies that followed exploited the available *F. hepatica* genome and transcriptome datasets, allowing for more comparative analyses between studies. These proteomic analyses highlighted that the composition of the two EV preparations, namely 15K microvesicle-like and 120K exosome-like EVs, are different and reflect the processes/locations of their release into the extracellular environment (Cwiklinski *et al.*, 2015b; de la Torre-Escudero *et al.*, 2016, 2019; Murphy *et al.*, 2020).

The EV subtype proteomes include a range of proteins typically found with the adult secretome, including various proteases, protease inhibitors, metabolic enzymes and cytoskeletal/structural proteins (Cwiklinski *et al.*, 2015b; Murphy *et al.*, 2020). Based on protein abundance, both EV subtype proteomes included representatives of the cathepsin L peptidases and the serine and cysteine protease inhibitors protein families; however, there are EV subtype preferences (15K EV: FhCL1_1, FhCL2, FhSrp1, FhSrp2, FhSrp3, FhSrp4, FhStf1; 120K EV: FhCL1_2, FhCL5, FhSrp6; multi domain cystatin, FhCys1). Similarly, the 15K EV proteome displayed abundant representation of leucine aminopeptidases, fatty acid-binding proteins (Fh_2 and Fh_3) and members of the thiol-dependent anti-oxidant system (FhPrx, FhTrx and FhTGR). In contrast, the 120K EV proteome displays a greater abundance of glutathione *S*-transferases of the mu, omega and sigma classes, ferritins and saposin.

Davis *et al.* (2020) have shown that, in addition to their role in manipulating the host immune response, *F. hepatica* EVs may also play a role in the sequestration of TCBZ and other toxic xenobiotic metabolites. Adult *F. hepatica* parasites cultured in the presence of TCBZ and its metabolites produced at least five times more EVs than the parasites cultured in the absence of drug. TCBZ and the TCBZ-sulfoxide metabolite were found to be present within these EVs by mass spectrometry analyses, which also detected negligible levels of TCBZ sulfone. Despite the known effects of TCBZ on the parasite tegument that lead to tegumental tissue damage, the morphology of the EVs and the route of EV release were not affected by the drug treatment. The uptake of TCBZ and its metabolites by the EVs was postulated by Davis *et al.* (2020) to be a method by which the parasite reduces the availability of the TCBZ compounds to enhance parasite survival.

The study of *F. hepatica* EVs has primarily focused on adult fluke, due to the availability of samples and the large quantity of ES products that can be recovered. However, a recent study by Sánchez-López *et al.* (2020) showed that EVs are secreted by other developmental stages, including eggs and NEJ cultured up to 24 days. Based on size exclusion chromatography, the recovered EVs display a diverse range of sub-populations that may play developmental stage-specific functions that require further investigation.

11.6.3 EV isolation methods

Within the EV research field a number of separation and concentration protocols have been described (Doyle and Wang, 2019; Stam *et al.*, 2021). To date the majority of helminth studies, including several of the *F. hepatica* EV studies, have relied on differential centrifugation as the main enrichment method (Sotillo *et al.*, 2020). However, limitations of the differential centrifugation-based protocols, including extremely high centrifugal forces that impact on EV integrity, and the potential for low EV yields of high purity suitable for

downstream applications, has driven recent *F. hepatica* studies to use other methods. Davis *et al*. (2019) showed that EVs isolated by size exclusion chromatography were of high purity but smaller in size (~76 nm) and represented a lower range of EV diversity compared with EVs isolated from the same source using differential centrifugation. Proteomic analyses identified 276 proteins that were shared by both samples with a consistent profile, as described above for EVs isolated from adult *F. hepatica* ES products. An additional 116 proteins unique to the differential centrifugation sample, and 45 proteins identified solely in the size exclusion chromatography sample, were also identified by Davis *et al*. (2019).

Recently, a gravity flow method that can be used for highly dilute samples (Konoshenko *et al*., 2018) has been used by Murphy *et al*. (2020) to isolate EVs from the adult *F. hepatica* secretome. This method recovered EVs that ranged in size from 30 nm to 200 nm, representing both the 15K and 120K EVs isolated by differential centrifugation. Proteomic analysis of the gravity flow-recovered EVs identified 618 proteins, of which 443 were shared with the proteomic analyses of the differential centrifugation EVs and 175 were found solely in the EVs isolated by gravity flow. Like the study by Davis *et al*. (2019), additional proteins were also identified to be unique to the differential centrifugation sample (132 proteins) (Cwiklinski *et al*., 2015b; Murphy *et al*., 2020).

These studies highlight the fact that different methods will isolate comparable EVs based on size and their proteomic signature. In addition, the data suggest that the 15K and 120K EV populations are not overtly damaged by the process of differential centrifugation if proper protocols are followed. The significant advances in EV research have highlighted the important role they play in *F. hepatica* biology. As such, to move forward in the field of parasite EVs, standardized protocols should be used to ensure comparable and reproducible results.

11.7 Glycomics Identify Sugars in Parasite–Host Interactions

Glycomics describes a set of methods used to study the total glycan (sugar) structures (glycome) of an organism. Glycosylation is a major post-translational modification by which glycoconjugates such as glycoproteins and glycolipids are formed. Glycans show vast structural diversity and, in association with proteins or lipids, generate an enormous biological complexity in these molecules, often changing their biochemical and antigenic properties. Unsurprisingly, glycans are the main stimulating agent of the host's immune system during parasitism. Since the sugar repertoire expressed by a parasite dictates its interactions with their hosts, such molecules must be taken into consideration to generate effective immune diagnostics and vaccines.

The current major approach to liver fluke experimental vaccinology in large animals remains immunization with one or more recombinant liver fluke antigens produced *in vitro*. This approach relies on reproducing the immune response generated by native parasite antigens that are frequently glycosylated; however, native antigens carrying glycosylation are not easily produced by standard protein expression systems (Dell *et al*., 1999; Toet *et al*., 2014; Wilbers *et al*., 2017). These difficulties have been compounded by the inability of bacteria, and even yeast and insect cells, to produce the specific glycan structures built by parasites, and also by the general absence of data on liver fluke glycan structures. The latter has now been addressed to a large degree by recent applications of sensitive mass spectrometry (MS) methods and improved sequence databases to liver fluke. These developments have allowed us to begin to characterize the protein and lipid-linked glycan structures produced by liver fluke, and the mechanisms by which these glycans are synthesized.

In eukaryotic cells, glycosylation is performed by a remarkably complex set of linked biochemical processes compartmentalized in the endoplasmic reticulum (ER) and Golgi apparatus. *N*- and *O*-glycans are the most common types of glycans found attached to proteins and lipids. Protein *N*-glycosylation initiates when the reducing end of an *N*-acetylglucosamine (GlcNAc) residue of the glycan block is linked to the asparagine (Asn) residue within an amino acid Asn-*X*-Ser/Thr triplet motif (where *X* is any amino acid but proline). Canonical *O*-glycosylation starts by attachment of an *N*-acetylgalactosamine (GalNAc) monosaccharide to a serine or threonine residue via an O-glycosidic bond.

Common to all glycosylation processes are the nucleotide sugar donors (UDP-sugar donors) required as building blocks. These are synthesized in the cytosol from monosaccharide precursors of endogenous or exogenous origin and then are actively transported into the lumen of the ER and Golgi apparatus, where they are used to build polysaccharide glycan structures. Vertebrates contain a relatively limited number of monosaccharides in their glycoconjugate repertoire; namely, glucose (Glc), *N*-acetyglucosamine (GlcNAc), galactose (Gal), *N*-acetylgalactosamine (GalNAc), mannose (Man), xylose (Xyl), glucuronic acid (GlcA), fucose (Fuc) and *N*-acetylneuraminic acid (NeuAc). Nonetheless, it is important to consider that organisms such as parasites, bacteria and plants are known to add different and even unique monosaccharides to their glycoconjugates (Seeberger, 2017).

N-linked glycan synthesis begins in the ER with the construction of a 14-sugar precursor glycan that is added to the nascent polypeptide, in a reaction catalysed by the oligosaccharyltransferase (OST) enzyme complex. This precursor glycan is subsequently trimmed and remodelled by a series of specific glycosidases in the ER and can be modified in the Golgi apparatus by the action of different glycosidases and glycosyltransferases. The final structure can be designated as one of the following structural types, in increasing order of structural complexity: (i) paucimannose; (ii) oligomannose; (iii) hybrid; or (iv) complex *N*-glycans (Fig. 11.5A). By contrast, *O*-glycosylation happens entirely in the Golgi apparatus and is initiated by UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferases (GalNAc-Ts), which link a GalNAc monosaccharide on the Ser/Thr residue of a protein, usually a mucin, backbone. Subsequently, four common subtypes of *O*-glycans (Cores 1 to 4) can be formed depending on the enzymes that perform the monosaccharide linkage reactions to the GalNAc residue (Fig. 11.5B). These glycans can be subsequently elongated and modified to form structures as complex as the *N*-glycans described above. However, *O*-glycans are often shorter than *N*-glycans (Varki *et al.*, 1999).

Glycosphingolipids, or glycolipids, consist of a lipid moiety (ceramide) of which the terminal primary hydroxyl group is glycosylated via the attachment of a residue of glucose or galactose in the form of a UDP-sugar precursor. The glucosyltranferase (GlcT-1) is responsible for this initial step that occurs in the Golgi apparatus. Additional glycosyltransferases are responsible for the stepwise addition of other monosaccharides to elongate the glycan structure.

Unlike proteins, which are primary gene products, glycans cannot be directly predicted from a DNA template. The glycan structure formed is a result of numerous possibilities generated by a variety of competing enzymes and availability of substrates, i.e. monosaccharides in the specific environment where the cell or organism is. Variation is also greatly introduced by different glycosidic linkage types that can produce glycans with diverse structural properties and biological functions, despite their identical composition in terms of monosaccharide residues. Finally, various specific modifications, namely phosphorylation, sulfation, methylation and *O*-acetylation, further enhance the glycans' diversity in nature and often serve to mediate specific biological functions. Thus, even with full knowledge of the expression levels of all relevant gene products (i.e. monosaccharides, monosaccharide transporters, enzymes involved in nucleotide sugar biosynthesis pathways, glycosidases and glycosyltransferases) it is still a major challenge to predict the glycan repertoire an organism will express (Varki and Kornfeld, 2017). Once glycoconjugates are synthesized they are either placed on the cell surface or secreted into the extracellular environment, where the glycans participate in modulating a variety of cell–cell and cell–molecule interactions.

11.7.1 *Fasciola hepatica* glycans

The glycome of *F. hepatica* is dynamic and changes significantly throughout parasite development. Mass spectrometry analyses of *N*-linked glycans of proteins from different fluke life cycle stages provided the first structural evidence of these changes. While *F. hepatica* adult worms carry a mix of high-mannose, complex and phosphorylated glycans on their surface, the tegument of NEJ contains mainly truncated and high-mannose structures (Garcia-Campos *et al.*, 2016; Ravida *et al.*, 2016). Using a different approach, Cancela *et al.* (2015) verified that mucin-encoding genes are upregulated in *F. hepatica* NEJ compared with other life stages. Interestingly,

mucins are highly *O*-glycosylated proteins and their enhanced expression on the invasive-stage parasite suggests that they play a significant role during invasion and migration processes.

A comprehensive *in silico* analysis explored the genetic composition of both *N*- and *O*-glycosylation pathways in various intra-mammalian stages of *F. hepatica* (McVeigh *et al.*, 2018a). The liver fluke glycogenome comprises 87 non-redundant genes. This dataset is relatively compact when compared with the human glycogenome of at least 190 genes, with the numeric simplicity reflecting the function of the glycogenome, where genes involved in production of more complex glycan structures appear to be absent in liver fluke. Glycotransferases responsible for the synthesis of elongated branches are largely absent, and only a single fucosyltransferase is present (McVeigh *et al.*, 2018a). Additionally, when the expression levels of glycosidases and glycosyltransferases were compared across different developmental stages, great variation was observed (McVeigh *et al.*, 2018a; De Marco Verissimo and Dalton, unpublished). Interestingly, these predictions are borne out by MS analyses of the glycoproteins from the surface of *F. hepatica* NEJ and adult parasites (Garcia-Campos *et al.*, 2016; Ravida *et al.*, 2016).

Recently, the profile of *N*- and *O*-glycans linked to glycoproteins within the somatic and excretory/secretory extracts of *F. hepatica* NEJ, 21-day juveniles and adult worms was analysed and compared (De Marco Verissimo and Dalton, unpublished). A total of 61 *N*- and 52 *O*-glycan structures were identified in these analyses and confirmed the presence of several *N*-glycan structures highlighted above. However, the glycan profile obtained for each life cycle stage was more complex than expected and indicated that the *F. hepatica* glycome is highly dynamic. Moreover, several glycan structures identified by MS studies could not be predicted based on the *F. hepatica* glycogenome unveiled by McVeigh *et al.* (2018a). The synthesis of the complex *N*-glycan containing bisected GlcNAc (Fig. 11.5), for example, would require the activity of enzymes that *F. hepatica* seems to lack based on *in silico* analysis of the genome. These discrepancies highlight an important limitation of the glycobiology field. Despite our understanding that parasites can use unique monosaccharides and linkages to build their glycoconjugates, we still rely on sequence homology with human enzymes to determine the glycogenome of these organisms. Taken together, these data suggest that *F. hepatica* expresses unique glycosidases and glycosytransferases or, alternatively, those homologues identified in the parasite genome and transcriptome have evolved to perform a broader range of functions and reactions, an avenue yet to be explored.

While glycolipids are mainly located on the outer leaflet of the plasma membrane, where they interact with the extracellular environment and regulate essential interactions of a given cell or organism, technical and methodological limitations have hindered detailed analyses of these macromolecules in parasites. Therefore, knowledge of lipid-linked glycans in liver fluke remains sparse. The few studies that aimed to characterize such molecules from *F. hepatica* found that adult worms express mammalian-type glycolipids as well as a unique Gal(β1-6)Gal terminating glycolipid. Interestingly, the Gal(β1-6)Gal motif was demonstrated to cause important cross-reactivity in immunodiagnostics, as numerous cestode parasites express the same motif in their glycoconjugates (Wuhrer *et al.*, 2003, 2004; Yamano *et al.*, 2009).

Varying the surface glycan coat is possibly one of the most important fluke strategies to evade the host immune-mediated killing mechanisms. Moreover, as *F. hepatica* migrates through different host tissues the changes in glycan expression and presentation might match the microenvironment conditions, including monosaccharide availability and local immune responses. One could suggest that such changes can function as a perfect disguise, which may explain the efficiency with which the parasite deals with different tissues and hosts. In fact, variations in glycosylation of cells and organisms were demonstrated to occur at the slightest change of microenvironment (Garcia-Campos *et al.*, 2016; Ravida *et al.*, 2016), an important aspect yet to be studied in *F. hepatica* parasites.

11.7.2 Function of glycans

The glycan repertoire that an invasive parasite displays on its surface or secretes into its host has a major impact on the success of the infection.

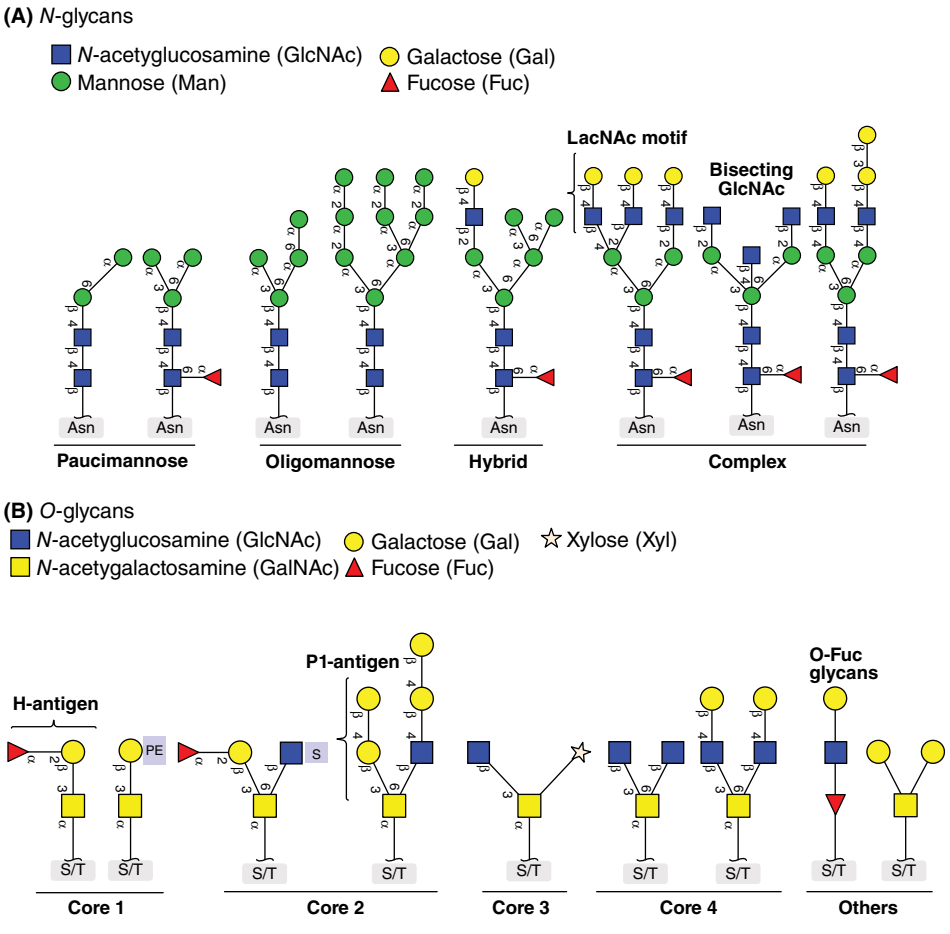

Fig. 11.5. Graphical representations of *N*- and *O*-glycans associated with *Fasciola hepatica* glycoproteins. **(A)** The four main *N*-glycan types formed from the core $Man_3GlcNAc_2$ (grey box) linked to glycoproteins through asparagine (Asn) residues are shown. (1) Paucimanose, in which three or fewer mannose residues are observed attached to the GlcNAc residues forming the core, which may or may not be fucosylated. (2) Oligomannose, in which only mannose residues are attached to the core. (3) Hybrid, in which only mannose residues are attached to the Manα1–6 arm of the core plus one or two antennae are on the Manα1–3 arm. (4) Complex, in which several 'antennae' initiated by GlcNAc residues are attached to the core. These antennae can be extended by the sequential addition of glycan residues to form specific terminal motifs (i.e. LacNAc motif). **(B)** The four classical *O*-glycan core structures linked to glycoproteins through serine or threonine (S/T) residues are represented by grey boxes. Core 1, Galβ1-3GalNAc; Core 2, GlcNAcβ1-6(Galβ1-3)GalNAc; Core 3, GlcNAcβ1-3GalNAc; Core 4, GlcNAcβ1-6(GlcNAcβ1-3)GalNAc; Others, non-mucin *O*-glycans, including α-linked *O*-fucose glycans formed when the *O*-fucosyltransferase 1 (POFUT1) transfers fucose from GDP-Fuc to a consensus motif C^2X_{4-5} (*S*/*T*)C^3, where Cs are conserved cysteines present in the protein backbone. These structures can be extended and form specific motifs (i.e. H-antigen and P1-antigen). Phosphoethanolamine (PE) and sulfated (S)-modified *O*-glycan modifications, shown in the light blue boxes, are also found in glycoproteins from *F. hepatica.*

F. hepatica characteristically induces a potent Th2/Treg immune response in its hosts. Glycans might contribute to this bias, preventing parasite detection and elimination, which ultimately results in the establishment of a chronic infection (Everts *et al.*, 2012; Meevissen *et al.*, 2012; Klaver *et al.*, 2013; Terrazas *et al.*, 2013; Vásquez-Mendoza *et al.*, 2013).

While *F. hepatica* builds some unique glycans, most sugars associated with the parasite glycoproteins are known as 'host-like glycans'. Oligomannose glycans, complex *N*-glycans containing LacNAc motifs (Galβ1,4GlcNAc) and *O*-glycans carrying the truncated T antigen (core 1, Galβ1-3GalNAc-Ser/Thr) or the blood group H-antigen (Fig. 11.5) are abundant in both *F. hepatica* and mammalian tissues. This glycan mimicry is considered an important immune evasion strategy in parasites, as their glycosylated antigens interact with receptors in host antigen-presenting cells (APCs), such as dendritic cells (DCs) and macrophages, as self-antigens (van Die and Cummings, 2010).

Parasite glycans, including those expressed by the liver fluke, manipulate host immune responses mainly by interacting with specific glycan receptors in APCs, i.e. Toll-like (TLR), C-type (CLRs) and other lectin receptors (Akira *et al*., 2006; Diebold, 2009; Terrazas *et al*., 2010; Rodríguez *et al*., 2015). Upon recognition via lectin receptors such as mannose receptors (MR), DCs were shown to internalize phosphorylated oligosaccharides from the tegument of *F. hepatica* adult flukes (Rodríguez *et al*., 2015; Ravida *et al*., 2016). This interaction with lectin receptors, which regulates the maturation of DCs and activates internal signalling pathways, subsequently influences their interaction with T cells and ultimately leads to a skewing of the immune response towards a Th2 response (van Liempt *et al*., 2007; Rodríguez *et al*., 2017).

Both host and parasite carbohydrates might impact on host specificity. Higher amounts of fucosylated glycan linked to proteins present in the haemolymph of *Biomphalaria glabrata* snails correlates with higher susceptibility to infection by *S. mansoni* (Lehr *et al*., 2010; Paschinger and Wilson, 2019). This is an avenue yet to be explored for *F. hepatica* to uncover the selectivity of the snail intermediate host. Further understanding of these interactions may result in successful strategies for parasite control through transmission blocking. Conversely, the importance of glycan interactions with lectins expressed by the vertebrate host during *F. hepatica* infection has been demonstrated. Garcia-Campos *et al*. (2017) found that NEJ migration through *ex vivo* rat intestine preparations is inhibited by specific lectins (GNL and ConA) that bind to mannosidic glycans on the surface of the larvae. The lectins may block surface or EV-derived glycan from interacting with the host tissues, suggesting that the adhesion of the NEJ's glycans to intestinal epithelial cells is important for the parasite to penetrate the intestinal wall. EVs possess surface-bound mannosidic glycoconjugates but these have a distinct profile from that on the tegumental surface of the parasite and thus both exposed surfaces can be easily targeted to hinder parasite infection (de la Torre-Escudero *et al*., 2019; Murphy *et al*., 2020).

The EV's glycans could also be of key importance in understanding the fluke's capacity for immunomodulation, given that the EVs can spread throughout the host, modulating cells remote from the parasite. Moreover, *N*- or *O*-glycans carrying terminal Gal(β1-3)GlcNAc (T antigen) or Gal(β1-4)GlcNAc are found in all fluke life stages and are specifically recognized by ruminant-specific lectins, i.e. galectin-11 and galectin-14. Swan *et al*. (2019) showed that the expression of these lectins is upregulated in the bile ducts of sheep infected with *F. hepatica* and suggested that galectins play an important role in host–parasite interactions during chronic infection.

While functional insights on fluke glycans are scarce, those outlined above highlight the critical role they play in host–parasite interactions. Moreover, *F. hepatica* glycans and glycoconjugates could be involved in the pathogenesis of both acute and chronic infections and thus are potential novel biomarkers for the development of improved diagnostics, as well as targets at which vaccines to combat fasciolosis could be directed.

11.8 Proteomic and Transcriptomic Analyses of Host Responses to *F. hepatica*

Omic technologies can be used to gain an unbiased and global picture of the effects that parasitic infection has on its host and can be compared and integrated with parallel studies on the parasite to understand stage-related immune responses and pathogenesis. The transcriptome/proteome profile, described in this section, of the host humoral and cellular responses that mirror each stage of infection can be attributed to the specific expression of genes by *Fasciola* spp., the release/

secretion of specific molecules and the temporally regulated post-translational modifications (particularly glycosylation) of parasite-specific proteins, as described above.

Transcriptomic studies have been performed on peripheral blood mononuclear cells (PBMC) obtained at various stages throughout infection from sheep (Alvarez Rojas *et al.*, 2016; Fu *et al.*, 2016) and cattle (Garcia-Campos *et al.*, 2019). Similarly, studies of the liver transcriptomic responses to the migrating parasite have been characterized from samples taken from sheep 8 weeks post *F. hepatica* infection (Alvarez Rojas *et al.*, 2015) and from cattle at 3–70 days post *F. gigantica* infection (Zhang *et al.*, 2017). These studies showed that gene transcription in the liver is highly regulated during *Fasciola* infection, particularly during acute infection (1–2 weeks pi). Rojas-Caraballo *et al.* (2015) reported similar findings using microarray studies of livers from *F. hepatica*-infected mice. Collectively, these studies describe upregulation of genes corresponding to fibrosis and tissue repair, consistent with the damaging migration of the parasite, where it begins its growth and development corresponding to increased parasite gene transcript expression (> 8000 genes are upregulated) (Cwiklinski *et al.*, 2015a, 2021; Zhang *et al.*, 2019b). These results mirror the proteomic analyses of liver tissue from buffalo infected by *F. gigantica* that show enrichment for proteins associated with wound healing and the immune system at 42 days pi (Hu *et al.*, 2020).

Changes in the liver transcriptomes reflect the host's response to the migrating parasite and reveals how the *Fasciola* spp. manipulates the host by inducing B cell activation and skewed Th2-driven responses, including genes encoding interleukin 27 receptor (alpha-IL27RA) B-cell CLL/lymphoma 6 (BCL6), Toll-like receptor 4 (TLR4) and transforming growth factor beta 1 induced transcript 1 (TGFB1) (Alvarez Rojas *et al.*, 2015; Zhang *et al.*, 2017). The study by Alvarez Rojas *et al.* (2015) also reported increased abundance of circulating reticulocytes due to the blood-feeding activity or haemorrhaging/anaemia caused by the migrating parasite. Increased transcription of haemoglobin-related genes and four genes putatively associated with Fanconi anaemia were also observed.

In keeping with the liver transcriptome data, PBMC transcriptome data exhibit suppression of genes associated with Th1 (e.g. inducible nitric oxide synthase (iNOS)) and Th17 responses (orphan retinoic acid nuclear receptor (ROR) family transcription factor RORγt, IL23/IL23R and IL17F/IL17RC) (Fu *et al.*, 2016). Upregulation of genes associated with transforming growth factor (TGF) β signalling, including the genes TGF β, collagen type 1 and the downstream SMAD signalling genes that play a major role in fibrosis and tissue repair, were also observed (Alvarez Rojas *et al.*, 2016; Fu *et al.*, 2016). Comparable upregulation of TGF β signalling and the Th2 pathway is also observed within the bovine PBMC transcriptome as early as 1 week pi (Garcia-Campos *et al.*, 2019). Moreover, genes associated with the KEGG complement and coagulation cascades, chemokine signalling pathway and cytokine–cytokine-receptor interaction pathway were also upregulated in sheep 8 weeks pi (Alvarez Rojas *et al.*, 2016). Similarly, in cattle at 14 weeks pi, the complement system, TLR signalling and antigen presentation canonical pathways were enriched (Garcia-Campos *et al.*, 2019).

As infection by *F. hepatica* progresses, the amount and composition of immune cells present both within the peritoneal cavity and circulating in the peripheral blood shifts to an abundance of eosinophils, which is associated with a polarization to Th2-type immune responses. Differential eosinophil cell counts were only reported in the Alvarez-Rojas *et al.* (2016) study and showed that infected animals had substantially more eosinophils than the control non-infected animals. At 4 weeks pi, the eosinophil count in the infected group ranged from 12% to 39% compared with the control group counts of 1–5%. In contrast, Garcia-Campos *et al.* (2019) reported no differences in eosinophil number between the infected and uninfected cattle. Therefore, the changes in the transcriptomic responses are reflective of both a change in transcription during infection as well as a change in the number and type of cells within the PBMC fraction, which may differ between species and must be interpreted accordingly.

Similarly, other cautionary notes for the interpretation of transcriptomic data include: (i) the variability between hosts (mice, sheep, cattle); (ii) the difference between animal breeds (e.g. Suffolk and Texel sheep); (iii) changes in host immune cell type and number altering

greatly at different stages of infection; (iv) many events, such as tissue damage and repair, occurring at the same time as the parasite migrates within the host tissues; and (v) techniques in tissue extraction sequencing and analysis/interpretation of data, which can differ greatly between laboratories.

Consistent with the transcriptomic analyses of PBMC (Alvarez Rojas *et al*., 2016; Fu *et al*., 2016), Rioux *et al*. (2008) showed that there were significant protein changes within the sera of infected hosts beginning within 3 weeks of infection. Two markers of particular interest, namely transferrin and apolipoprotein A-IV (Apo A-IV), were upregulated during this early period. The elevation of transferrin is associated with anaemia caused by the blood-feeding parasites, whereas Apo A-IV is associated with regulation of appetite within the intestine of mammals. In comparison, levels of transferrin detected within the bile by Morphew *et al*. (2007) were reduced compared with levels in the serum, indicating that data can vary significantly depending on the sample type (serum, bile, peritoneal fluid etc) and time of infection.

To get an idea of the immunopathogenesis caused by parasites migrating through the peritoneal cavity, Ruiz-Campillo *et al*. (2017) performed a proteomic analysis of the peritoneal fluid from sheep infected by *F. hepatica* at 18 days pi. They identified an abundance of proteins associated with the complement system and with the liver extracellular matrix (ECM), such as collagen VI, fibronectin and fibrocystin, likely due to the damage caused by the parasite as it invades and migrates through the liver. This study also detected two ECM-related molecules, periostin and vascular cell adhesion protein 1 (VCAM-1), that mediate leucocyte infiltration and are associated with marked eosinophilia and are of value as biomarkers of liver fluke infection.

Although primarily used to investigate the mammalian host responses, the Gourbal group have used omics sequencing technologies to investigate the susceptibilities of the snail intermediate host to *F. hepatica* infection (Alba *et al*., 2019). This study focused on *Pseudosuccinea columella*, a snail found throughout the world but predominantly in South America and the Caribbean, that displays both susceptible and resistant phenotypes to *Fasciola* spp. infection (Alba *et al*., 2018). Analysis of the transcriptome and proteome data revealed an enrichment of genes/proteins related to the defence and stress responses in the resistant snails, including immune-related molecules such as pathogen recognition/interacting proteins and cytokines involved in proliferation, recruitment and activation of immune cells. In contrast, metabolic processes related to protein synthesis were under-represented, which, coupled with the lower reproductive rate of the resistant *P. columella* snails, suggests a trade-off between survival and reproduction (Alba *et al*., 2019).

11.9 Concluding Remarks

The exciting and rapidly advancing omics technologies are increasingly shedding light on the complex relationship between *Fasciola* spp. and their hosts. The improved methodologies and increased sensitivity of the techniques are allowing us to probe parts of the life cycle that were inaccessible to scrutiny just two decades ago, particularly the NEJ and the stages that live inside the intermediate snail host. As technologies such as single-cell sequencing develop (Kashima *et al*., 2020), we will have a greater abundance of omics information to interrogate liver fluke biology in novel ways. Furthermore, comparative genomic/transcriptomic studies will allow us to place our knowledge of liver fluke biology within the wider context of helminth biology and evolution, host interaction, immunology and pathogenesis.

While a large proportion of *Fasciola* spp. genes are categorized solely as sharing homology with uncharacterized genes of other Platyhelminthes, these will eventually be identified and their functions revealed. Post-genomic tools for knocking down gene expression (RNA interference (RNAi)) or editing the genes themselves (clustered regularly interspaced short palindromic repeats (CRISPR)) will become increasingly important to probe the expression and function of these uncharacterized genes. This growing repository of omics data for both *F. hepatica* and *F. gigantica* will allow us to profile the similarities and dissimilarities between these related parasites and elucidate how they survive within their hosts and induce different immune responses, and will be critical to understanding the biology and pathogenesis of emerging hybrid parasites.

We have found that many genes transcribed by *Fasciola* spp. are members of large families that are highly regulated throughout the life cycle within the mammalian host. But we are only beginning to understand the role miRNAs play in the regulation of gene transcription at different stages of parasite development and how these may be pivotal in host–parasite interaction, such as hijacking host immune responses. The availability of well-characterized genome, transcriptome and proteome data has also made it possible to explore the importance of post-translational processes, particularly glycosylation. Novel glycan structures on the parasite surface and EVs are being uncovered and now we need to determine how these alter antigen structure, function and immunogenicity, as well as their role in countering host immune attack. Bringing all these different but connected omics datasets together should eventually lead us to new ways of combatting fasciolosis.

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12 Vaccines for *Fasciola*: New Thinking for an Old Problem

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12.1 Introduction

The liver flukes *Fasciola hepatica* and *F. gigantica* are ancient parasites that diverged as separate species about 5.3 million years ago (Choi *et al.*, 2020). *F. hepatica* is thought to have arisen in sheep and goats in Eurasia whereas *F. gigantica* arose in ruminant groups related to bovines in Africa (Mas Coma *et al.*, 2009; Choi *et al.*, 2020). These species infected ruminants before the domestication of wild herbivores around 10,000 years ago (Mas Coma *et al.*, 2005, 2009). Fasciolosis can therefore be considered an old problem that continues to cause significant economic losses and impact the welfare of livestock and humans worldwide (Mas Coma *et al.*, 2005, 2009; Piedrafita *et al.*, 2010; Charlier *et al.*, 2014; Mehmood *et al.*, 2017).

The prevalence of fasciolosis in the tropical regions varies between countries in Asia and Africa and ranges up to 73% in sheep, 68% in goats, 91% in cattle and 68% in buffalo; it is considered to be a major helminth infection of cattle and buffalo (Dargie, 1987; Fabiyi, 1987; Spithill *et al.*, 1999; Mehmood *et al.*, 2017). In non-tropical countries recent reports show that the prevalence of liver fluke in dairy cattle is substantial, with a prevalence of 73–88% in the UK (Howell *et al.*, 2015) and up to 80% in south-east Australia, where some farms show 100% prevalence (Kelley *et al.*, 2020). Production losses in livestock occur as decreases in milk, meat and wool production, reductions in fertility and, in the tropics, reduced draught capacity of working animals; other costs accrue due to the cost of drugs used to treat fasciolosis and secondary bacterial infections that can occur with liver fluke infection. Annual economic losses due to fasciolosis in dairy cattle have been recently estimated at over £333 per cow in the UK (Howell *et al.*, 2015), €52 million in Switzerland (Schweizer *et al.*, 2005) and AU$129 million in Australia (Kelley *et al.*, 2020). Losses in different countries vary and conservatively range up to US$20–107 million/year (Spithill *et al.*, 1999; Mehmood *et al.*, 2017). Overall global economic losses due to fasciolosis have been conservatively estimated at over US$3000 million per year with more than

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600 million animals infected (Hillyer and Apt, 1997; Spithill *et al.*, 1999; Piedrafita *et al.*, 2010).

The importance of fasciolosis is further highlighted by the increased prevalence of triclabendazole resistance in livestock, as triclabendazole is the preferred drench for controlling fasciolosis in farm animals (Kelley *et al.*, 2016; Fairweather *et al.*, 2020). Moreover, fasciolosis is now recognized as an important human disease with about 2.4 million people infected with *Fasciola* and a further 180 million at risk of infection (Anonymous, 1995; Mas Coma, 2005). There are several human cases where drug-resistant liver fluke infections were reported; these studies further emphasize the zoonotic importance of fasciolosis as a global one-health problem in animals and humans (Kelley *et al.*, 2016).

Multiple studies have reported attempts to vaccinate laboratory animals, sheep and cattle with crude somatic extracts and excretory/secretory (ES) products from *Fasciola* spp., dating back to at least 1966. The results of these early studies, which have often been variable, have been previously reviewed (Rickard and Howell, 1982; Reddington *et al.*, 1984; Haroun and Hillyer, 1986; Hughes, 1987). There have been more recent reviews of the development of subunit liver fluke vaccines over the past 3–4 decades (Toet *et al.*, 2014; Molina Hernandez *et al.*, 2015; Carmona and Tort, 2017; McManus, 2020). The literature clearly indicates that the development of a liver fluke vaccine is also a timeworn challenge.

Here, we focus on progress in liver fluke vaccine research since our review in 2014 (Toet *et al.*, 2014) and suggest new ideas for future areas of research that may accelerate vaccine discovery. We suggest that some novel approaches need to be taken if fluke vaccines with efficacy > 50% are to be achieved in livestock. These include:

- an evaluation of tegument proteins on the surface of juvenile flukes as vaccines;
- an assessment of fluke glycans as potential vaccines;
- the potential for vaccine strategies aimed at interfering with exosome function;
- a better assessment of adjuvants and vaccine formulations that will enhance the immune effector mechanisms in livestock that kill juvenile flukes;
- a deeper understanding of the actual mechanism of killing of juvenile flukes in ruminants *in vivo*, where these flukes are killed; and
- how we can tip the host immune response in favour of fluke killing and reduce fluke-induced host immunosuppression.

We also suggest that vaccine evaluation should rely not just on mean percentage efficacy/reduction in fluke counts but also on the ability of vaccines to reduce the intensity of fluke burdens in livestock below the threshold for significant clinical pathology and, by extension, economic loss. Finally, we discuss the commercial considerations in liver fluke vaccine development and the characteristics of a commercial vaccine product.

12.2 Recent Vaccine Studies in Livestock (2014–2020)

Since our review (Toet *et al.*, 2014), there have been relatively few new advances in vaccine development against liver fluke. Most of the recently reported vaccine trials conducted in livestock (cattle, sheep, goats and buffalo) have focused on previously tested candidates, with limited results. These include known immunomodulatory proteins such as the cathepsin proteases, primarily cathepsin L1 (FhCL1), as well as leucine aminopeptidase (FhLAP) and glutathione *S*-transferase (GST). Vaccine trials conducted in livestock and the vaccine efficacy observed from the past 7 years are summarized in Table 12.1. Notably, recent efforts in vaccine development using a range of adjuvants have resulted in protection less than the highest levels previously achieved using Freund's adjuvant, such as 89% using native LAP in sheep (Piacenza *et al.*, 1999) and 72% using CatL2 in combination with haemoglobin (Hb) in cattle (Dalton *et al.*, 1996; Mulcahy *et al.*, 1998).

12.2.1 Cathepsin L1

FhCL1, in combination with other *Fasciola* proteins, is considered to be a lead candidate for a fasciolosis vaccine (Dalton *et al.*, 2013; Toet *et al.*,

Table 12.1. Efficacy of liver fluke vaccines evaluated in livestock and published from 2013 to 2020 (excluding results reported in Toet *et al.*, 2014).

Antigen	Abbreviation	Form	Adjuvant	Delivery	Host	*Fasciola* species	Vaccine efficacy (%)	References
Single vaccines								
Cathepsin L1	rFhpCL1	recombinant	Quil A	s.c.	Sheep	*F. hepatica*	0	Orbegozo-Medina *et al.,* 2018
	rFhCL1	recombinant	Montanide™ ISA70 VG	s.c.	Sheep	*F. hepatica*	11	Pacheco *et al.,* 2017
	rmFhCL1	recombinant	Montanide™ ISA 70 VG	ND	Sheep	*F. hepatica*	0	Fu *et al.,* 2016
	rFhCL1	recombinant	Quil A	s.c.	Goat	*F. hepatica*	0	Zafra *et al.,* 2013a.b
Cysteine protease (cathepsin)	CPFhW	recombinant	None	oral	Sheep	*F. hepatica*	35.5	Wesołowska *et al.,* 2018
	CPFhW	recombinant	None	oral	Cattle	*F. hepatica*	56.2*	Wesołowska *et al.,* 2018
Glutathione *S*-transferase	rGST-S1	recombinant	Quil A	s.c.	Goat	*F. hepatica*	0	Zafra *et al.,* 2013c
Helminth-defence molecule	MF6p/FhHDM-1	synthetic	Quil A	s.c.	Sheep	*F. hepatica*	6	Orbegozo-Medina *et al.,* 2018
	MF6p/FhHDM-1	native	Quil A	s.c.	Sheep	*F. hepatica*	15	Orbegozo-Medina *et al.,* 2018
Phosphoglycerate kinase	cDNA-FhPGK/pCMV	cDNA	None	i.m.	Sheep	*F. hepatica*	5.8	Wesołowska *et al.,* 2016
	cDNA-FhPGK/pCMV	cDNA primed/protein boost	Montanide ISA 206	i.m.	Sheep	*F. hepatica*	0	Wesołowska *et al.,* 2019
	cDNA-FhPGK/pCMV	cDNA	CTLA-4	i.m.	Sheep	*F. hepatica*	0	Wesołowska *et al.,* 2019
Thioredoxin glutathione reductase	rFhTGR	recombinant	IFA	s.c.	Cattle	*F. hepatica*	8.2	Maggioli *et al.,* 2016
	rFhTGR	recombinant	Adyvac 50	s.c.	Cattle	*F. hepatica*	3.8	Maggioli *et al.,* 2016
	rFhTGR	recombinant	Alum	s.c.	Cattle	*F. hepatica*	23	Maggioli *et al.,* 2016

Continued

Table 12.1. Continued.

Antigen	Abbreviation	Form	Adjuvant	Delivery	Host	*Fasciola* species	Vaccine efficacy (%)	References
14-3-3z	r14-3-3z	recombinant	Montanide™ ISA 71 VG	s.c.	Sheep	*F. hepatica*	0	Pérez-Caballero *et al.,* 2018
Combination vaccines								
Cathepsin L1 and L3	rmFhCL1/ rmFhCL3	recombinant	ZA1	s.c.	Cattle	*F. hepatica*	37.6	Garza-Cuartero *et al.,* 2018
	rmFhCL1/ rmFhCL3	recombinant	ZA1	s.c.	Cattle	*F. hepatica*	0	Garza-Cuartero *et al.,* 2018
Cathepsin L5 and B2	CatL5 & CatB2	recombinant	CpG-ODN2135 & ISC adjuvant	i.n.	Sheep	*F. hepatica*	40.5* (11.4)	Norbury *et al.,* 2018
	CatL5 & CatB2	recombinant	Quil A	i.m.	Sheep	*F. hepatica*	20.9 (8.5)	Norbury *et al.,* 2018
Leucine aminopeptidase & Cathepsin L1	rFhLAP-CL1	recombinant chimera	Quil A	ND	Sheep	*F. hepatica*	25.5, 30.7*, 46.5*	Ortega-Vargas *et al.,* 2019
60 kDa fraction (Enolase)	60 kDa	native	FCA/IFA	i.m.	Sheep	*F. gigantica*	41.3*	Mahana *et al.,* 2016
32 kDa fraction	32 kDa	native	FCA/IFA	i.m.	Sheep	*F. gigantica*	14.1	Mahana *et al.,* 2016
28 kDa fraction	28 kDa	native	FCA/IFA	i.m.	Sheep	*F. gigantica*	19.2	Mahana *et al.,* 2016
Fasciola tegument proteins	Fhteg1 and Fhteg5	recombinant	FCA/IFA	i.m.	Cattle	*F. hepatica*	4.6	McCusker *et al.,* 2020

*, significant efficacy; FCA/IFA, Freund's complete adjuvant/Incomplete Freund's adjuvant; i.m., intramuscular; i.n., intranasal; ND, not defined; s.c., subcutaneous; ZA1, Zoetis Adjuvant 1.

2014). FhCL1 is produced by both immature flukes in the liver and adult parasites that have matured in the bile ducts of the host and has been shown to actively cleave the peptide bonds of haemoglobin, presumably to assist parasite feeding (Lowther *et al.*, 2009). Recent studies with vaccine formulations testing recombinant FhCL1 (rFhCL1) as a single antigen in sheep and goats have shown poor to no protection (Zafra *et al.*, 2013a,b; Fu *et al.*, 2016; Pacheco *et al.*, 2017; Orbegozo-Medina *et al.*, 2018). Combinations of rFhCL1 and rFhCL3 with the use of a novel adjuvant ZA1 (Zoetis Adjuvant) in cattle (Garza-Cuartero *et al.*, 2018) and a chimeric protein consisting of rFhLAP and rFhCL1 in conjunction with Quil A in sheep (Ortega-Vargas *et al.*, 2019) reduced fluke burdens by 0–37.6% and 25.5–46.5%, respectively. This is lower than previous trials of FhCL1, such as those by Villa-Mancera *et al.* (2014) using FhCL1 phage mimotopes in goats (46.9–79.5% efficacy) and Piacenza *et al.* (1999) consisting of a mixture of native FhCL1, FhCL2 and LAP in sheep (79% efficacy) (Toet *et al.*, 2014).

12.2.2 GST

Similar to a previous trial conducted by Buffoni *et al.* (2010) using native GST in combination with FCA/IFA in goats, recombinant GST Sigma class 1 (rGST-S1) delivered with Quil A was unsuccessful in protecting goats against infection with *F. hepatica* (Zafra *et al.*, 2013c). This suggests that both native and recombinant GST may not be suitable for vaccination of goats against *F. hepatica*, despite varied evidence of good protection with native GST in both cattle and sheep (reviewed in Toet *et al.*, 2014).

12.2.3 Other antigens

Notably, there have been very few publications focusing on new antigens as vaccine candidates in livestock since 2013. Thioredoxin glutathione reductase (rFhTGR) has previously been tested with promising results of 96.7% protection in vaccinated rabbits (Maggioli *et al.*, 2011a). However, vaccination of cattle with recombinant rFhTGR using three different adjuvants (FIA only, Adyvac 50 and Alum) resulted in reduced fluke burdens of only 8.2%, 3.8% and 23%, respectively (Maggioli *et al.*, 2016). Additional new single antigens tested included a helminth-defence molecule (FhHDM-1) (Orbegozo-Medina *et al.*, 2018), a phosphoglycerate kinase (FhPGK) (Wesołowska *et al.*, 2016, 2019) and a 14-3-3z protein (Pérez-Caballero *et al.*, 2018). All were unsuccessful in significantly reducing fluke burdens in vaccinated sheep (0–15%).

New combinations tested included novel tegument membrane proteins, termed Fhteg1 and Fhteg5. These proteins were found in the tegument of adult *F. hepatica* (Wilson *et al.*, 2011); they were also expressed in the juvenile fluke within 2 days of excystment and were shown to be surface exposed on 7-day-old juveniles by immunolocalization. Collectively, these observations suggested that Fhteg1 and Fhteg5 are potential targets for killing juvenile flukes by antibody-dependent cell cytotoxicity (ADCC) (McCusker *et al.*, 2020). Analysis of proteomic datasets showed that these proteins were also found to be associated with extracellular-like vesicles (Cwiklinski *et al.*, 2015b; McCusker *et al.*, 2020). However, a combination subcutaneous vaccination with FCA/IFA induced only 4.6% protection in cattle, despite moderate protection (48%) in rats (McCusker *et al.*, 2020).

Lastly, poor to moderate results were obtained using purified native fractions of *F. gigantica* Triton soluble surface membrane and tegument proteins (TSMTPs) for vaccination of sheep (Mahana *et al.*, 2016), with 41.3% reduction in fluke burden achieved using a native 60 kDa protein fraction enriched in Enolase and 14% and 19% efficacy using a 32 kDa and 28 kDa fraction, respectively. Enolase has been reported as an excretory/secretory protein with plasminogen-binding ability (Bernal *et al.*, 2004) and has been evaluated as a vaccine candidate for other parasitic diseases, such as *Clonorchis sinensis* (Wang *et al.*, 2014). Enolase was found in the soluble S2SS fraction and membrane UTCS fraction from the tegument of adult *F. hepatica* (Wilson *et al.*, 2011).

12.2.4 Mucosal vaccine delivery

Livestock vaccines have generally been administered by the intramuscular or subcutaneous route to induce IgG isotypes, IgG2 in particular,

targeting the migrating fluke, although there is limited supporting evidence that IgG2 itself is a protective antibody isotype (Toet *et al.*, 2014). It is possible that alternative vaccination protocols could be utilized to target the newly excysted juvenile (NEJ) flukes earlier as they cross the mucosal barrier of the intestinal wall. A method to target the early migration of NEJs might be to develop a mucosal vaccine, delivered either intranasally or via the oral route.

Norbury *et al.* (2018) compared the delivery of a CatL5/CatB2 combination by both intranasal and intramuscular methods in sheep. Interestingly, intranasal co-administration of CatL5/CatB2 with CpG-ODN 2135 (a TLR9 agonist) and ISC adjuvant improved the efficacy of the vaccine (40.5% reduction in worm burden) compared with intramuscular administration with Quil A (20.9% reduction) relative to the unvaccinated control group. Despite the ability of the intramuscular vaccination to induce strong IgM and IgG responses against both CatL5 and CatB2, it still failed to induce significant protection against *F. hepatica* in sheep. A significant reduction in both worm burden ($P = 0.006$) and parasite egg viability (> 92%, $P = 0.028$) was observed for the intranasal vaccinated group, relative to the unvaccinated control group, despite only anti-CatL5 IgG responses being induced post-challenge and a lack of IgA response detected in serum and nasal washings. However, relative to their respective intramuscular and nasal controls, no protection was observed (8.5% im, 11.4% i.n.). The induction of strong IgG responses to CatL5 post-challenge in the intranasal group compared with the vaccination controls suggests that nasal vaccination was still able to prime the sheep immune system in the absence of any significant systemic or nasal localized response.

A novel alternative method for vaccination was trialled by Wesołowska *et al.* (2018) in livestock using oral administration of a freeze-dried transgenic plant (lettuce) expressing the cysteine protease CPFhW fused to a hepatitis B virus core antigen carrier (HBcAg) in the absence of an adjuvant. This oral method of targeting mucosal immunity was able to induce 56.2% protection ($P < 0.05$) in cattle and 35.5% protection (not significant) in sheep against *F. hepatica*. IgG levels were not induced after oral vaccination, although significant levels of IgG were present in the CPFhW-vaccinated animals from 6 weeks post-infection (pi) and peaking at 10 weeks pi. No serum IgA responses were observed in any vaccinated animals. A major benefit of oral vaccines is the reduced time and costs of purification and processing the vaccine for formulation and storage (Lugade *et al.*, 2010; Wesołowska *et al.*, 2018). Additionally, plant protein expression includes post-translational modifications required for biological activity of parasite antigens, such as protein folding, assembly and glycosylation (Wesołowska *et al.*, 2018), allowing for the potential development of protective antibodies against conformational epitopes. The use of such edible vaccines is also deemed favourable due to the lack of potential human and animal pathogens involved in their production, providing vaccines that are both safe and biosecure (Wesołowska *et al.*, 2018). The lack of sufficient systemic antibody responses induced by mucosal vaccines, such as oral and edible vaccines, prior to challenge suggests that they may be more effective as booster vaccinations as part of a mixed intramuscular/mucosal vaccination programme.

12.2.5 IgG1 and IgG2 responses in vaccinated cattle and sheep

It has been shown that *Fasciola* spp. are able to immunomodulate the host, driving a non-protective Th2 immune response (Dalton *et al.*, 2013). To date, vaccine research has been based on protection against *Fasciola* spp. driven by an antibody-mediated Th1 response and characterized by a negative correlation between antigen-specific IgG2 responses and fluke burdens (Dalton *et al.*, 1996; Mulcahy *et al.*, 1998; Toet *et al.*, 2014; Molina-Hernández *et al.*, 2015). Therefore, induction of *Fasciola* antigen-specific antibodies is deemed important for a protective vaccine.

In the more recent vaccine trials, four papers investigated the kinetics of the IgG1 and IgG2 isotype responses to determine the type of immune response stimulated by vaccination. Garza-Cuartero *et al.* (2018) reported that both IgG1 and IgG2 antibodies were induced by vaccination of cattle with a combination of rFhCL1/

FhCL3 with ZA1 adjuvant in two trials, despite mixed results of 37.6% protection in Trial 1 and 0% protection in Trial 2. Protection was significant in Trial 1 only with stronger IgG1/2 responses observed and IgG2 levels maintained during the infection, supporting the suggestion that IgG2 antibodies are an important indicator of immunity against liver fluke (Dalton *et al.*, 1996; Mulcahy *et al.*, 1998). Ortega-Vargas *et al.* (2019) found that both IgG1 and IgG2 antibodies increased rapidly 2 weeks after the first vaccination with a chimeric rFhLAP-CL1 protein/Quil A formulation in sheep, indicating a mixed Th1/Th2 response. However, statistically significant negative correlations were only observed between both IgG1 and IgG2 levels with worm burdens when all vaccinated animals were combined, but not for individual vaccinated groups. No correlations with IgG levels were observed by Orbegozo-Medina *et al.* (2018) when sheep were vaccinated with rFhCL1, or with the native (n) or synthetic (s) forms of MF6p/FhHDM-1 despite strong total IgG responses. Quil A induced mixed IgG1/IgG2 responses in each vaccinated group, where higher IgG1 responses were noted over IgG2, and the kinetics of the IgG1 responses were similar to the observed total IgG responses. Likewise, Maggioli *et al.* (2016) found that vaccination with rFhTGR using three different adjuvants (FIA, Adyvac50 and Alum) induced a mixed IgG1/IgG2 response with no significant correlations between antibody levels and worm burdens.

12.3 A Potential Way Forward

12.3.1 Adjuvants

It is well understood that adjuvants are important in driving the immune system in the desired direction for protection of the host. After identifying antigens that induce moderate to good repeatable protection against fluke, the next step would be to identify a suitable adjuvant that stimulates the immune response and improves protection (further discussed in section 12.8). It is interesting to note that only one recent study investigated the effect of different adjuvants on the efficacy of vaccination with the same antigen, where Alum performed slightly better (23%) than Adyvac50 and IFA for rFhTGR in cattle (Maggioli *et al.*, 2016).

12.3.2 *In vitro* models to screen vaccine candidates

As no experimental fluke vaccine has yet reached the commercial phase, research is still ongoing to find a reliable, efficacious vaccine against fasciolosis. The majority of recent research into screening possible candidates still focuses on the reliance of small animal models (about 30 papers) with fewer studies published on vaccine trials in livestock, such as cattle (three papers), sheep (ten papers), goats (two papers) and buffalo (one paper). Various papers discussed here have shown that vaccine research in small animal models is not directly translatable in target livestock (Maggioli *et al.*, 2016; Wesołowska *et al.*, 2016; Norbury *et al.*, 2018; McCusker *et al.*, 2020). It is clear that the cost of livestock vaccine trials is hindering advances in the field and this raises two questions:

1. Are potential livestock vaccine candidates being disregarded due to negative vaccine data obtained from small animal models?
2. Are we testing candidates in livestock based on positive data from small inappropriate animal models and thus wasting scarce monetary resources needed for vaccine development?

These concerns strongly indicate that a reliable screening model is needed to test antigens for their suitability as livestock vaccine candidates prior to testing in large animals.

It has been proposed that the immune mechanism that kills invading flukes in livestock is ADCC mediated and occurs during the early stages (within 2–4 weeks pi) of infection (Roberts *et al.*, 1997; Piedrafita *et al.*, 2007; Toet *et al.*, 2014; Sulaiman *et al.*, 2016). Previously, *in vitro* ADCC killing assays have been conducted using sheep and bovine macrophages, showing that polyvalent immune sera can induce ADCC killing at the surface of juvenile flukes (Piedrafita *et al.*, 2007; Sulaiman *et al.*, 2016). Additionally, a culture system utilizing chicken serum that is able to maintain NEJ flukes that can develop to an immature and adult-like stage has been established (McCusker *et al.*, 2016). Using such an *in vitro* ADCC system, it may be feasible to screen sera from livestock vaccinated with a specific fluke protein to provide insights into a protein's potential efficacy as a vaccine candidate prior to

conducting full-scale large-animal vaccine trials (discussed further in section 12.4).

12.3.3 Combination vaccines

Most of the *F. hepatica* antigens that have been tested to date are immunomodulators. Since ADCC is a proposed killing mechanism for NEJ fluke in the livestock host (Piedrafita *et al.*, 2007; Toet *et al.*, 2014; Sulaiman *et al.*, 2016), it follows that antigen-specific antibody is needed to target the surface of the fluke; therefore it is likely that surface exposed and tegument-associated proteins are potential key vaccine targets. Immunomodulation is a major issue that needs to be prevented to induce protection and it is possible that a vaccine utilizing one or more immunomodulating proteins, in conjunction with tegumental proteins, will improve the efficacy of either type of antigen alone. For example, protection was not achieved by vaccination with the Fhteg1/Fhteg5 combination in cattle although 48% efficacy was observed in rats (McCusker *et al.*, 2020). However, it is possible that early immunomodulation by *F. hepatica* due to the slower fluke migration in cattle may have affected the ability of cattle to target the NEJ by ADCC. Protection with Fhteg1 and Fhteg5 was induced in rats where juveniles invade the liver within 5 days (Sukhdeo and Sukhdeo, 2002). In contrast, in cattle, only 10% of juveniles invade the liver at 7 days pi (Doy and Hughes, 1984). This difference in the timing of juvenile fluke migration may be a key factor determining the efficacy of Fhteg1 and Fhteg5 in rats. Identification of potential tegument-associated proteins are addressed in section 12.4.

12.3.4 Conclusion to the above discussion

Repeatability of protection with specific fluke antigens in livestock vaccine trials still remains an issue that needs to be resolved for the development of a commercially viable vaccine. The use of a relevant *in vitro* screening method, replacing small-animal models, may allow us to better focus on selected antigens, leading to an increase in the rate of testing of promising antigens in large animals. It is likely that an efficacious fluke vaccine will consist of a combination of antigens. Once suitable vaccine candidate combinations have been found, the development of an efficacious edible vaccine would be a potentially favourable approach for vaccine delivery, due to the reduced processing time, lower cost of manufacturing and ease of delivery to livestock. However, the possible lack of significant systemic antibody production following oral delivery of an edible vaccine in host species may need to be counteracted by using an intramuscular or subcutaneous vaccine (with a suitable adjuvant) to boost responsiveness.

12.4 Identifying New Protein Vaccine Candidates on the Surface Tegument of Juvenile Flukes

12.4.1 Immunity to *Fasciola* associated with killing of juvenile flukes early after infection

Although cattle and sheep do not acquire natural immunity to fluke infection, they do acquire good immunity to *F. hepatica* after vaccination using irradiated parasites (Haroun and Hillyer, 1986), drug-abbreviated infections (Hoyle *et al.*, 2003) or single proteins (reviewed in Toet *et al.*, 2014; Molina-Hernandez *et al.*, 2015). Data from several vaccine studies show that, in protected animals, many young parasites are killed before reaching the bile ducts, suggesting that juvenile-stage flukes in the peritoneum or liver are the targets of acquired immunity in cattle (Dalton *et al.*, 1996) and sheep (Roberts *et al.*, 1997; Piacenza *et al.*, 1999). A detailed study of the establishment of flukes in Indonesian Thin Tail (ITT) sheep resistant to *F. gigantica* showed that the killing of juvenile flukes begins within 2 weeks of infection (Roberts *et al.*, 1997). Early studies *in vitro* observed an antibody–antigen precipitate forming on the surface when newly excysted juveniles (NEJ, i.e. juveniles up to 24 h post-excystment) were incubated with immune sera, highlighting that antibody can bind to the surface of NEJ flukes (Lang, 1976; Howell *et al.*, 1977; Lang and Hall, 1977; Howell and Sandeman, 1979; Hanna, 1980a). Vaccination with these crude antigen extracts has elicited 75–85% protection in mice (Lang, 1976; Lang and

Hall, 1977) and 50% protection in rats (Howell and Sandeman, 1979), demonstrating the potential of a surface-directed juvenile-specific anti-*Fasciola* vaccine in small-animal models. However, no protection was observed in sheep, which likely reflects the lack of acquired immunity to *F. hepatica* in sheep (Sandeman *et al.*, 1980).

12.4.2 Surface protein profile on surface of young *F. hepatica* changes after flukes enter the liver

The tegument is a layer of cytoplasm covering the fluke that is bounded by a plasma membrane and a glycoprotein/glycoconjugate layer called the glycocalyx. Important tegumental functions include renewal of the surface membrane after immune attack and uptake of nutrients for the fluke (Dalton *et al.*, 2004; Halton, 2004). Interestingly, the surface tegument ultrastructure changes as the fluke matures from a juvenile parasite to an immature (liver stage) parasite and adult fluke (Bennett and Threadgold, 1975; Hanna, 1980b; Dalton and Joyce, 1987). Surface labelling of juvenile, immature and adult flukes identified about 14 surface proteins of size 10–200 kDa and showed that the surface protein profile of the juvenile fluke differed from the immature fluke profile, which itself was more similar to the adult profile (Lammas *et al.*, 1985; Dalton and Joyce, 1987). Other studies used mild detergent extraction to attempt to release the tegument but the data revealed few membrane proteins and were consistent with contamination of the 'tegument' preparations with soluble proteins from the fluke gut (Haçariz *et al.*, 2012; Morphew *et al.*, 2013). Four studies have cloned surface tegument proteins in *F. hepatica* (Trudgett *et al.*, 2000; Gaudier *et al.*, 2012; Morphew *et al.*, 2013; McCusker *et al.*, 2020).

12.4.3 Proteomic analysis of the adult *F. hepatica* tegument identified several novel tegument proteins

In 2011, using strong detergent fractionation of the adult fluke tegument and proteomic analysis of these proteins, 229 tegument proteins were identified which included 129 membrane proteins such as tetraspanins (TSP) and annexins (Anx) (Wilson *et al.*, 2011). Key proteins of interest include multiple novel proteins that we termed Fhteg (Fhteg 1, 3, 5, 8, 10, 19, 21) (McCusker *et al.*, 2020). The Fhteg proteins are a group of tegument proteins observed by Wilson *et al.* (2011) as being specific to *Fasciola* or trematodes (i.e. no orthologous genes are found in gene databases at NCBI) and are thus thought to be key proteins in trematode tegument biology. Fhteg1 and Fhteg5 are each highly transcribed 2–4 days after excystment and may play key roles in the early liver stage of infection (McCusker *et al.*, 2020) (Fig. 12.1). Fhteg21 is highly transcribed early after juvenile excystment, then constitutively expressed and may play a key role in the juvenile establishment early in the host (Fig. 12.1). Fhteg19 is constitutively transcribed from the metacercaria stage and may represent a tegument housekeeping protein.

12.5 Potential Mechanisms of Immunity Against *Fasciola hepatica*

In terms of potential mechanisms of immunity, NEJs of *Fasciola* were shown to be susceptible to ADCC *in vitro* mediated by rat, sheep and cattle antibodies and macrophages bound to the parasite surface tegument (Piedrafita *et al.*, 2001, 2007; Toet *et al.*, 2014; Sulaiman *et al.*, 2016). These results suggest that ADCC may be one of the effector mechanisms that kills juvenile flukes *in vivo* and that antigens on the surface tegument of juvenile flukes, recognized by antibodies in immune sera, may be the targets for ADCC killing *in vivo*. Surface-directed antigens show promise as vaccine targets in other helminth parasites such as *Schistosoma mansoni*, with phase 1 clinical trials in progress against tegument antigens such as tetraspanin-2 (TSP2) and calpain (Sm-p80) (Fonseca *et al.*, 2015; Merrifield *et al.*, 2016; Siddiqui and Siddiqui, 2017; Molehin, 2020). We propose that tegument proteins represent potential novel vaccine antigens for *F. hepatica* control (Toet *et al.*, 2014). The key step now is to identify the juvenile surface proteins targeted by an effective immune mechanism. We suggest three key approaches to identify new candidate vaccine proteins that can be fast-tracked for evaluation in livestock.

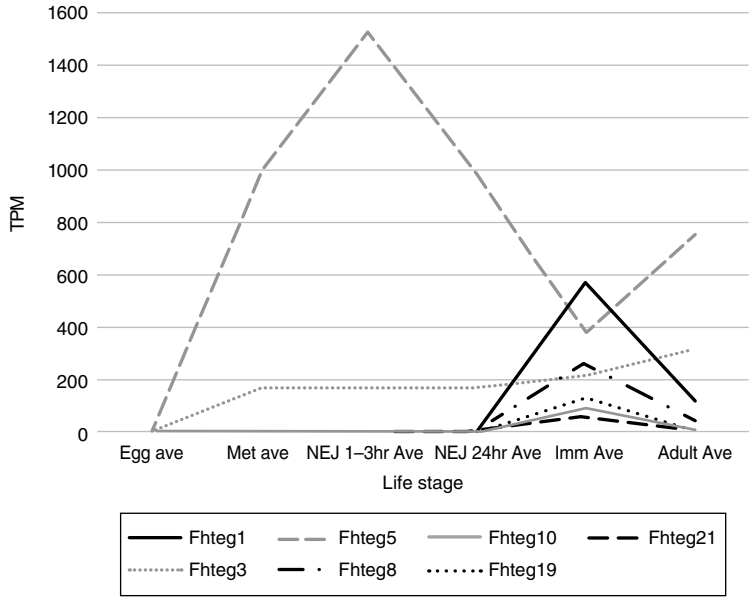

Fig. 12.1. RNA expression patterns of several *F. hepatica* teg sequences during fluke development from juvenile to immature and adult stages. Data from Cwiklinski *et al*. (2015a). FhTeg1, 3, 5, 8, 10 are all highly transcribed in immature (Imm) liver stage flukes. TPM (transcript copies (RNA copies) per million) is shown as the average (Ave) of replicate samples and is a metric to attempt to normalize RNASeq data for sequencing depth and gene length.

12.5.1 Approach 1: Proteomic analysis of antigens immunosloughed from the juvenile surface to identify new vaccine candidates

As discussed above, *F. hepatica* juveniles shed (slough) their surface antigens following binding of immune sera (IgG) to the fluke surface *in vitro,* with a half-life of about 1 h, in a process termed immunosloughing (antigen shedding) (Howell and Sandeman, 1979; Hanna, 1980a). This shedding of bound IgG is thought to be a defence mechanism against antibody-mediated attack by the host. Recently, using IgG from resistant ITT sheep infected for 4 weeks with *F. gigantica*, the immunosloughing technique was established using adult flukes as the experimental model. This study identified 38 sloughed antigens recognized by ITT sheep, including six novel membrane proteins specifically recognized by immune sheep IgG (Cameron *et al.*, 2017). Notably, 21 of the 38 proteins matched with proteins recently reported to be associated with the proposed small exosome-like EVs of adult *F. hepatica* (Marcilla *et al.*, 2012; Cwiklinski *et al.*, 2015b). In addition, 28 of these proteins are known to be expressed in juvenile RNAseq datasets, suggesting that these 28 proteins are also expressed in juvenile flukes. Clearly, these antigens are of interest as possible vaccine candidates: the challenge now is to identify the best candidates from this large number of shed antigens.

12.5.2 Approach 2: Determine the rate of turnover of individual antigens on the surface of juvenile flukes

It would be of interest to compare the immunosloughing of individual antigens from the surface of cultured juveniles to determine whether antigens vary in their shedding rate, as this may impact the ability of the host to mount effective ADCC against particular proteins on invading flukes. The rate of sloughing of individual antigens shed from the surface may be a key factor that determines whether ADCC can be

effective against juvenile flukes, since killing of flukes *in vitro* occurs over a 1–3-day period (Piedrafita *et al.*, 2001, 2007; Sulaiman *et al.*, 2016) whereas the half-life for antigen shedding is about 1 h (Hanna, 1980a). By using IgG specific to recombinant tegument proteins, it should be possible to examine the sloughing rates of individual proteins during incubations *in vitro* using fluorescent labelled secondary antibodies and confocal microscopy. For example, do some proteins shed within 1–2 h whereas others are shed over a longer period? It is also possible that other antigens may be bystander proteins that form a complex and are released together with the specific antigen that is the target of the IgG (discussed in Cameron *et al.*, 2017). These types of experiments will better inform our understanding of the behaviour of juvenile fluke surface proteins after exposure to specific IgG and whether proteins vary in their rate of sloughing. Such data could allow us to identify a refined list of key tegument proteins that may be new efficacious vaccine candidates. ADCC assays using sera to individual antigens (Approach 3, below) will then allow us to assess whether the turnover rate influences the degree of killing by ADCC *in vitro*.

12.5.3 Approach 3: Using the tegument proteins identified in Approaches 1 and 2, evaluate whether these are good ADCC targets and potential lead candidates for testing as vaccines *in vivo*

We suggest that evaluation of the level of ADCC *in vitro* to juvenile flukes, induced by specific IgG to individual recombinant tegument proteins, may identify new lead proteins for downstream vaccine evaluation. Such ADCC methods are now well described (Piedrafita *et al.*, 2001, 2007; Sulaiman *et al.*, 2016). Evidence suggests that killing of juvenile flukes occurs in the liver in resistant sheep, or vaccinated sheep or cattle, after 2–6 weeks of infection, suggesting that there may be a 'window for fluke killing' in the liver within 6 weeks of infection; data in ITT sheep resistant to *F. gigantica* suggest killing of this parasite occurs as early as 14 days after infection (Roberts *et al.*, 1997). Interestingly, a precedent exists with *Taenia ovis* where there is a 'window for susceptibility' of onchospheres to complement-mediated killing at 3–9 days of *in vitro* culture, due to time-regulated expression of the target antigens To16, To18 and To45W (Jabbar *et al.*, 2010).

Although we do not know which individual juvenile antigen(s) is/are the target of the ADCC mechanism observed *in vitro*, surface tegument proteins and glycoconjugates expressed in 1–6-week-old juvenile flukes are certainly good candidates. It may be that certain antigens are better ADCC targets than others due to their level of exposure on the surface. For example, using qPCR or RNA-seq analysis, we know that cultured juveniles upregulate the expression of Fhteg1 and Fhteg5 by 4–13-fold within 2–4 days of culture *in vitro* (McCusker *et al.*, 2020). Using specific antisera we have shown that Fhteg5 (Fig. 12.2) and Fhteg1 (not shown) are localized on the juvenile surface after 7 days of culture (McCusker *et al.*, 2020).

These data suggest that we could use specific antisera to single recombinant tegument proteins to evaluate whether certain fluke antigens are actual targets of ADCC mechanisms *in vitro*. Experimentally, it would be simpler to use rat antisera to specific proteins with rat peritoneal macrophages to screen a number of antigens for susceptibility to ADCC killing of juvenile flukes (Piedrafita *et al.*, 2001); however, studies with specific cattle sera/macrophages are also potentially feasible (Sulaiman *et al.*, 2016).

It is important to note that McCusker *et al.* (2016) developed an *in vitro* culture system whereby juvenile flukes develop over time in chicken serum (CS) media to more immature/adult forms. Given the fact that it is not clear which age of juvenile is targeted for killing *in vivo*, it would be useful to compare the killing of juveniles cultured in CS *in vitro* for, say, 4–28 days to determine if there is an optimal time during fluke development for ADCC killing. By using specific sera to a range of proteins we can determine whether sera to single antigens induce good ADCC killing or whether combinations of sera to more than one protein induce better killing than single sera *in vitro*, i.e. that synergy may occur at the fluke surface during ADCC killing. In addition, with the availability of transcriptomic datasets (Cancela *et al.*, 2010; Young *et al.*, 2010, 2011; Cwiklinski *et al.*, 2015a; Haçariz *et al.*, 2015) it should be feasible to determine

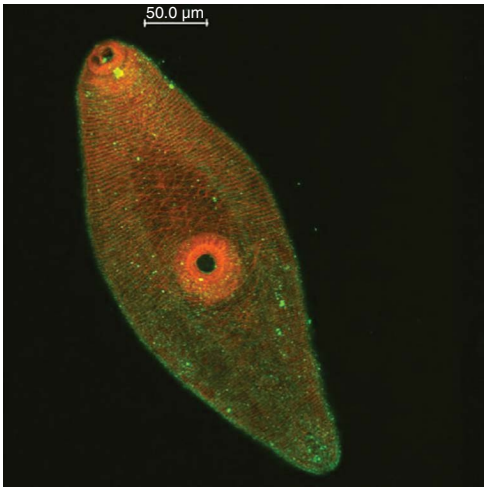

Fig. 12.2. Immunolocalization of Fhteg5 on juvenile flukes cultured for 7 days *in vitro*. The green signal shows the broad distribution of specific IgG binding to Fhteg5 on the tegument surface. The red staining shows the muscle layers stained with phalloidin-tetramethylrhodamine isothiocyanate (TRITC) (see McCusker *et al*., 2020).

whether there is an association between the level of antigen expression (as measured by RNA-seq data) and ADCC killing of juveniles.

These types of experiments may answer several questions:

- Which individual antigens are targets of ADCC killing *in vitro*?
- Are certain combinations of antigens superior ADCC targets *in vitro*?
- Is there a time window during fluke development when ADCC killing is optimal?
- Is there any correlation with sloughing rate and efficacy of ADCC observed *in vitro*?

Any proteins showing promise from these *in vitro* studies could then be fast-tracked for evaluation as recombinant protein vaccines in cattle.

12.6 Glycans and Glycoconjugates as Vaccine Targets

A new approach to develop novel vaccines for *F. hepatica* could involve the use of recombinantly engineered fluke glycoproteins or synthetic fluke glycoconjugates but relatively little is known about the glycobiology of liver flukes. Recently, the glycoproteins and glycoconjugates of *F. hepatica* have been demonstrated to play a role in stimulating a Th2/Treg immune response via interaction with several C-type lectin receptors, including DC-SIGN, MGL, dectin-1 and mannose receptors, in either human or mouse models (Guasconi *et al*., 2015, 2018; Rodríguez *et al*., 2015, 2017a,b; Ravidà *et al*., 2016a). These fluke glycoproteins and glycoconjugates have also been shown to interact with ruminant galectin 14 but the consequence of this interaction for the parasite is currently unknown (Swan *et al*., 2019). The *F. hepatica* tegument that is responsible for the majority of the host–parasite interactions is thought to be made up of approximately 370 glycoproteins, based on lectin enrichment and mass spectrometry studies (Ravidà *et al*., 2016b). The role these glycoproteins/glycoconjugates play as particular vaccine candidates and their antigenicity during infection is yet to be elucidated, but their importance in fluke biology should not be overlooked, although they are difficult to study. Glycosylation is not template driven and, due to the inevitable glycan micro-heterogeneity, many different glycoforms of a single native protein are highly possible (Astronomo and Burton, 2010). This results in specific glycoprotein vaccine candidates being difficult to identify and design.

Glycan epitopes have been demonstrated to be highly antigenic in a number of different parasitic helminth species. Currently, one of the only successful helminth vaccines commercially available to combat *Haemonchus contortus* in sheep is Barbervax® (Bassetto *et al*., 2018), which contains a crude extract of lectin-enriched glycoproteins/proteins (Smith *et al*., 1999). To date, it is still unknown if the carbohydrates in this extract play a role in antigenicity of the vaccine. In saying that, the protection elicited by vaccination of sheep with adult *H. contortus* ES is, to a significant extent, thought to be contributed by the glycoconjugates present, in particular, the GalNAcβ1,4(Fucα1,3)GlcNAc (LNDF) motif (Vervelde *et al*., 2003). This glycan motif has also been demonstrated to be antigenic in *S. mansoni* infections in mice (Nyame *et al*., 2000). Carbohydrate larval antigen (CarLA) is another carbohydrate-based antigen in *Trichostrongylus colubriformis* that has been demonstrated to induce a strong immune

response in sheep. CarLA is thought to be a glycolipid, as no protein is detectable following its purification, although no composition or structural information is known for either the glycan or lipid components of this antigen (Harrison *et al.*, 2003). Its effectiveness as a vaccine in sheep is yet to be tested; however, CarLA has been shown to stimulate a strong protective antibody response in sheep and cattle that have developed resistance to *Trichostrongylus* spp. (Harrison *et al.*, 2008; Merlin *et al.*, 2017).

Apart from the initial chemical and lectin staining experiments conducted in the 1970s and 1980s (Threadgold, 1976; Rogan and Threadgold, 1984), the number of studies investigating the glycans of *F. hepatica* has only recently begun to increase. From these newer datasets, previous protein vaccine candidates can be re-evaluated. Many different cathepsins from *F. hepatica* have been trialled in a number of different host species, as both native or recombinant proteins expressed in several expression systems (Toet *et al.*, 2014; Molina-Hernandez *et al.*, 2015). Interestingly, a substantial number of cathepsin L (FhCatL) and cathepsin B (FhCatB) proteases have appeared in multiple glycan affinity purification studies (lectin enriched or using glycan biotinylation), suggesting that they are glycosylated (Garcia-Campos *et al.*, 2016; Ravidà *et al.*, 2016b; Swan *et al.*, 2019). More specifically, particular sites on FhCatB1 and FhCatL3 have been determined to be occupied by paucimannose structures (Garcia-Campos *et al.*, 2016). Other vaccine candidates identified in glycosylated fractions include LAP, Fh14-3-3, paramyosin and GST (Garcia-Campos *et al.*, 2016; Ravidà *et al.*, 2016b; Swan *et al.*, 2019). If these candidates are in fact glycosylated, the glycans have every possibility to be playing a role in the protein characteristics, such as conformation, functionality and antigenicity. A lack of glycosylation in current recombinant versions of these proteins may reduce immunogenicity; further investigation into site-specific glycosylation of these candidates is therefore required.

Recently, the glycome of adult and NEJ *F. hepatica* has been shown to be dominated by oligomannose and paucimannose structures; in addition, less abundant complex and hybrid structures have been observed (Garcia-Campos *et al.*, 2016; Ravidà *et al.*, 2016a.b). A number of generic plant lectins have also shown binding to both NEJ and adult fluke, suggesting the presence of other more complex glycan structures in *F. hepatica* (McAllister *et al.*, 2011; Garcia-Campos *et al.*, 2016; Ravidà *et al.*, 2016b). Additionally, *in silico* data have highlighted a broad range of glycosyltransferases and glycosidases expressed in these *F. hepatica* life stages (McVeigh *et al.*, 2018). These data will be beneficial in the future if new or known fluke vaccine candidates are ever glyco-engineered in alternative expression systems.

Previously, individual components of the Barbervax vaccine have been trialled. One component, the H11 protein, has been expressed in *C. elegans* in a hope to have a more 'native' nematode-like glycosylation structure; although some glycan motifs were present, others were lacking and no protection was observed with this recombinant protein in a vaccine study in sheep (Roberts *et al.*, 2013). Similarly omega-1 and kappa-5, two major glycoprotein components of the soluble egg antigen (SEA) of *S. mansoni*, have been glyco-engineered in the leaves of *Nicotiana benthamiana* to be post-translationally modified with Lewis X (Galβ1,4 [Fucα1,3]GlcNAc) and LDN/LNDF glycan structures (Wilbers *et al.*, 2017). The glycosylation pattern of these recombinant proteins was quite heterogeneous but omega-1 was shown to stimulate a Th2-type immune response *in vitro*, using human cells, as did the native protein, as well as *in vivo* in mice (Everts *et al.*, 2009; Wilbers *et al.*, 2017). Although this plant expression system has potential for its application for *S. mansoni*, it may be less suitable for expression of *F. hepatica* glycoproteins, as the current opinion is that *F. hepatica* does not modify its *N*-glycans with core α1,3-fucose and the modification of β1,2-xylose has not been investigated, which are two common modifications in plants that are shared with *S. mansoni* (Garcia-Campos *et al.*, 2016; Ravidà *et al.*, 2016a; McVeigh *et al.*, 2018).

The two mentioned expression systems, *C. elegans* and *N. benthamiana*, have not yet been successfully evaluated for use with *F. hepatica* glycoproteins. A recombinant expression system for *F. hepatica* vaccine production will be needed in the future, as the production of native liver fluke antigens will not be commercially viable (Geldhof *et al.*, 2007). If a lead *F. hepatica* candidate antigen is glycosylated, expression systems with the capability to produce glycosylated recombinant proteins should be considered (Geldhof

et al., 2007). More strategies are becoming available to do this, including Glycoswitch technology, which engineers *Pichia pastoris* to prevent the natural hyper-mannosylation of *N*-glycans: instead *N*-glycosylation can be optimized by introducing the unique glycosidases and glycosyltransferases necessary for the desired glycosylation (Jacobs *et al.*, 2009). Likewise, a similar process has been used to manipulate the glycosylation machinery of the baculoviral insect cell system, which has the additional advantage over a mammalian cell system of producing recombinant protein much more quickly (Palmberger *et al.*, 2012). Interestingly, FhCatL3, previously mentioned to be modified by paucimannose structure, has also previously been expressed recombinantly in both *Saccharomyces cerevisiae* and a baculoviral insect cell system, without prior manipulation of the glycosylation machinery (Reszka *et al.*, 2005). When these two recombinant proteins were used to vaccinate rats, both produced comparable serological responses; however, the baculovirus-expressed FhCatL3 reduced the fluke burden by 52%, compared with no protection provided by the *S. cerevisiae* version. *F. hepatica* GST is potentially *O*-glycosylated (Garcia-Campos *et al.*, 2016) and, if the *O*-glycan could be structurally elucidated, it may be possible to be synthetically synthesized and attached to GST and potentially increase the levels of protection observed with GST (Morrison *et al.*, 1996; Toet *et al.*, 2014). These mentioned methods could potentially provide promising expression systems in the future as greater detail of the *F. hepatica* glycosylation pathways are revealed and site-specific glycosylation of the vaccine candidates is achieved.

The exact antigenicity of many of the native *F. hepatica* glycoproteins has yet to be demonstrated and it is difficult to study specific antibody responses to these glycans, considering their micro-heterogeneity. However, if glycan epitopes on fluke vaccine candidates could be identified this would provide an incentive to pursue this pathway of glyco-engineering vaccine candidates in the future (Geldhof *et al.*, 2007). As mass spectrometry and recombinant protein technologies continue to improve, a more thorough understanding of *F. hepatica* protein glycosylation is on the horizon and hopefully glycans will be considered in the future when designing novel *F. hepatica* vaccine candidates.

12.7 Whole Exosomes, Exosome Extracts and Exosome Antigens as Vaccines

Recently, attention has turned towards the description of parasite exosome-like extracellular vesicles (EVs) and their vital interactions with the definitive host in assisting parasite infection. The description of EVs in helminth parasites first occurred in *F. hepatica* and *Echinostoma caproni* (Marcilla *et al.*, 2012), and EVs are being seen as a 'new' interface between host and parasite. EVs are membranous vesicles released by numerous cell types into their immediate environment and have been implicated in intercellular communication, extracellular transport of molecules, physiological maintenance and, in terms of parasite EVs, modulation of the host immune system (Kalra *et al.*, 2012; Raposo and Stoorvogel, 2013; Marcilla *et al.*, 2014; Coakley *et al.*, 2015, 2016; de la Torre-Escudero *et al.*, 2016).

Exosomes are generated in parasite cells and encapsulated within multivesicular bodies (MVBs) which fuse with the tegument surface membrane, releasing the vesicles into the extracellular environment (Coakley *et al.*, 2015; de la Torre-Escudero *et al.*, 2016). These vesicles have the capability to interact with, or even be internalized by, host cells to modulate cell function (Marcilla *et al.*, 2012; Buck *et al.*, 2014; Wang *et al.*, 2015), but are also thought to have a function in inter-parasite communication. One example of this is with *Plasmodium falciparum* EVs, which can move between infected erythrocytes to stimulate malarial sexual development and can also transfer genetic material between infected erythrocytes to stimulate parasitic growth (Mantel *et al.*, 2013; Regev-Rudzki *et al.*, 2013). A bias in the literature focusing on the immunomodulatory capabilities of EVs means that functional descriptions of inter-parasite communication are rarely given, but this is also an important aspect of EVs to investigate as the potential disruption of these mediators of communication may also identify novel targets of immunotherapeutic potential (Coakley *et al.*, 2015).

12.7.1 EVs as immunomodulators of host responses

EVs have been identified in numerous helminth species, such as the nematode *Heligmosimoides*

polygyrus (Buck *et al.*, 2014; Coakley *et al.*, 2017) and trematodes such as *Echinostoma caproni* (Marcilla *et al.*, 2012), *Dicrocoelium dendriticum* (Bernal *et al.*, 2014), *Opisthorchis viverrini* (Chaiyadet *et al.*, 2015), *Schistosoma mansoni* (Nowacki *et al.*, 2015; Sotillo *et al.*, 2016), *Schistosoma japonicum* (Wang *et al.*, 2015; Zhu *et al.*, 2016) and *F. hepatica* (Marcilla *et al.*, 2012; Cwiklinski *et al.*, 2015b). It is the inherent ability of EVs to modulate the definitive host's immune response to enable parasite survivability through immunomodulatory proteins and microRNAs (miRNAs) that has garnered a greater focus on this host–parasite interface (Coakley *et al.*, 2015; Fromm *et al.*, 2017; Eichenberger *et al.*, 2018; Kuipers *et al.*, 2018; Tritten and Geary *et al.*, 2018; Zakeri *et al.*, 2018).

Several examples of host immunomodulation by parasite EVs have been reported. *H. polygyrus* EVs have been shown to suppress the host inflammatory response and increase parasite survival by interacting with host targets such as IL-33, a cytokine known to be involved in the inflammatory response (Buck *et al.*, 2014), as well as suppress the activation of macrophages (Coakley *et al.*, 2017). Mouse macrophages can be polarized to an M1 phenotype via an unknown mechanism when incubated *in vitro* with *S. japonicum* EVs (Wang *et al.*, 2015) while EV cargo miRNA molecules, which are known to play a regulatory role in host–pathogen interactions (reviewed in Sotillo *et al.*, 2020), can be internalized to target host mammalian cells such as mouse liver cells (Zhu *et al.*, 2016).

F. hepatica EVs have been shown to modulate immune responses and could even potentially be used as immunotherapeutics to treat autoimmune diseases such as colitis by reducing proinflammatory cytokines in a T cell independent manner (Roig *et al.*, 2018). Functional descriptions of these parasite EVs are still to be reported and further elucidation of the functionality of EVs will provide a greater understanding of the host–parasite biology and potentially highlight key novel targets for therapeutic control (Eichenberger *et al.*, 2018; Tritten and Geary, 2018; Sotillo *et al.*, in press).

12.7.2 Parasite EVs as vaccines

Parasite EVs have been proposed as a potential vaccine target in order to disrupt host–parasite interactions (Kifle *et al.*, 2017; Mekonnen *et al.*, 2018; Drurey *et al.*, 2020). EVs isolated from protozoan parasites have demonstrated an ability to elicit protection in mice when whole purified parasite EVs were used as a vaccine antigen source against *Toxoplasma gondii* (Beauvillain *et al.*, 2007, 2009) and *Leishmania major* (Schnitzer *et al.*, 2010). Chickens vaccinated with EVs from a mixture of *Eimeria* spp. showed reduced mortality rates and less severe disease symptoms when infected with a combination of *Eimeria tenella*, *E. maxima* and *E. acervulina* (del Cacho *et al.*, 2012), while a follow-up study showed that vaccination with only *E. tenella* EVs had a similar impact in protection from disease (del Cacho *et al.*, 2013).

Focusing on helminths, a recent vaccination study with EVs of the nematode *H. polygyrus* in mice elicited an 82% reduction in worm burden and a significant reduction in egg production and stimulated a strong EV-specific antibody response for IgM, IgA, IgE and IgG1 (Coakley *et al.*, 2017). Although no significant reductions in worm burden were elicited when mice were vaccinated with EVs isolated from the trematode *Echinostoma caproni*, the vaccinated group showed reduced severity of disease symptoms and lower mortality rates than the control group post-infection (Trelis *et al.*, 2016).

A vaccine trial in hamsters using EVs or recombinant EV-localized tetraspanins (TSPs) of the human liver fluke *O. viverrini* elicited significant protection of 21–34% (Chaiyadet *et al.*, 2019). TSP1 is a protein known to be EV-associated and has a role in the uptake of EVs by recipient cells (Raposo and Stoorvogel, 2013). When *O. viverrini*-derived EVs were incubated with anti-sera to TSP1, EV uptake by host cholangiocytes was significantly inhibited and the production of pro-inflammatory cytokines such as IL-6 was also reduced, thus highlighting the potential of an EV-directed immune response to inhibit EV functionality (Chaiyadet *et al.*, 2015). A follow-up study showed that antisera directed against *O. viverrini* TSP2 and TSP3 was also successful in reducing the uptake of EVs by host cholangiocytes *in vitro* by 91% and 77%, respectively (Chaiyadet *et al.*, 2019). The ability to successfully direct an immune response towards parasite EVs and inhibit their host interaction and functionality suggests that EV-directed targets have a high

level of promise (Marcilla *et al.*, 2014; Coakley *et al.*, 2016; de la Torre-Escudero *et al.*, 2016).

12.7.3 Characterization of EVs from *F. hepatica*

Marcilla *et al.* (2012) first described EVs secreted from *F. hepatica* and posited that the identification of these vesicles explains a new active mechanism for secretion of atypically secreted proteins such as annexins and heat shock proteins which lack a classical secretory signal. Cwiklinski *et al.* (2015a) provided a more thorough proteomic analysis of *F. hepatica* EVs, identifying large and small EVs and analysing the components of EV lumen (cargo) and EV membrane proteins separately, although there is a high degree of overlap observed with the 180 proteins identified between these fractions. For example, a large number of known ES proteins that are likely to be cargo-associated, such as the cathepsin B and L proteases, leucine aminopeptidase (LAP) and legumains, were also identified in the membrane fraction; conversely, membrane-associated proteins such as annexins, tetraspanins and integral multipass membrane transporters were identified in the lumen fraction, indicating an incomplete separation of the cargo and membrane components during their isolation and fractionation.

Bennett *et al.* (2020) investigated the biogenesis of *F. hepatica* EVs and determined that EVs are predominantly derived from the gut rather than the tegument. The addition of the chemical inhibitor of sphingomyelinase (SMase) (GW4869) was shown to inhibit EV biogenesis, suggesting that SMase has a critical role in EV biogenesis (Bennett *et al.*, 2020). SMase was also identified as a target antigen in the immunosloughing study of Cameron *et al.* (2017), proposing this protein, or other proteins associated with EV biogenesis, as attractive vaccine targets (de la Torre-Escudero *et al.*, 2019).

There are currently no reported studies using *Fasciola* spp. EVs as a vaccine. Although the use of whole EVs would not be feasible from a commercial viewpoint, initial vaccination studies using whole purified EVs would prove invaluable as a proof of the principle that *Fasciola* EVs are a suitable target, similar to the trials described above using protozoan parasite EVs. Fractionation of EVs into cargo and surface membrane proteins and their evaluation as whole extract vaccines may prove informative as to which components of the EV to target, and candidate antigen selection could proceed from there (Mekonnen *et al.*, 2018). Two different subpopulations of *F. hepatica* EVs (termed 15K and 120K) have been described which differ in size and cellular origin (Cwiklinski *et al.*, 2015b; de la Torre-Escudero *et al.*, 2019). Murphy *et al.* (2020) used a gravity flow method to isolate EVs from adult *F. hepatica* and showed that this EV population exhibited a comparable proteomic profile to the 15K and 120K EVs described above. They concluded that the EVs isolated by gravity flow essentially consist of the same populations as the combined 15K and 120K EVs. Further examination into the immune response and host–parasite interactions of these different subpopulations is required.

Known vaccine candidate antigens of *S. mansoni* have been identified within EVs such as annexins, tetraspanins, calpain and GST, and it is thought that the presence of these proteins in EVs is a potential mechanism by which some or all of these antigens are having a protective impact as vaccines (Sotillo *et al.*, 2016). Previously identified vaccine candidates are present within the *F. hepatica* EVs, including the above-mentioned cathepsin L/B proteases and LAP, as well as other proteins proposed as candidates based on orthologues in other species, such as the annexins and tetraspanins; this suggests that the EV proteome may reveal further novel candidate antigens (Cwiklinski *et al.*, 2015b). In one novel study, Cameron *et al.* (2017) incubated adult liver flukes with IgG from infected ITT sheep resistant to *F. gigantica* and analysed the proteins sloughed from the tegument surface: they found that 55% of immunogenic proteins identified were associated with the *F. hepatica* EV membrane or were cargo proteins. These results show that the IgG response in a resistant host includes specificities that target EV proteins directly and suggest that the ITT sheep's ability to target the EV may be a factor in establishing protection from *Fasciola* infection.

Targeting EV biogenesis is proposed as a way to inhibit EV function; hence, targeting proteins known to be associated with this pathway such as Rab GTPases, SMases and ALIX, to name but a few, may be feasible (reviewed in de la

Torre-Escudero *et al.*, 2016); orthologous versions of each of these proteins were all identified in the *F. hepatica* proteome. Identification of the proteome of juvenile *Fasciola* spp. EVs would also provide fascinating insights about proteins unique to EVs within that life stage and would help us understand the functionality of *Fasciola* spp. EVs, and whether the different life stages produce specific EVs which will interact with the host in different ways.

12.8 New Adjuvants to Enhance Liver Fluke Vaccine Efficacy

An optimal vaccine can be defined as an antigen–adjuvant formulation that is considered commercially acceptable, can generate long-lived immunity after a single dose (or at least with one booster) and has minimal to nil side effects in the host. Each component within a vaccine formulation must be compatible to generate significant efficacy, as potency is not purely based on the antigen alone. It is critical to identify the antigen and adjuvant combination that stimulates the desired immune response in the human or animal host to control the target pathogen. However, there has been limited comparative research defining the optimal adjuvant for an antigen to develop a vaccine towards liver fluke (*F. hepatica* and *F. gigantica*). Selection of a beneficial adjuvant is paramount when assessing the efficacy of recombinant proteins as vaccine candidates, since they often lack the immunogenicity of their native protein counterpart. Given that the use of recombinant proteins is a practical and cost-effective pathway to vaccine commercialization, the choice of adjuvant will be key in driving protection. In order to choose a suitable adjuvant, an understanding of the protective immune mechanisms in a host is needed. Based on current understanding, an effective fasciolosis vaccine adjuvant would be able to induce a type 1 helper T cell response (Th1), with certain immunomodulatory adjuvants known to be capable of eliciting this type of immune response (Dalton *et al.*, 2013; Molina-Hernández *et al.*, 2015).

Helminth parasites are known modulators of the host's immune system, in general stimulating a dominant Th2-like anti-inflammatory immune response (Anthony *et al.*, 2007; McNeilly and Nisbet, 2014). During infection with *F. hepatica*, this results in parasite-induced differentiation of alternatively activated M2 macrophages in the host, with polarization of the Th2 immune response and active suppression of the Th1 immune response (Dalton *et al.*, 2013; Molina-Hernandez *et al.*, 2015). It is suggested that dampening the effects of the dominant parasite-induced Th2 immune response could be critical to elicit significant protection in livestock (Dalton *et al.*, 2013; Toet *et al.*, 2014; Molina-Hernandez *et al.*, 2015). This idea has been strongly supported by studies showing that ITT sheep, which are naturally resistant to *F. gigantica* (but susceptible to *F. hepatica*), exhibit elevated levels of Th1-associated cytokines (IL-2 and INFγ) and elevated IgG2 levels when displaying resistance during the early stages of infection (Piedrafita *et al.*, 2004; Pleasance *et al.*, 2011). In addition, it has been shown that juvenile *F. hepatica* are susceptible to ADCC *in vitro* and are killed in a nitric oxide-dependent manner, which is a response created by classically activated macrophages within a Th1 cytokine environment (Piedrafita *et al.*, 2007; Toet *et al.*, 2014). These observations have resulted in the prediction that a vaccine containing an adjuvant capable of stimulating a strong Th1 immune response could induce protection against liver fluke.

An adjuvant can enhance the immune response in a vaccine through different mechanisms: increasing antigen stability, enhancing presentation and uptake, recruitment of immune cells, promoting trafficking to secondary lymph nodes and modulating the immune system (Singh and O'Hagan, 2003; Awate *et al.*, 2013; Bastola *et al.*, 2017). To produce long-lasting immunity through a primed adaptive immune response, the innate system has to be stimulated first. Innate immune cells are activated through binding of their pattern-recognition receptors, often by conserved bacterial structures that adjuvants can mimic, leading to the activation of antigen-presenting cells (APCs) (O'Hagan and Valiante, 2003; Brunner *et al.*, 2010). Adjuvants can increase antigen phagocytosis and presentation via the major histocompatibility complex (MHC), with some adjuvants influencing presentation via either MHC I or MHC II that differentially activate cytotoxic T cells or T helper cells,

respectively (Spickler and Roth, 2003; Bastola *et al.*, 2017). Immunomodulating adjuvants can alter the cytokine repertoire and influence the overall Th1/Th2 dichotomy (Singh and O'Hagan, 2003; Awate *et al.*, 2013). Veterinary vaccinology has the added difficulty of varied responses and effectiveness of adjuvants in different host species. To combat a parasite such as liver fluke that infects numerous host species, reliance on one cross-species adjuvant may not be sufficient; therefore, a single universal fasciolosis vaccine may not be possible and species-specific vaccine formulations may be required.

12.8.1 Adjuvants showing efficacy for vaccines against *Fasciola* spp.

12.8.1.1 Comparative screening of adjuvants

Fasciolosis vaccine development is still at the stage of assessing candidate antigens, with adjuvant selection a secondary consideration, resulting in limited studies investigating the comparative immunogenicity of a vaccine using multiple adjuvants. The two most comprehensive publications assessing the effects of adjuvants show that protection elicited by an antigen is adjuvant dependent. Cattle immunized with native glutathione *S*-transferase (nGST) exhibited an efficacy range of −14% to 68% using ten different adjuvants (Morrison *et al.*, 1996); and sheep vaccinated with recombinant leucine aminopeptidase (rFhLAP) had significant reductions of fluke burdens, but displayed an efficacy range of 49.5–86.9% using five different adjuvants (Maggioli *et al.*, 2011b). Currently, the highest protection against liver fluke infection in cattle (69.5%) was observed using Freund's Complete Adjuvant (FCA) followed by Freund's Incomplete Adjuvant (IFA) using native cathepsin L1 (nFhCL1) (Dalton *et al.*, 1996). In sheep the highest protection was observed with the same adjuvant and native LAP (nLAP) (89.6%) (Piacenza *et al.*, 1999). FCA is a viscous water-in-oil (W/O) emulsion which stimulates strong antibody production due to the presence of inactivated mycobacteria (Stills, 2005). Although FCA/IFA is a good starting point for determining potential efficacies of new antigens, it can cause granulomas at the site of injection as an unwanted side effect in livestock, making it unacceptable for commercialization (Stills, 2005). An adjuvant selected for use in production animals needs to be safe, cause minimal to nil side effects, and be available commercially at an acceptable price, approved by regulatory authorities and accepted by farmers and consumers.

12.8.1.2 Oil emulsions

Emulsions are frequently used adjuvant formulations that induce a depot effect, where antigens are trapped at the injection point and slowly released to APCs (Awate *et al.*, 2013). Emulsions can vary in composition, oil type and the aqueous/oil-phase ratio. Mineral oil-based adjuvants gained comparatively suboptimal results in the nGST study of Morrison *et al.* (1996); however, those adjuvants have since evolved into products such as the Montanide™ series (Seppic™, Air Liquide Healthcare). Montanide™ ISA 70VG (W/O) and ISA 206 (W/O/W, i.e. water-in-oil-in-water) have been tested in cattle with rCatL1, inducing efficacies around 48% and a mixed IgG2/IgG1 response following a natural infection (Golden *et al.*, 2010): however, neither formulation alone induced a significant reduction in fluke burden. ISA 70VG has also been evaluated in buffalo against *F. gigantica* infection, gaining significant protection (35%) using rGST and rFABP in combination as antigens (Kumar *et al.*, 2012). Another mineral oil-based W/O adjuvant that warrants further analysis is Adyuvac 50 (Lebak Trading), which gained 74.4% efficacy using rLAP in sheep (Maggioli *et al.*, 2011b), which is proportional to the highest efficacy established with the nLAP using FCA/IFA in sheep (Piacenza *et al.*, 1999). There is a substantial gap in the literature describing this adjuvant, with the Th1/Th2 balance largely unknown. This was partially explored in Maggioli *et al.* (2011b) who, using rLAP as an antigen, showed similar IgG1/IgG2 ratios in sheep as that seen with FCA/FIA after vaccination, indicating a potential preference for the desirable Th1 response. However, Adyuvac 50 attained no protection (3.5% efficacy) with rTGR in cattle, showing how the efficacy of a formulation can vary dramatically between host species (Maggioli *et al.*, 2016).

A promising oil-in-water (O/W) adjuvant is MF59® (Novartis), which displays a unique mechanism of action and a capability to induce

both Th1 and Th2 cytokine responses (Brunner *et al.*, 2010; Sahly, 2010). MF59® is a microfluidized emulsion with uniform droplets of squalene oil and two ionic surfactants (Tween 80 and Span 85) (O'Hagan and Valiante, 2003). MF59® can be easily mixed with an antigen solution and injected and is currently used in the human influenza vaccine Fluad® (Novartis). MF59® is not believed to bind antigens and create the depot effect but is co-administered and independently stimulates muscle cells to recruit APCs that envelop the antigen, transport it to lymph nodes and present it to B and T cells (O'Hagan and Valiante, 2003; Awate *et al.*, 2013). MF59® shows promise as a fluke vaccine adjuvant, gaining a significant efficacy of 41% using nGST in cattle (Morrison *et al.*, 1996). The protection gained with MF59® was greater than the mineral oil-based and Th1-stimulating adjuvants within the same study, and comparable to the mean efficacy (49%) of the lead combination, nGST in Quil A/Squalene Montanide 80 (Morrison *et al.*, 1996). Both lead adjuvants in that study were squalene oil-based, indicating its potential preference as a fluke vaccine adjuvant over mineral oil-based adjuvants. This is noteworthy because squalene oil used in O/W adjuvants is less viscous and easier to formulate. Squalene is a precursor to cholesterol, is naturally produced by all animals and is a known component of an adjuvant system (Fox, 2009).

The RIBI adjuvant system® (Sigma-Aldrich) is another squalene O/W adjuvant that contains immunomodulatory bacterial cell wall components and increases both humoral and cellular immune responses, with preferential induction of Th1-associated cytokines in mice (Stills, 2005; Cargnelutti *et al.*, 2013). RIBI gained significant protection (49.8%) in sheep vaccinated with rLAP but induced a relativity low IgG2/IgG1 ratio and lower protection compared with other adjuvants tested simultaneously (Maggioli *et al.*, 2011b; Toet *et al.*, 2014). With the potential capacity to elicit significant protection, RIBI is yet to be tested as a fluke vaccine adjuvant in cattle. A single antigen comparative study in cattle of squalene oil (MF59®, RIBI) and mineral oil (ISA 70, ISA 206) adjuvants would confirm if one type surpasses the other for a commercial fluke vaccine.

12.8.1.3 Alum

Aluminium salt-based adjuvants, such as aluminium hydroxide, stimulate a Th2-like immune response (Spickler and Roth, 2003). Alum absorbs soluble antigens to its surface via hydrophobic interactions, creating insoluble alum–antigen particles that are readily internalized by APCs (Brunner *et al.*, 2010; Awate *et al.*, 2013). Furthermore, alum has the ability to induce innate immune cells to the site of injection and stimulate a significant humoral IgG1 response (Kool *et al.*, 2012). Alum has elicited protection comparable to FCA/IFA in cattle when vaccinating with a crude adult fluke extract, gaining 85.9% protection relative to Freund's adjuvant (96.8%) (Guasconi *et al.*, 2012); similarly, in sheep vaccinated with rLAP, the alum formulation induced 86.9% protection relative to 83.8% observed with Freund's adjuvant (Maggioli *et al.*, 2011b). Alum has also been used alongside other adjuvants (IFA and Adyuvac 50, both W/O emulsions), showing the highest (albeit non-significant) protection at 23% compared with 8.2% and 3.8%, respectively, when vaccinating with an antigen that elicits low levels of protection in cattle: thioredoxin glutathione reductase (rFhTGR) (Maggioli *et al.*, 2016). The perplexing aspect of alum is the production of a higher IgG1/IgG2 ratio and lower fluke-specific antibodies compared with Th1-stimulating adjuvants, yet it outperforms those adjuvants in eliciting protection in livestock, suggesting that alum should not be discounted as an adjuvant, even though it stimulates a Th2-like immune response (Maggioli *et al.*, 2011b, 2016). This highlights our need to better understand host protective mechanisms against *Fasciola* spp. and the role of participating antibodies and cell-mediated mechanisms (see Chapter 9).

12.8.1.4 Quil A

Quil-A is a common veterinary adjuvant shown to elicit moderate to no protection with a range of key fluke molecules. Quil-A is a complex extract of saponins purified from the tree bark of *Quillaja saponaria.* The mechanism of action for Quil-A is not yet fully described but it is known to increase a humoral response while stimulating a mixed Th1/Th2 response and cytotoxic T lymphocytes (Spickler and Roth, 2003; Rajput

et al., 2007). In cattle, Quil-A mixed with squalene and Montanide 80 (which is no longer commercially available) notably gained reproducible efficacy (mean 43%, range 19–69%) using nGST across five cattle trials (Morrison *et al.*, 1996; Toet *et al.*, 2014).

This adjuvant combination is comparable to the adjuvant adaptation (ADAD) system that uses the saponin extract from *Q. saponaria* and a non-mineral oil, Montanide ISA 763A, to form a W/O micelle encapsulating additional immunomodulators. This ADAD adjuvant, containing the immunomodulator PAL, a hydroalcoholic extract of the fern *Phlebodium pseudoaureum* (syn. *Polypodium leucotomos*), induced a significant 24.3% protection in sheep vaccinated with native Fh12 fatty acid-binding protein (Martínez-Fernández *et al.*, 2004). Other immunomodulators have been tested using the Fh12 antigen with ADAD in sheep: OA0012, a synthetic lipidic aminoalcohol, induced 25% (non-significant) protection but elicited 42% (non-significant) protection when combined with PAL (López-Abán *et al.*, 2008, 2012; Vicente *et al.*, 2014, 2015, 2016). With recombinant Fh15 antigen, the ADAD system induced 43% (non-significant) protection against *F. hepatica* in sheep (López-Abán *et al.*, 2007). Although validation in livestock is needed, mouse studies indicate that this system induces a proinflammatory immune response, with prior indications that OA0012 is capable of increasing nitric oxide production (del Olmo *et al.*, 2006; López-Abán *et al.*, 2008; Vicente *et al.*, 2014). This system has the intriguing concept of an initial 'adaptation' of the immune system, since the first vaccination solely contains the ADAD system while the subsequent vaccination contains the antigen within the ADAD system. Although an initial adjuvant immune priming likely increases the response to the later vaccinated antigen, it may require an additional dose of ADAD and antigen to achieve sustainable efficacy, i.e. a three-dose protocol. However, the synergetic effects of Quil-A with other immunomodulators show promise and warrant further investigation with other antigens.

Additionally, Quil-A alone in goats and sheep has elicited protection with vaccines comprising FhCL1 (Villa-Mancera *et al.*, 2014; Ortega-Vargas *et al.*, 2019), along with no protection elicited in other studies using rFhCL1 (Pérez-Écija *et al.*, 2010; Zafra *et al.*, 2013a,c; Orbegozo-Mendina *et al.*, 2018) or other antigens (Mendes *et al.*, 2010; Orbegozo-Medina *et al.*, 2018). Interestingly, Orbegozo-Mendina *et al.* (2018) showed that Quil-A was superior to alum in aiding production of specific IgG in sheep after parasite challenge following immunization of the adjuvants alone. They suggested that Quil-A can increase antibody levels without prior antigen stimulation by increasing total lymphocytes and expansive drainage of the lymphatic system, resulting in a lower threshold for T cell activation when the host is later exposed to the parasite.

Considering that Quil-A shows selective protection, future use could instead be steered towards QS-21. QS-21 is a less toxic, further purified form of Quil-A that has retained adjuvant properties with the ability to induce Th1-biased cytokines (Kensil *et al.*, 1991; Sun *et al.*, 2009). The Th1-inducing abilities of QS-21 (also known as QA-21) was employed in the CaniLeish® vaccine for canine leishmaniasis, with prior determination that Th1-type immunity is needed to counteract the parasite's ability to immunomodulate the host towards a Th2-like immune response (Moreno *et al.*, 2012; Martin *et al.*, 2014). This principle proved effective and induced long-lasting immunity, with dogs protected against experimental *Leishmania* challenge one year after vaccination, displaying a dominant Th1 profile and macrophages still expressing nitric oxide synthase, leading to the production of nitric oxide derivatives (Martin *et al.*, 2014; Moreno *et al.*, 2014). In cattle, the addition of QS-21 to a commercially available foot-and-mouth vaccine (containing Montanide ISA 206) induced neutralizing antibodies earlier than the commercial vaccine (Çokçalışkan *et al.*, 2016).

Another potential Quil-A-containing adjuvant is ISCOM (Immune Stimulating COMplex), a cage-like structure of cholesterol, phospholipids and Quil-A that attaches antigens through hydrophobic interactions (Hu *et al.*, 2001). Importantly, ISCOMs have fewer side effects than Quil-A, as the Quil-A is bound within the ISCOM and has minimal lytic effects since it can no longer interact with cell membranes (Rönnberg *et al.*, 1995; Sun *et al.*, 2009). ISCOMs can induce humoral immunity and a balanced cellular Th1/Th2 response (Hu *et al.*, 2001; Lövgren Bengtsson *et al.*, 2011). ISCOMs can also establish

mucosal immunity, with sheep showing some protection from *F. hepatica* after intranasal administration of CatB2 and CatL5 with ISCOM and CpG (Norbury *et al.*, 2018). Notably, the group vaccinated intranasally with CatL5/CatB2 and CpG-ODN2135/ISC adjuvant were better protected (45.5%) than the intramuscularly vaccinated CatL5/CatB2 and Quil-A group (20.9%). Uniquely, the intranasal antigen group showed significantly reduced egg viability (92%) and induced a Th1 response post-vaccination (Norbury *et al.*, 2018). Interestingly, although this study did not identify increased IgA levels after intranasal vaccination, it does highlight the promise of ISCOMs as mucosal adjuvants. It should be noted that not every antigen easily associates with ISCOMs, with non-amphipathic molecules potentially having to be modified to expose hydrophobic regions or chemically coupled to ISCOMs for adequate incorporation (Barr *et al.*, 1998; Lövgren-Bengtsson and Morein, 2000; Stills, 2005).

12.8.1.5 Strong Th1-inducing vaccine formulations

It appears that adjuvants currently available on the market might not be capable of inducing the substantial Th1 immune response potentially required to elicit protection against liver fluke. Strong immunomodulatory molecules are produced by other pathogens and these molecules have been tested in current commercial vaccine formulations to establish a Th1-like immune response. An example pathogen is *Bordetella* spp. that cause respiratory infections; *B. pertussis* causes whooping cough in humans, whereas *B. bronchiseptica* has a broad host range and causes kennel cough in canines (Mattoo and Cherry, 2005). Clearance of *Bordetella* infection requires both the stimulation of cell-mediated (Th1/Th17) and antibody-mediated immune response (Solans and Locht, 2019). Inactivated whole-cell *Bordetella* spp. vaccines are known to induce a strong Th1 response, while acellular vaccines induce both the Th1 and Th2 responses (Mattoo and Cherry, 2005; Mills *et al.*, 2014; Ellis, 2015). Less virulent live *Bordetella* mutants have been used as a vaccine carrier of foreign antigens. Intranasal vaccination of a live attenuated recombinant *B. pertussis* with *Schistosoma mansoni* GST (Sm28GST) fused to the adhesin molecule, filamentous haemagglutinin (FHA), stimulated specific IgG and IgA at the mucosal surface and induced 56% protection against the parasite in mice (Renauld-Mongenie *et al.*, 1996; Mielcarek *et al.*, 1998). *B. pertussis* FHA antigen acts as an adjuvant when co-administrated intranasally in mice with Sm28GST, showing an increase in antigen-specific antibodies and increased levels of mRNAs coding MHC II (Poulain-Godefroy *et al.*, 2003). Interesting findings were observed by Lee *et al.* (2020) showing that acellular *B. bronchiseptica* increased dendritic cell activity with higher MHC II expression and IL-12 production which was greater than that induced by LPS stimulation, indicating that *B. bronchiseptica* possesses adjuvant capabilities.

These data raise the interesting possibility of using the immune-modulating abilities of current commercially available vaccine formulations as the 'adjuvant' to deliver a candidate liver fluke protein for inducing a Th1 response. Examples of possible vaccines include the Protech BB *B. bronchiseptica* vaccine for dogs, the Canigen KC *B. bronchiseptica*/para influenza virus vaccine for dogs and the Canileish dog vaccine. It would be interesting to determine the immunological effect and safety of co-administration of candidate fluke antigens with such existing commercially available formulations in cattle.

12.8.1.6 Mucosal adjuvants

Stimulation of mucosal immunity could protect livestock from liver fluke, as the parasite establishes infection at the mucosal surface. Parenteral administration of antigen has a limited ability to stimulate mucosal immunity and IgA production, while mucosal immunization can induce the mucosal and systemic immune systems (Chen, 2000; Jin *et al.*, 2019). Needle-free mucosal delivery strategies, such as oral or intranasal administration, are often safer to administer but optimal delivery approaches are needed to prevent protein degradation (Chen, 2000). Two potent mucosal adjuvants are bacterial AB_5 toxins: the cholera toxin (CT) and the *Escherichia coli* heat-labile enterotoxin (LT). Of particular interest here are non-toxic forms of LT that can activate both the Th1 and Th2 arms of immunity, compared with CT's restricted Th2 stimulation (Williams *et al.*, 1999; da Hora *et al.*,

2011). Toxicity of the catalytic A domain can be eliminated in LT mutants with the non-toxic B subunit (LTB) alone showing adjuvanticity (Ma, 2016; Seo *et al.*, 2020). The binding between LTB and its ganglioside-GM1 receptor stimulates the immune system, with LTB implicated in increasing antigen presentation to lymphocytes, T cell activation/differentiation and immunoglobulin class switching (de Haan *et al.*, 1998; Rappuoli *et al.*, 1999; Boyaka *et al.*, 2003). Mucosal administration of LTB preferentially induces a Th1 immune bias in mice, showing elevated levels of IFNγ and/or IgG2 production (Conceição *et al.*, 2006; Su *et al.*, 2019). Pigs intranasally administered with LTB fused to three *Mycoplasma hyopneumoniae* antigens had increased specific IgG and IgA levels, with the chimeric protein retaining ganglioside-GM1 binding activity and antigenicity of all linked proteins (Marchioro *et al.*, 2014). No current literature has explored the Th1/Th2 cytokine response to LTB after mucosal delivery in cattle and sheep. CTB and LTB can induce the production of bovine antibodies to linked antigens after intranasal or subcutaneous vaccination in cattle (Ayalew *et al.*, 2009; Cunha *et al.*, 2014; Ghazali-Bina *et al.*, 2019). Moreover, Pelosi *et al.* (2012) found that plant-based delivery methods for LTB via oral consumption protects LTB from the sheep ruminant digestive system, with the plant material used critical to antigen release and immunogenicity.

12.8.1.7 Oral vaccine delivery

Oral vaccines have also been explored for the delivery of virus-like particles, which repetitively present an abundance of antigens on the non-replicating virion surface. Wesołowska *et al.* (2018) tested transgenic lettuce expressing a fluke cysteine proteinase cathepsin (CPFhW) fused to the hepatitis B core antigen in cattle and sheep, gaining 56.2% and 35.5% protection against fluke challenge, respectively. This novel adjuvant and route combination achieved comparable efficacy to previous studies testing CatL1 (Dalton *et al.*, 1996; Golden *et al.*, 2010; Toet *et al.*, 2014). Virus-like particle platforms should be further explored for fasciolosis, as they induce strong humoral responses by directly interacting with B cells and have the potential to activate Th cells and cytotoxic T lymphocytes (Liu *et al.*, 2012). Virus-like particles act as antigen carriers and can require an adjuvant for complete protection, as seen with the human papillomavirus vaccine, Gardasil® formulated with alum (Cimica and Galarza, 2017). Given the relatively restricted level of efficacy gained with currently used antigens, it may be informative to use a combination of adjuvants or viral display mechanisms as a promising way forward to deliver liver fluke vaccines.

In summary, we suggest that further assessment of a liver fluke antigen's immunogenicity should take place in combination with a range of adjuvants with differing mechanisms of action, as antigen effectiveness can be adjuvant-dependent (Morrison *et al.*, 1996; Maggioli *et al.*, 2011b). Ideally, candidate antigens should not be dismissed unless tested with multiple or a combination of adjuvants, as the synergistic effects of the antigen–adjuvant pair may stimulate good protection. It would be beneficial for future research in cattle to supplement prior work on underexplored protection-inducing adjuvants (such as MF59®, RIBI, Alum and Adyuvac 50) along with further exploring mucosal administration delivery vehicles (such as LTB and ISCOM) or oral delivery methods. Having a common repertoire of adjuvants to screen an antigen, or antigen combination, may be useful in fully evaluating the vaccine efficacy of new candidates. The specific antibodies and immune responses that contribute to liver-fluke killing in livestock remain unknown, making selection for the optimal adjuvant difficult. The current modus operandi with assessing candidate antigen efficacy is that adjuvants are a secondary consideration; this could be resulting in certain antigens being discarded as potential candidates before thorough evaluation. This thinking might be negating the potential of certain fluke antigens to establish commercially adequate levels of protection in livestock.

12.9 Assessing Liver Fluke Vaccine Efficacy in Cattle Based on Reduction in Intensity of Fluke Burden

Traditionally, liver fluke vaccine efficacy has been determined by comparing the mean fluke

burdens in vaccinated animals with the mean burden in control animals expressed as a (significant) percentage (%) efficacy or % protection of the vaccine. A vaccine with > 70% efficacy has been a generally acceptable goal for a commercial livestock vaccine. However, the key question for any fluke vaccine is: does the vaccine reduce fluke counts in a herd to such an extent (below the threshold for economic losses) that the productivity of the herd is enhanced sufficiently to justify the cost of vaccination, i.e. to maximize profit on the investment? The threshold for economic losses in cattle due to fasciolosis is estimated at 30–40 flukes (Hope Cawdery *et al.*, 1984; Vercruysse and Claerebout, 2001); production losses occur in sheep with burdens of 30–54 flukes (Hope Cawdery *et al.*, 1977; Hawkins and Morris, 1978; Dargie, 1987). So, a vaccine that can reduce mean fluke burdens in a herd to below 30–40 flukes would be of practical economic benefit to cattle producers.

12.9.1 Intensity of fluke counts in naturally infected cattle

Given a threshold of 30 flukes, it is clear that the mean efficacy required by a fluke vaccine to achieve that threshold will vary with the local intensity of infection. From a producer's point of view, it is irrelevant if a vaccine is 50% or 80% effective: they simply want the fluke vaccine to minimize productivity loss. The intensity of fluke counts in natural infections varies between countries and regions, due to factors such as seasonal differences, weather patterns, snail numbers and nutrition levels between animals; some representative examples of fluke counts are shown in Table 12.2. From 1966 to 2019, mean fluke counts in cattle have ranged from four to 126 in various countries, although the actual number of flukes per animal varies considerably. In general, based on the available mean fluke burden data, the mean burden in naturally infected cattle appears to be relatively low, with a high proportion of animals having < 50 flukes.

However, in addition to the intensity of infection, productivity losses also relate to the percentage of highly infected animals in a herd. As shown in Table 12.2, Dargie (1987) reported that, in Glasgow, over 90% of bovine livers had fewer than ten flukes and only 3% of cattle livers had > 50 flukes. More recently, Charlier *et al.* (2008) reported that 49–60% of cattle in Belgium had a low infection (< 10 flukes) and only 28–29% of animals had > 30 flukes. Turner *et al.* (2017) modelled fluke infections in calves and predicted a skewed fluke intensity with high fluke burdens (maximum about 300 flukes) in a few animals while most animals had low burdens close to the mean (19.33), which is comparable to the mean infection of 16 flukes observed by Golden *et al.* (2010) in a natural infection in Ireland.

Kelley *et al.* (2020) reported the prevalence of liver fluke in 1669 dairy cattle grazing irrigation regions in south-east Australia. Using faecal egg count (FEC) data as an indicator of the intensity of infection, they showed that 18.3% of cattle had an FEC at > 5 eggs per gram (epg), which is considered to indicate economic losses, and only 2.6% of cattle had an FEC at > 40 epg, which is considered to indicate clinical disease (Vercruysse and Claerebout, 2001). These results suggest that, although the overall mean prevalence in this region was 39%, the mean intensity of infection sufficient to cause economic losses appears to be relatively low (18.3%). Overall, the data show that a vaccine of 50–70% efficacy would likely be of benefit in a variety of countries due to the high proportion of animals (about 60% or more) in a herd with relatively low (< 50) fluke burdens.

12.9.2 Other factors impacting cattle vaccine trial design and interpretation

In experimental trials with livestock, the cost of trials (currently in Australia, for 24 cattle, about AU$100,000 or US$70,000) usually leads to the use of small group sizes to evaluate the vaccine, often of the order three to eight cattle and four to ten sheep (Table 12.3). A common feature of experimental fluke infections is the variation in the fluke counts in infected controls, as seen in natural infections, despite the general use of a controlled single challenge dose of metacercariae, usually given orally. Table 12.3 shows the fluke counts in livers from

Table 12.2. Intensity of liver fluke counts in cattle from various countries.

Reference	Year of study	Country	No. animals studied	Parasite	Mean fluke count	Range in fluke counts	Comments
Ross (1966)	1964–65	N Ireland, UK	54	*F. hepatica*	98	31–176	
Sahba *et al*. (1972)	1971	Iran	25	*F. gigantica*	68	Up to 324	
Schillhorn van Veen *et al*. (1980)	1974–75	Nigeria	14	*F. gigantica*	99	8–548	
McCausland *et al*. (1980)	1978	SE Australia	87	*F. hepatica*	24	0–324	
Malone *et al*. (1982)	1979–81	USA	36	*F. hepatica*	4–33	n/a	
Dargie (1987)	1986	UK Scotland	n/a	*F. hepatica*	n/a	n/a	> 90% of bovine livers had < 10 flukes; only 3% of livers had > 50 flukes (citing Armour, unpublished)
Anderson *et al*. (1999)	1995–96	Vietnam	92	*F. gigantica*	10 (2yo cattle); 60–80 (> 3yo cattle	< 2yo: 1–80: > 3yo: 1–500	88% of livers had < 100 flukes
Reichel (2002)	2001	SE Australia	41	*F. hepatica*	23	2–72	
Mezo *et al*. (2004)	2004	Spain	80	*F. hepatica*	Not reported	1–154	72% of livers had < 20 flukes; 91% of livers had < 50 flukes
Sothoeun *et al*. (2006)	1998–99	Cambodia	44	*F. gigantica*	100	2–250 adult flukes; 0–1000 immature flukes	Mean counts: 40 adult flukes; 60 immature flukes
Phiri *et al*. (2006)	2002–3	Zambia	102	*F. gigantica*	24–126	0–912	63% of livers had < 50 flukes
Murphy *et al*. (2006)	2002–3	Ireland	83	*F. hepatica*	Not reported	1–>100	40% of livers had < 10 flukes; 57% of livers had < 100 flukes
Charlier *et al*. (2008)	2006	Belgium	200	*F. hepatica*	n/a	1–446	49–60% of livers had < 10 flukes; 28–29% of livers had > 30 flukes
Golden *et al*. (2010)	2009–10	Ireland	13	*F. hepatica*	16	4–32	
Mochankana (2014)		Botswana	78	*F. gigantica*	30.5	3–213	53% of livers had < 20 flukes; 86% of livers had < 50 flukes
Mazeri *et al*. (2016)	2013–14	Scotland, UK	619	*F. hepatica*	8.5	1–86	
Brockwell *et al*. (2014)	2014	SE Australia	6	*F. hepatica*	24	20–31	
Kelley *et al*. (2020)	2019	SE Australia	10	*F. hepatica*	30	9–72	

controls in 29 experimental groups of cattle indicating the degree of variability observed. Note that five control groups of cattle in Table 12.3 were challenged by intraruminal injection of metacercariae and show a variation in fluke uptake of 2.02–2.72-fold, suggesting that there is natural variation in the uptake of adult fluke despite intraruminal experimental challenge. Another 24 cattle groups were challenged orally and show a 1.28–6.13-fold variation in fluke uptake. Such variation confounds interpretation of trial data.

In addition, in Table 12.3 the mean fluke count in all the 105 control cattle is 125 and only 7/105 (6.6%) or 4/105 (3.8%) cattle have < 40 or < 30 flukes, respectively; in contrast, based on the observations from field studies above, up to 70% of naturally infected cattle can have < 30 flukes. These data suggest that the average challenge dose used in experimental trials is too high and does not reflect the 'real world' field challenge in most cattle, i.e. we are overdosing cattle with metacercariae. One possibility would be to adjust the dose and do a one-off challenge at about 200–260 metacercaria/animal, which would generate a mean control fluke count of about 30–50, which better reflects the natural challenge for most animals in a herd.

Moreover, a careful analysis of the data reveals that in many, if not most, trials the fluke counts in the vaccinates will often contain 'outliers' of one to three animals where the fluke count is higher than the trend observed in the rest of the animals in the group. Such outliers also confound the assessment of efficacy. This fact, together with the use of small group sizes, may actually lead us to underestimate the potential beneficial effects of a vaccine, due to the innate variation in the fluke counts in control animals.

A key factor impacting fluke vaccine development is repeatability, as this is required to inform any commercialization decisions. In this regard, the best demonstration of repeatability was reported by Morrison *et al.* (1996) where the GST vaccine, at two doses of 400 µg and 200 µg, showed 38.5%, 49%, 52% and 69% efficacy with a single point challenge and 45% efficacy with a trickle challenge (mean of all data: 50.7% efficacy).

12.9.3 Re-analysis of vaccine data based on fraction of vaccinates with low fluke counts

We propose that there is an alternative approach to evaluate vaccine efficacy. Using data from Table 12.3, comprising 29 experimental vaccine groups and 166 vaccinated cattle, we analysed the proportion of vaccinated animals that showed fluke burdens less than the lowest fluke count in the control group, i.e. were deemed to be protected (Table 12.3 and Fig. 12.3). As anticipated, there is a very strong association between efficacy and the fraction of animals in a vaccine group with low fluke counts ($r^2 = 0.7142$). Figure 12.3 shows that there are 7/29 (24%) groups where the % efficacy is well below the trendline, i.e. the % reported mean vaccine efficacy is lower than might be expected from the overall trend – in other words, the efficacy is potentially underestimated. These seven groups are asterisked in Table 12.3 and their % efficacy is shown in Fig. 12.3.

Interestingly, Turner *et al.* (2016) recently modelled the vaccine efficacy needed to control fluke infections in livestock and found that a vaccine with only 43% efficacy would bring benefit to a cattle herd if 90% of the herd was protected for a full season. They concluded that:

> ... given that the distribution of fluke burden is heavily skewed, with the majority of animals having just a few flukes, it is particularly important that the proportion of the population protected by a vaccine is high, if the vaccine is to be effective ...

and

> ... delivering a vaccine that may have imperfect efficacy but can protect >90% of a herd is more likely to be commercially successful than one with greater efficacy but a low level of (herd) protection.

This modelling suggests that we need a vaccine with a good duration of immunity but there has been no detailed assessment of the longevity of vaccine induced protection in cattle. As an example, Morrison *et al.* (1996) showed that one group of cattle challenged 26 weeks after vaccination with GST still showed a significant 48.5% protection. Golden *et al.* (2010) showed that cattle vaccinated with recombinant cathepsin L1 showed 48% efficacy when challenged naturally over a 13-week period.

Table 12.3. Data from eight publications reporting *F. hepatica* cattle vaccine trials where individual fluke counts/animal were available.

Report	Antigen	Adjuvant	% Efficacy	Fraction of animals with low counts	No. outliers/ vaccine group	Control fluke no.(fold range in counts)	Vaccinate fluke no. (fold range in counts)
Morrison, 1996	nGST/	BCG	0	0	0/5	115, 127, 245, 257, 310 (2.70)	115, 180, 209, 273, 294 (2.56)
Morrison, 1996	nGST/	CGP11637/ SM80	0*	0.2*	1/5	115, 127, 245, 257, 310 (2.70)	***58***^, 218, 231, 277, 330 (5.69)
Morrison, 1996	nGST/	Auspharm	0	0	0/8	134, 136, 156, 165, 166, 174, 179, 238 (1.78)	138, 146, 152, 197, 211, 228, 230, 251 (1.82)
Dewilde, 2008	rHbF2/	QA/Aphigen/ cholesterol	0*	0.22*	2/9	68, 73, 74, 90, 108, 116, 121, 147, 197 (2.90)	***52***, ***65***, 96, 145, 153, 170, 193, 222^, 238^ (4.58)
Maggioli, 2016	rFhTGR/	Adyuvac	3.8*	0.17*	1/6	72, 83, 90, 91, 102, 110 (1.53)	***58***, 75, 83, 90, 100, 121^ (2.09)
McCusker, 2020	Fhteg1+ Fhteg5	FCA/IFA	4.6*	0.29*	0	82, 84, 87, 89, 122, 130, 159 (1.94)	***63***, ***81***, 97, 104, 115, 118, 141 (2.24)
Maggioli, 2016	rFhTGR/	IFA	8.2*	0.33*	0/6	56, 72, 87, 91, 96, 126 (2.25)	***23***, ***42***, 87, 99, 105, 129 (5.61)
Morrison, 1996	nGST/	QSM	19	0.33	0/6 (IR)	26, 31, 32, 35, 41, 55 (2.12)	***10***, ***10***, 34, 36, 41, 48 (4.80)
Morrison, 1996	nGST/	ISA50D	22*	0.6*	0/6	115, 127, 245, 257, 310 (2.70)	***101***, ***109***, ***113***, 224, 281 (2.78)
Maggioli, 2016	rFhTGR/	Alum	23	0.33	0/6	59, 88, 94, 108, 111, 126 (2.14)	***35***, ***45***, 63, 67, 104, 127 (3.63)
Morrison, 1996	nGST/	QSM	26	0.17	1/6 (IR)	84, 98, 118, 127, 145, 211 (2.51)	***29***, 90, 96, 100, 113, 151^ (5.21)
Morrison, 1996	nGST/	DEAE	28	0.2	0/6	115, 127, 245, 257, 310 (2.70)	***102***, 138, 162, 178, 182 (1.78)
Morrison, 1996	nGST/	ISA70	32	0.2	1/6	115, 127, 245, 257, 310 (2.70)	***34***^, 124, 158, 193, 214 (6.29)
Morrison, 1996	nGST/	ISA50	37	0.4	0/6	115, 127, 245, 257, 310 (2.70)	***40***, ***106***, 144, 192, 234 (5.85)
Dalton, 1996	nCatL1/	FCA/IFA	38	0.66	1/3	141, 142, 145, 180 (1.28)	***43***, ***91***, 148^ (3.44)
Morrison, 1996	nGST/	QSM	38.5	0.66	2/6 (IR)	106, 176, 196, 204, 214, 229 (2.16)	***44***, ***81***, ***90***, ***102***, 170^, 207^ (4.70)
Morrison, 1996	nGST/	MF59	41	0.4	1/5	115, 127, 245, 257, 310 (2.70)	***103***, ***112***, 122, 124, 164^ (1.59)
Morrison, 1996	nGST/	QSM	45*	0.88*	0/8 (IR)	82, 101, 105, 110, 112, 112, 134, 166 (2.02)	***44***, ***53***, ***55***, ***58***, ***58***, ***74***, ***79***, 87 (1.98)
Morrison, 1996	nGST/	QuilA/ SM	48.5	0.66	2/6 (IR)	123, 142, 206, 243, 293, 334 (2.72)	***54***, ***67***, ***76***, ***111***, 180^, 203^ (3.76)
Morrison, 1996	nGST/	QuilA/ SM	49	0.8	1/5	115, 127, 245, 257, 310 (2.70)	***75***, ***97***, ***106***, ***108***, 151^ (2.01)
Morrison, 1996	nGST/	QSM	52	0.25	0/8	54^, 118, 136, 145, 159, 294, 298, 331 (6.13)	***43***, ***53***, 60, 71, 97, 97, 105, 124 (2.88)
Dalton, 1996	nCatL1/	FCA/IFA	52.5	1	1/4	141, 142, 145, 180 (1.28)	***12***, ***67***, ***70***, ***140***^ (11.7)

Wedrychowicz, 2007	rCPFhW/	none; i.n.	54	1	0/3	48, 59, 81 (1.69)	***18***, ***22***, ***46*** (2.56)
Dalton, 1996	nCatL1/	FCA/IFA	54.9	0.75	1/4	141, 142, 145, 180 (1.28)	***5***, ***17***, ***105***, 147^ (29.4)
Hillyer, 1987	nFABP/	FCA/IFA	55	1	0/5	27, 27, 30, 45, 71 (2.63)	***15***, ***17***, ***18***, ***19***, ***20*** (1.33)
Wesołowska, 2018	rCPFhW/	none; oral	56.2	1	0/6	48, 56, 59, 59, 81, 101 (2.10)	***16***, ***20***, ***24***, ***36***, ***39***, ***42*** (2.63)
Morrison, 1996	nGST/	PLG	59	0.88	1/8	134, 136, 156, 165, 166, 174, 179, 238 (1.78)	***0***, ***2***, ***10***, ***10***, ***77***, ***110***, ***115***, 163^ (81.5)
Morrison, 1996	nGST/	QSM	69	1	0/8	134, 136, 156, 165, 166, 174, 179, 238 (1.78)	***17***, ***18***, ***44***, ***62***, ***63***, ***66***, ***67***, ***84*** (4.94)
Dalton, 1996	nCatL1/	FCA/IFA	69.5	1	0/3	141, 142, 145, 180 (1.28)	***17***, ***52***, ***70*** (4.12)

Notes: There are 29 cattle vaccine groups (n = 105 controls, n = 166 vaccinates). Mean fluke count in all control cattle is 125. Animals in each vaccine group with fluke counts less than the lowest count in the control group shown in italics. Fold range in fluke counts in each group shown in parentheses. * indicates the seven cattle groups (n = 46) where % efficacy is lower than might be expected from overall trend. ^, outliers; n, native protein; r, recombinant protein; s, synthetic protein. FCA/IFA, Freund's complete/incomplete adjuvant (note this adjuvant is not commercially acceptable, due to site reactions in animals); QSM, Quil A/Squalene Montanide. IR, intraruminal infection.

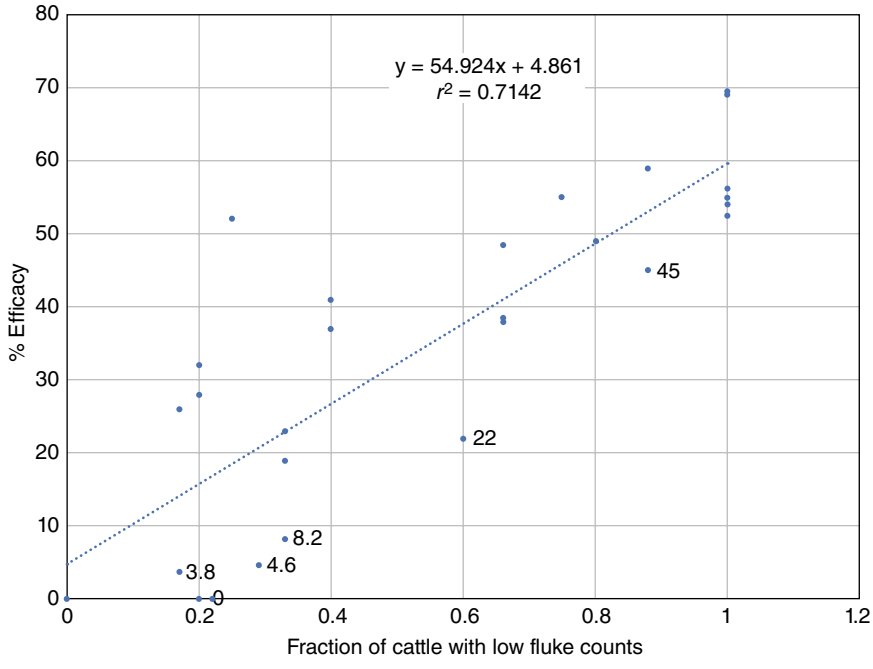

Fig. 12.3. Relationship of mean percentage vaccine efficacy and the fraction of cattle with fluke counts lower than the lowest count in control animals. The raw data are shown in Table 12.3. The % efficacy of the seven groups where the % efficacy is well below the trendline is shown (note: there are two groups at 0% efficacy). A very high association $r^2 = 0.7142$ is observed.

We used the equation shown in Fig. 12.3 to estimate the vaccine efficacy needed to reduce the fluke counts to below the lowest control count in > 80% of animals, as recommended by Turner *et al.* (2016), since low fluke counts correlate with production benefits. The analysis shows that 80% of animals will have low fluke counts using a vaccine with about 49% mean efficacy; 90% of the animals will have low counts with a vaccine of about 54% mean efficacy; whereas 100% of animals will have low counts at about 60% mean efficacy. These data suggest that several current experimental vaccines (Table 12.3) may be able to reduce the fluke counts below the lower limit of counts in controls in 80% of animals in a herd and, in some cases, in 90% of animals. This would likely be very economically beneficial to a producer, even though the mean efficacy of the vaccine may be only about 49–54%.

In summary, for cattle vaccine trials, we suggest that our use of small group sizes, unrealistically large single-point challenge doses, the inherent variation in fluke counts in controls, the presence of outliers and the use of % mean efficacy for vaccine evaluation may lead us to underestimate the potential economic benefit of a vaccine, raising the question: are we therefore eliminating antigens before a robust evaluation of efficacy? Perhaps future studies should use larger group sizes, with lower and more realistic challenge doses (200–260 metacercariae to give control fluke counts of about 30–50 flukes), or focus on using natural challenge from grazing contaminated pasture (Golden *et al.*, 2010), and then examine the proportion of animals with low fluke counts as an additional parameter to assess efficacy. A high percentage of animals with low fluke counts will result in lower herd production losses, which is the key parameter for a vaccine (Turner *et al.*, 2016). Overall, these data suggest that a commercial, repeatable vaccine of about 54% efficacy is technically feasible, which would likely be sufficient to bring production benefits to 90% of a herd if the efficacy was sustained for a grazing season (Turner *et al.*, 2016).

12.10 Commercial Considerations in Liver Fluke Vaccine Development

There are currently only a few commercial vaccines against helminth parasites, including vaccines using whole organisms (Difil; Bovilis Huskvac® originally 'Dictol'), native extracts (Barbervax®) and, more recently, recombinant proteins (Providean Hidatil EG95®; Cysvax®) (reviewed in Stutzer *et al.*, 2018). The general lack of success with helminth vaccines is testament to the complexity of eukaryotic pathogens and how parasites have evolved and adapted themselves to their host. Evasion of the host's adaptive immunity is common to many parasitic infections and this alone makes parasite vaccine development a particular challenge.

In cases where hosts acquire immunity to reinfection, then conventionally researchers have utilized that immune response to identify antigens of interest. However, with *Fasciola* there is essentially no acquired immunity: cattle can be reinfected with metacercariae at any stage of their life (Piedrafita *et al.*, 2004; Molina-Hernanadez *et al.*, 2015). Evidence exists that *Fasciola* diverts the immune system away from a protective Th1-like response towards a strong Th2-like response (Dalton *et al.*, 2013). Developing a successful vaccine will rely on both understanding and controlling this immunomodulation. The conventional approaches adopted by researchers to identify relevant antigens have been logical and laudable but have yet to yield consistent levels of protection of > 60–70% in cattle.

There are several factors that will influence the development and commercial success of subunit fluke vaccines, including: (i) market; (ii) product development; (iii) scalability and manufacturing; (iv) safety; (v) efficacy; (vi) regulatory requirements; (vii) producer expectations; and (viii) pricing.

12.10.1 Market

With the ongoing globalization and centralization of R&D efforts in the animal health industry, products with niche applications or small markets are unlikely to be pursued. However, the fluke market is global and in some regions the incidence of *F. hepatica* and *F. gigantica* overlap. It is likely that initial success will be with *F. hepatica*, due to the source of research funding and a focus on existing medicalized cattle where the payback is more lucrative and more likely. The major markets for a fluke vaccine will be those countries where farmed medicalized cattle are routinely drenched with commercial flukicides. The greatest penetration would be expected in those countries where drug resistance is significant and new alternatives for controlling flukes would be readily adopted. Drug resistance is now widespread in the UK, Europe, Ireland and Australia (Kelley *et al.*, 2016; Fairweather *et al.*, 2020). Table 12.4 shows the major *F. hepatica* markets and can be used to evaluate various scenarios, but the role of the potential Indian market is significant, at least in terms of potential doses.

12.10.2 Product development

Although most vaccine research to date has been directed at *F. hepatica,* ideally a vaccine

Table 12.4. An example of a simple market estimation for a *F. hepatica* vaccine.

Country	Estimated total no. of cattle	Estimated average incidence of fasciolosis	Estimated realistic penetration of vaccine in Year 3	Estimated potential vaccine doses sold
Australia	29,290,769	39%	50%	5,711,700
France	19,095,797	20%	40%	1,527,664
India	214,350,000	50%	20%	21,435,000
Ireland	1,140,800	100%	90%	1,026,720
South Africa	14,000,000	20%	30%	840,000
Turkey	13,916,924	10%	20%	278,338
United Kingdom	9,844,000	80%	80%	6,300,160
TOTAL				**37,119,582**

would be effective against *F. gigantica* as well. This could be achieved through the discovery and utilization of cross-protective antigens or as a combination vaccine. The mode of vaccine action should target juvenile and immature flukes, because they will be most at risk as they migrate through the peritoneal cavity and because most of the economic loss comes from damage to the liver by flukes 2–6 weeks of age (Toet *et al.*, 2014). The lack of representative small-animal models for *Fasciola* infection and immunity means that vaccine trials must generally be conducted in cattle or sheep. This makes the cost of trials expensive, which consequently limits the number of putative antigens and immunomodulators that are tested. Challenge trials are most often designed so that immunity is at its peak at time of challenge. Additionally, for practical purposes, animals are usually challenged at a single time point. Neither of these aspects of the clinical trial matches the on-farm reality. A more relevant test of efficacy would be to vaccinate animals and allow them to graze known contaminated pasture along with relevant controls over a year or two while measuring productivity parameters (e.g. Golden *et al.*, 2010). Such trials will probably yield a wider spread of infection at post-mortem, so larger group sizes will be required and they will be more expensive, take longer and be logistically more difficult.

The finished product should fit with normal farm practice. The number of vaccinations should ideally not exceed two for the first year, followed by annual boosters.

12.10.3 Scalability and manufacturing

While subunit vaccines can be straightforward and cheap to manufacture (assuming yields from fermentation are good), any complexity added through the need for multiple antigens and/or other active ingredients (such as immunosuppressants) increases both the risk of batch failure and the cost. Vaccines that are simple to manufacture will have a lower cost of goods. As the number of antigens increases, so does the cost of goods, as well as the development and manufacturing risk. For instance, a vaccine requiring five antigens cannot be sold if just one antigen is not available due to manufacturing problems. Recombinant organisms should ideally produce a high yield of recombinant protein that requires little downstream processing. Recombinant proteins should be stable, soluble and, preferably, should not require complex refolding chemistry. The cost of the efficacious dose of the protective immunogen used in the final formulation must not be prohibitive. The chosen adjuvant should be safe, registrable and commercially available at an appropriate cost. Immunomodulators should ideally meet the same criteria. Novel adjuvants or immunomodulators may face the same regulatory hurdles as a new active pharmaceutical ingredient, which pushes up cost and risk significantly.

12.10.4 Safety

Vaccines must not generate unacceptable local or systemic reactions in the host. The experimental use of small doses of Freund's complete adjuvant may be justified for proof-of-concept studies but cannot be used in a commercial product. Novel adjuvants or immunomodulators will require more extensive safety testing.

12.10.5 Efficacy

Parasite vaccine developers strive to generate products that induce high levels of protection (normally > 80%). However, with liver fluke it is known that cattle can tolerate 30–40 flukes without a concomitant reduction in productivity (Hope Cawdery *et al.*, 1984; Vercruysse and Claerebout, 2001). For this reason, vaccines consistently generating lower levels of protection (> 60–70%) may deliver similar productivity benefits to existing flukicides. The level of efficacy needed is related to the percentage of highly infected cattle in a herd. The fraction of cattle with high fluke burdens in Belgium and Australia is only about 18–29%, suggesting that a vaccine with moderate efficacy (60%) may be beneficial to the majority (70–80%) of cattle in a herd in those countries (Charlier *et al.*, 2008; Kelley *et al.*, 2020). Vaccines should have a short

onset of immunity and a long duration of immunity (Turner *et al.*, 2016). No vaccine will compete with the immediate benefit of a flukicide but longer durations of immunity should mean fewer flukicide treatments.

12.10.6 Regulatory requirements

The registration dossier containing the safety, efficacy, chemistry, manufacturing and control data for a vaccine will ideally be developed for all the countries of interest. This will probably mean conducting studies (safety and efficacy in cattle, stability, etc.) to comply with internationally recognized guidelines. However, it is likely that individual country regulators will require confirmatory efficacy studies to demonstrate cross-protection against local fluke isolates. Demonstrating safety and efficacy in young as well as pregnant *Bos taurus* and *Bos indicus* cattle will probably be required. The final formulation will need to be easily administered using existing vaccinating equipment. A freeze-dried presentation may be required in countries with unreliable refrigeration, while those with refrigeration will prefer a vaccine that is ready to use.

12.10.7 Producer expectations

There has been a long history of highly effective flukicides (Fairweather *et al.*, 2020). Farmers will expect a new vaccine to completely replace the use of flukicides; however, this is unlikely. It is doubtful that a vaccine will kill existing adult flukes and so a flukicide may be required to 'clean out' existing infections, followed by vaccination to prevent new infections. It is also likely that vaccination will be used as part of an ongoing integrated approach to parasite management. 'Selling' an integrated approach will be a challenge for the marketing company's employees and will impact on the payback of the vaccine investment. Resistance to change is not limited to purchase decisions by farmers. Even more change will be required by farmers using broad-spectrum products to control nematodes and trematodes. How a vaccine is incorporated into the parasite management plan will vary depending on the intensity of the local fluke challenge in a herd, the degree of fluke drug resistance and access to drenches, but most importantly the technology transfer and technical support available. Benefits from a reduction in flukicide treatments include fewer musters (reduced labour costs), less money spent on flukicides and less exposure to active ingredients which, in turn, should theoretically slow the rate of drench resistance. Adoption of a vaccine should be welcome on properties with existing fluke resistance to triclabendazole, such as in Ireland, the UK and Australia (Kelley *et al.*, 2016; Fairweather *et al.*, 2020).

12.10.8 Pricing

A fluke vaccine would be priced appropriately for different markets. Pricing would depend on the cost of goods and the vaccine's claims, including (and most importantly) the percentage efficacy and duration of immunity. However, pricing would also be influenced by the current cost of alternative treatments, the market's ability to pay (especially in developing countries) and the sales and marketing strategy. Animal health companies will also seek a return on their R&D investment, which will be built into an appropriate margin above the cost of goods.

In summary, a successful *Fasciola* vaccine should ideally have the following characteristics:

- 80% protection against infection from juvenile flukes (> 60–70% is probably acceptable);
- efficacious against both *F. hepatica* and *F. gigantica*;
- demonstrable animal production benefits equivalent to triclabendazole for non-resistant strains;
- effective against drug-resistant flukes;
- safety equivalent to existing clostridial vaccines;
- > 18 months shelf life; and
- cost of vaccine and application per year lower than existing treatments (this requirement incorporates the vaccine's duration of immunity).

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13 *Fasciola gigantica* and *Fasciola* Hybrids in Southeast Asia

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13.1 Introduction

Fasciola gigantica, the cause of tropical fasciolosis, is primarily found in less developed countries where livestock ownership is a significant contributor to household income. Research interest in *F. gigantica* lags considerably behind that in *F. hepatica*, despite posing both human and animal health risks across Asia, Africa and the Middle East - an area comprising nearly 80% of the global human population. This research gap has continued to widen over the past decade and is representative of the continued neglect of zoonotic parasites in the less developed world (Fig. 13.1). A considerable proportion of information about the prevalence and impacts of this parasite is conducted by government departments or published in local journals, leaving it inaccessible to the greater scientific community. Moreover, rarely is this information produced in a form available to local doctors, veterinarians, extension workers or farmers. The few publications that do make it into peer-reviewed national or international journals each year are commonly hidden behind paywalls, resulting in restricted access by scientists in the areas most often affected.

The impacts of fasciolosis on livestock production are considered to be one of the primary limitations to ruminant productivity in a region where many farmers live below the poverty line. The production-limiting nature of fasciolosis is particularly concerning given the threats posed by global food insecurity and a rising demand for animal-derived protein. A significant difference between fasciolosis caused by infection with *F. hepatica* and *F. gigantica* is the association of the latter with farming in less developed tropical countries reliant on subsistence agriculture, which is often closely tied to aquatic rice production. This combination of livestock husbandry and aquatic rice production provides the ideal habitat for maintenance of the *Fasciola* spp. life cycle and has profound effects on the seasonality of infection, routes of transmission, and control options likely to be employed by smallholder farmers.

Human cases of *F. gigantica* infection are under-reported, largely due to the limited medical and diagnostic facilities available in areas where the disease is likely to be most prevalent (WHO, 2006; Mas-Coma *et al.*, 2018). While the human and animal health impacts due to infection with *F. gigantica* are cause for concern in their own right, an increase in reports of possible hybridization events between *F. hepatica* and *F. gigantica* suggest that a somewhat alarming scenario may be occurring in regions

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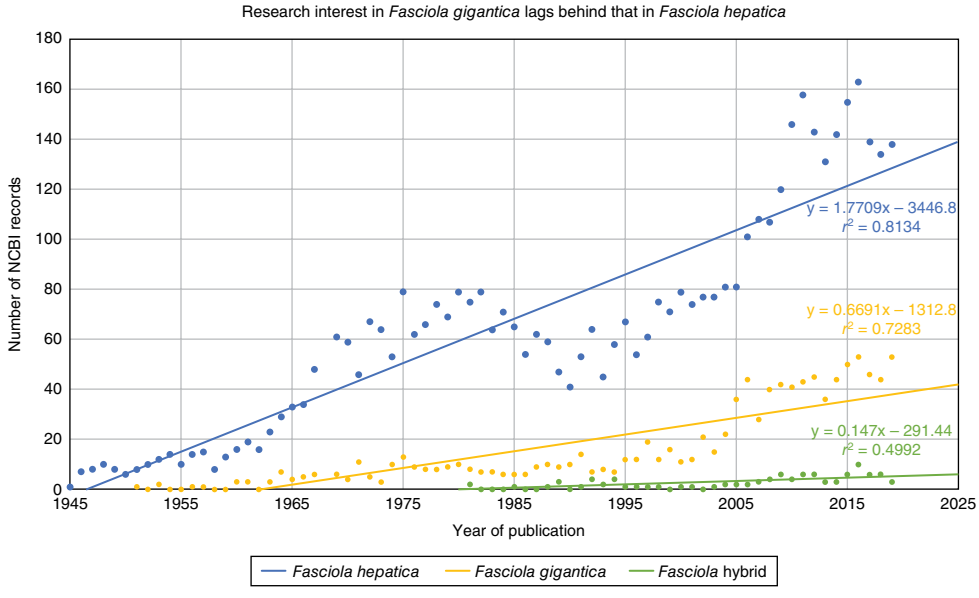

Fig. 13.1. Research interest in *Fasciola gigantica, Fasciola hepatica* and *Fasciola* hybrids since 1945. Data show the total number of PubMed records per year until 2019 projected through to 2025, from the National Centre for Biotechnology Information (NCBI).

where both species coexist. Under these conditions, genetic crossover events in animals co-infected with both species could result in the generation of parasite strains where the epidemiological and control outcomes remain unknown, and that poses a greater health risk than that of either species alone (Calvani and Šlapeta, 2021).

This chapter outlines the life cycle of *F. gigantica* and the differences compared with *F. hepatica*, including the economic significance and control options available, with particular emphasis on the importance of the smallholder farmer and the role of rice paddies on the maintenance of the life cycle in Southeast Asia. Diagnostic options available and their suitability for use in the context of less developed countries are summarized in order to provide updated estimates of parasite prevalence and species distribution. Recent reports of *Fasciola* hybrids and the possible functional implications of these forms are discussed, and appropriate methods for the molecular characterization of these events are described. Recommendations for the future of *F. gigantica* and *Fasciola* hybrid research are proposed, with an emphasis on inclusivity and application in the field.

13.2 *Fasciola gigantica* Life Cycle

13.2.1 Introduction

The life cycle of *F. gigantica* consists of the same developmental stages as *F. hepatica*, yet the length of each stage and factors affecting the exposure and infection of intermediate and definitive hosts vary greatly (Table 13.1). While some of the differences between the life cycles of these two parasites are the result of variations in geographical and climatic factors, the contrasting socio-economic and agricultural production systems in the more-versus less-developed world explain many of the epidemiological disparities between them. The distribution of *F. gigantica* is primarily limited to less developed tropical countries where smallholder and subsistence farmers account for the overwhelming majority of livestock owners. Of an estimated 525 million smallholder farms globally (farms with a production area < 2 ha), 87% are found in Asia and the Pacific – a large proportion of which rely on a combination of large-ruminant husbandry and aquatic rice production (Nagayets, 2005; Rapsomanikis, 2015; Lowder *et al.*, 2016). The interface

Table 13.1. Fundamental differences between *Fasciola hepatica* and *F. gigantica*.

	Fasciola hepatica	*Fasciola gigantica*
Egg size (EW × EL)	61.7–102.1 µm × 105.3–159.5 µm	61.6–123.6 µm × 129.6–204.5 µm
Adult size (BW × BL)	4.88–14.07 mm × 11.64–29.00 mm	6.03–11.84 mm × 28.82–52.30 mm
Length of prepatent period	8–12 wpi	12–16 wpi
Temperature required for egg development	10–37°C	15–43°C
Commencement of cercarial shedding	4–7 wpi	5–7 wpi
Temperature for metacercarial survival	Up to 25°C	Up to 35°C
Primary parasite distribution	Temperate regions	Tropical regions
Main definitive hosts	Sheep, goats, and cattle	Cattle and water buffalo
Main intermediate hosts	*Galba truncatula*	*Radix natalensis*, *R. auricularia*, *R. rubiginosa*

BL, body length; BW, body width; EL, egg length; EW, egg width; wpi, weeks post-infection. See text for references.

between the management of large ruminants and rice-growing activities, in conjunction with seasonal flooding or irrigation of rice paddies, contributes to the maintenance of the *F. gigantica* life cycle and is the key to understanding risk factors for both human and animal fasciolosis. Thus, despite the similarities in their life cycles, the management of *F. gigantica* is distinct from that of *F. hepatica* and therefore, their epidemiologies should be considered separately. Although production systems where *F. gigantica* is prevalent vary between cultures, countries and continents, here we have chosen to focus on the importance of smallholder farmers and the role of the rice paddy in the management of *F. gigantica* in Southeast Asia.

13.2.2 Definitive hosts

Large ruminants, particularly cattle and water buffalo, are the species most at risk of infection with *F. gigantica* due to their predominance across farming systems in Asia, Africa, and the Middle East (FAO, 2019). Infections in sheep, goats, donkeys, yaks, and camels are also known to occur, especially in countries or cultures where these animals are commonplace (Haroun *et al.*, 1989; Nooruddin and Islam, 1996; Haridy and Morsy, 2000; Getachew *et al.*, 2010; Qin *et al.*, 2019). Several studies have suggested that *F. gigantica* is better adapted to cattle than to sheep, while the reverse appears to be true for *F. hepatica* (Hammond and Sewell, 1975; Zhang *et al.*, 2005b,c). This difference in host susceptibility is expected to be related to the origins of each species, with *F. hepatica* having evolved in Eurasian ovicaprines and *F. gigantica* in east African bovids (Mas-Coma *et al.*, 2009a).

Indonesian Thin Tail (ITT) sheep demonstrate both an innate and acquired resistance to infection with *F. gigantica* and have been extensively studied as a result, due to their potential role in the development of new methods of *Fasciola* spp. control (Wiedosari and Copeman, 1990; Roberts *et al.*, 1997a,b,c; Hansen *et al.*, 1999; Pleasance *et al.*, 2011). Water buffalo are less susceptible to infection with *F. gigantica* than cattle, often housing fewer mature flukes, significantly lower faecal egg counts, and delayed or less severe clinical signs (Prasitirat *et al.*, 1996; Molina and Skerratt, 2005; Molina *et al.*, 2005a,b,c; Wiedosari *et al.*, 2006). An array of wild herbivores (for example, antelope and giraffes) may also act as reservoir and incidental hosts, particularly in Africa where they are more likely to share grazing areas with domesticated ruminants (Hammond, 1972; Mucheka *et al.*, 2015; Malatji *et al.*, 2020). The successful infection of laboratory animals is more difficult for *F. gigantica* than for *F. hepatica*, and while hamsters and guinea pigs appear to be more susceptible to *F. gigantica* infection, Wistar rats are the most commonly employed species (Mango *et al.*, 1972; Itagaki *et al.*, 1994; Prasad *et al.*, 1999; Itagaki *et al.*, 2011). The number of studies comparing their susceptibility is limited, however,

and it is likely that the strain of *F. gigantica* used plays an important role in the susceptibility and mortality of laboratory animals, as is seen in lab-maintained *F. hepatica* (Phalee *et al.*, 2015). Outside known hyper-endemic regions in Vietnam, documentation of human cases of infection with *F. gigantica* is sporadic and expected to be under-reported (WHO, 2006; Nguyen *et al.*, 2011; Bui *et al.*, 2016). Human fasciolosis has been extensively reviewed and therefore a summary of zoonotic *F. gigantica* infections is covered later in this chapter, as well as elsewhere in this volume (Mas-Coma, 2005; Mas-Coma *et al.*, 2014; Bui *et al.*, 2016).

There are conflicting reports on the difference in pathogenicity between *F. hepatica* and *F. gigantica* (Raadsma *et al.*, 2007; Valero *et al.*, 2016). These differences appear to be related to the variation in susceptibility of the definitive host species and the establishment of higher worm burdens by *F. hepatica* compared with *F. gigantica* during experimental infections, along with the capacity of the different species to evade the immune system of infected hosts (Roberts *et al.*, 1997a; Piedrafita *et al.*, 2004; Zhang *et al.*, 2005a; Raadsma *et al.*, 2007; Pleasance *et al.*, 2011). Due to its larger size and the greater amount of time spent migrating through the liver, *F. gigantica* is considered more pathogenic in hosts that are equally susceptible to infection with both species (Valero *et al.*, 2016).

As with *F. hepatica*, infection of definitive hosts occurs via ingestion of infective metacercariae, after which excystment begins in the small intestine in the absence of oxygen and in the presence of carbon dioxide (Hanna and Jura, 1976). Newly excysted juvenile flukes burrow through the intestinal wall into the abdominal cavity before eventually reaching the liver. Here they penetrate the liver capsule and migrate through the hepatic parenchyma before entering the bile ducts where, as adults, they reach sexual maturity and commence egg-laying (Fig. 13.2). Eggs are shed via the bile ducts into the gastrointestinal tract, where they exit the definitive hosts into the environment. The time from ingestion of metacercariae to the excretion of eggs in faeces (the pre-patent period) of *F. gigantica* is longer than that of *F. hepatica* and differs depending on the species and breed of definitive host. Various studies in cattle and sheep have described the occurrence of eggs in faeces from 12–13 weeks post-infection (pi), although eggs were detected as early as 8 weeks pi in two zebu calves (Bitakaramire and Bwangamoi, 1969; Hammond and Sewell, 1974; Haroun and Hussein, 1976; Valero *et al.*, 2016). Eggs appear in faeces from 13–14 weeks pi in experimentally

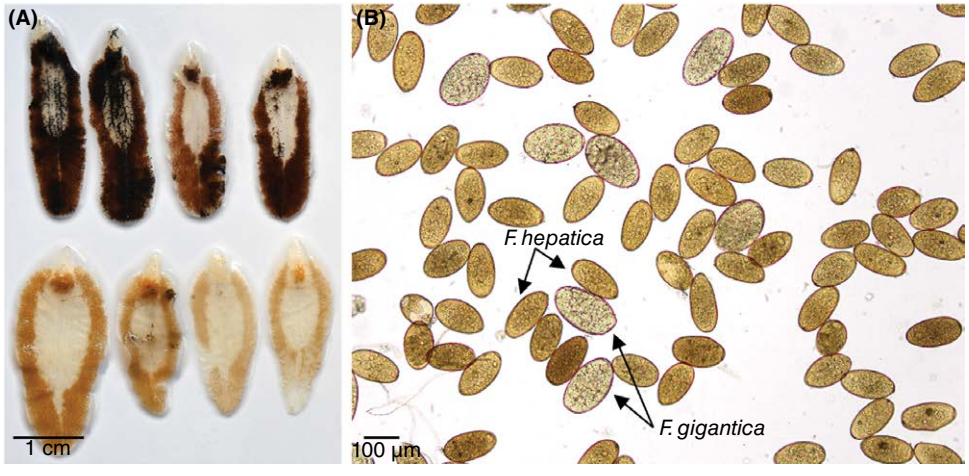

Fig. 13.2. (A) Adult *Fasciola gigantica* (top) and *Fasciola hepatica* (bottom). **(B)** A mix of *Fasciola gigantica* and *Fasciola hepatica* eggs. Adult *Fasciola* specimens and their eggs are from the collection at the Laboratory of Veterinary Parasitology at the University of Sydney, Australia. Adult *F. hepatica* were collected during post-mortem examination from sheep in Australia, while adult *F. gigantica* were collected from cattle in Indonesia. Eggs were artificially mixed after removal from the uteri of adults.

infected buffalo, indicating that the maturation of flukes is delayed in this species (Yadav *et al.*, 1999; Zhang *et al.*, 2006; Edith *et al.*, 2010a,b). In almost all cases, egg output increases for the first few months before gradually falling (Bitakaramire and Bwangamoi, 1969; Prasitirat *et al.*, 1996; Yadav *et al.*, 1999). This presents an issue for the diagnosis of chronic or low-level *F. gigantica* infections in large ruminants in particular, where the increased faecal volume in comparison with small ruminants further dilutes out the remaining eggs (Sewell, 1966; Calvani *et al.*, 2017).

Embryonation and the subsequent hatching of eggs occur when they are free from faeces and exposed to light and must be in the presence of water in order to facilitate infection of the intermediate aquatic snail hosts (Grigoryan, 1958; Guralp *et al.*, 1964). Prior to hatching, the development of *F. gigantica* miracidium in eggs is temperature-dependent, occurring between 10 to 11 days at 30°C, and can take up to 59 days in temperatures as low as 15°C, although this is not common (Guralp *et al.*, 1964). Under laboratory conditions development occurs between 13 to 15 days at room temperature (Hussein *et al.*, 2010). Egg mortality occurs above 43°C, with the eggs highly sensitive to desiccation (Grigoryan, 1958; Suhardono *et al.*, 2006c). Up to 9% of eggs collected from cattle faeces and stored in the sun for up to 14 weeks were shown to remain viable (Suhardono *et al.*, 2006c). Hatching occurs over a series of days, a process that is also temperature dependent, and can occur for as long as 116 days at room temperature (Guralp *et al.*, 1964). Miracidia are almost immediately infective to suitable intermediate snail hosts and have a life span of 9–12 h, but may survive in water under optimal conditions for up to 26 h (Asanji, 1988; Hussein *et al.*, 2010).

13.2.3 Intermediate hosts

The various species of intermediate snail hosts susceptible to infection with *F. gigantica* miracidium have been extensively reviewed and thus will only be covered here in brief, with their distribution summarized in Table 13.2 (Dawes, 1960b; Guralp *et al.*, 1964; Kendall, 1965; Massoud and Sadjadi, 1980; Bargues and Mas-Coma, 2005; Walker *et al.*, 2008; Correa *et al.*, 2010; Kaset *et al.*, 2010; Dung *et al.*, 2013). A variety of lymnaeid snails act as intermediate hosts for *F. gigantica* globally, all of which require an aquatic or semi-aquatic environment (Kendall, 1965). The morphological identification and phylogenetic relationships between these species are complicated by the extensive variation in shell appearance and homogeneity of other anatomical traits commonly used for species differentiation (Kendall, 1965; Bargues and Mas-Coma, 2005). This has led to controversy regarding the identity of several species capable of infection with either *Fasciola* spp. and has caused their subsequent renaming throughout the literature as their phylogenetic relationships and morphologies are resolved (Bargues *et al.*, 2001; Bargues and Mas-Coma, 2005; Correa *et al.*, 2010).

Miracidia do not feed, but seek out suitable snail hosts via a rapid swimming motion in response to chemical, light and gravitational signals within 12 h of hatching (Hussein *et al.*, 2010). Host attachment and penetration occur via the foot, body, mantle or tentacles, during which they shed their epithelial plates before transforming into young mother sporocysts inside the snail host (Dinnik and Dinnik, 1956; Dawes, 1960a,b; Kendall, 1965). The time taken to complete penetration varies according to the site of attachment and is aided by the secretion of proteolytic enzymes (Dawes, 1960a; Kendall, 1965). As with other trematodes, the mother sporocyst gives rise to several generations of rediae, the first of which are fully developed 6–8 days after infection when maintained at 26°C (Dinnik and Dinnik, 1956, 1964; Kendall, 1965; Rakotondravao *et al.*, 1992). The production of the first wave of cercariae commences as early as 26 days after infection of the snail host, after which several cycles of redial and cercarial development occur. The development of sporocysts and successive generations of rediae and the switch to cercarial production is temperature-dependent (Dinnik and Dinnik, 1964; Rakotondravao *et al.*, 1992). Cercarial production appears to cease in *Radix natalensis* (*Lymnaea natalensis*) below 20°C (Dinnik and Dinnik, 1964).

Several studies have demonstrated the impacts of temperature and of definitive and intermediate host species on the production and

Table 13.2. *Fasciola gigantica* infections in intermediate snail hoses.

Region	Country	Snail species infected with *F. gigantica*	References
Africa	Egypt	*Radix natalensis* (*Lymnaea natalensis*)	Kuntz (1957)
		Pseudosuccinea columella (Lymnaea columella)	Ahmed and Ramzy (1999)
		Galba truncatula (Lymnaea truncatula) *Pseudosuccinea columella**	El-Shazley *et al*. (2002)
		R. natalensis (L. natalensis)	Grabner *et al*. (2014)
	Kenya	*R. natalensis (L. natalensis)*	Bitakaramire (1968a)
	Uganda	*R. natalensis (L. natalensis)*	Ogambo-Ongoma and Goodman (1976)
	Malawi	*R. natalensis (L. natalensis)*	Mzembe and Chaudhry (1979)
	Madagascar	*G. truncatula** (*L. truncatula*)	Da Costa *et al*. (1994)
	Tanzania	*R. natalensis (L. natalensis)*	Walker *et al*. (2008)
Asia	Malaysia	*Radix rubiginosa* (*Lymnaea rubiginosa*)	Palmieri *et al*. (1977)
	Nepal	*Radix rufescens* (*Lymnaea auricularia rufescens*)	Morel and Mahato (1987)
	Lao PDR	*R. rubiginosa**	Ditrich *et al*. (1992)
	India	*R. Auricularia** (*L. auricularia*)	Velusamy *et al*. (2004)
	Thailand	*R. rubiginosa**, *Austropeplea viridis**, *Radix swinhoei**	Kaset *et al*. (2010)
	Vietnam	*A. viridis** (*Lymnaea viridis*), *R. swinhoei* (*Lymnaea swinhoei*), *R. auricularia**, *R. rubiginosa**	Nguney *et al*. (2012), Dung *et al*. (2013)
Middle East	Iran	*Radix peregra* (*Lymnaea viridis*), *R. auricularia* (*L. auricularia*)	Massoud and Sadjadi (1980)
	Iraq	*R. auricularia* (*L. auricularia*)	Al-Kubaisee and Altaif (1989)
	Turkey	*R. auricularia* (*L. auricularia*)	Guralp *et al*. (1964)
Laboratory infections	N/A	*R. rufescens* (*L. a. rufescens*) *R. natalensis* (*L. natalensis*)	Dawes (1960b), Dinnik and Dinnik (1964)
		Lymnaea stagnalis, *R. peregra*, *R. auricularia* (*L. auricularia*), *Lymnaea tomentosa*, *G. truncatula L. truncatula*), *Stagnicola palustris* (*Lymnaea palustris*)	Boray (1965)
		R. auricularia (*L. auricularia*)	Yadav and Gupta (1988)
		G. truncatula (*L. truncatula*)	Rakotondravao *et al*. (1992)
		Lymnaea tomentosa	Dreyfuss and Rondelaud (1995)
		G. truncatula (*L. truncatula*), *R. natalensis* (*L. natalensis*)	Dar *et al*. (2002, 2003a,c, 2004)

Current names provided, followed (in parentheses) by names used in original references.
*Snail species molecularly identified

shedding of *F. gigantica* cercaria (Bitakaramire, 1968a; Massoud and Sadjadi, 1980; Yadav and Gupta, 1988; Al-Kubaisee and Altaif, 1989; Da Costa *et al*., 1994; Dreyfuss and Rondelaud, 1995; Dar *et al*., 2004). In brief, cercariae are shed from infected snails in up to 15 waves from 36 to 45 days pi, primarily at night (Dinnik and Dinnik, 1964; Guralp *et al*., 1964; Bitakaramire, 1968a; Da Costa *et al*., 1994). Waves of cercariae are shed 1–8 days apart, with 50–70 cercariae released in each wave (Da Costa *et al*., 1994). Several larval stages may occur within a single snail at any given time and thus shedding occurs continually over an extended period and has been recorded under laboratory conditions for up to 175 days pi (Kendall, 1965; Da Costa *et al*., 1994). More recent studies suggest that parasite origin also plays a significant role in cercarial production, with Egyptian *F. gigantica* isolates demonstrating reduced survival rates in *Galba truncatula* when compared

with Chinese isolates (60.2% at 30 days post-exposure versus 90.0%), but with significantly increased cercarial output (275.5 versus 29.0 mean cercariae per snail), and a reduced prepatent period (48.0 versus 55.6 days) (Al-Kubaisee and Altaif, 1989; Dar *et al.*, 2003a,b).

13.2.4 Production and survival of metacercariae

The encystment of cercariae into metacercariae occurs within a few hours of emergence from infected snails. Up to 35% of metacercariae float, due to the presence of an air-filled outer ring surrounding the external wall of the cyst (Da Costa *et al.*, 1994). Of the non-floating cysts, 98% attach to vegetation within 10 cm of the base of the plant (Ueno and Yoshihara, 1974; Mahato and Harrison, 2005; Suhardono *et al.*, 2006e). This is likely due to the material being submerged at some point, as several studies have shown that the cercariae of *F. gigantica* tend to encyst on objects at or below water level (Ueno and Yoshihara, 1974; Dumag *et al.*, 1976; Suhardono *et al.*, 2006e). The proportion of floating cysts decreases with each wave of cercarial output and is positively correlated with snail size, with 38% of cysts from large snails floating compared with only 18.2% from smaller snails (Da Costa *et al.*, 1994; Vareille *et al.*, 1994). In one instance it was shown that *G. truncatula* (*L. truncatula*) produce more floating metacercariae when infected with *F. gigantica* than with *F. hepatica* and thus contaminated water bodies may play a more important role in the transmission of *F. gigantica* than occurs with *F. hepatica* (Dreyfuss and Rondelaud, 1997). Floating cysts may also act as a source of infection in water bodies considered unsuitable for habitation by intermediate hosts and hence the role of irrigation in the distribution of this parasite should not be discounted (ACIAR, 2008).

Metacercariae are the most environmentally robust stage of the *Fasciola* spp. life cycle and, as the infective stage to mammalian hosts, play an important role in the epidemiology of infection. As with *F. hepatica,* both direct sunlight and low humidity have been shown to rapidly accelerate metacercarial death; however, the metacercariae of *F. gigantica* appear to be more tolerant of higher temperatures. A combination of direct sunlight and low humidity killed 100% of *F. gigantica* metacercariae within 6 h (Suhardono *et al.*, 2006a). Temperatures at or below 20°C, combined with a relative humidity of 95%, appear to provide the most favourable conditions for the survival of *F. gigantica* metacercariae, with 11% shown to survive up to 23 weeks when maintained in water at 13°C (Suhardono *et al.*, 2006a). As with *F. hepatica,* the viability decreases with increasing temperature, with temperatures above 35°C considered almost completely lethal to *F. gigantica* metacercariae even when maintained fully submerged in water (Suhardono *et al.*, 2006a). At 30°C only 5% and 7% maintain normal movement and appearance at 95% and 75% humidity, respectively (Suhardono *et al.*, 2006a).

13.2.5 The role of the rice paddy in *F. gigantica* life cycle

Livestock production systems in less developed tropical countries differ significantly from those where *F. hepatica* is prevalent. In Southeast Asia, rice paddies and rice straw are the main sources of *F. gigantica* infection in livestock, due to the role of rice as the primary cereal crop throughout the region (Roberts and Suhardono, 1996; Tum *et al.*, 2004; Mahato and Harrison, 2005; Molina *et al.*, 2005a; Suon *et al.*, 2006b; Bhutto *et al.*, 2012; Nguyen *et al.*, 2012). Irrigation channels, roadside canals, and the edges of ponds, rivers and lakes also play a role in the maintenance of the *F. gigantica* life cycle in Southeast Asia, albeit to a lesser extent (Tum *et al.*, 2004; Nguyen *et al.*, 2012, 2014; Portugaliza *et al.*, 2019). Aquatic rice production provides the ideal habitat for lymnaeid snail proliferation, which thrive in well-oxygenated stagnant or slow-moving water. The *F. gigantica* life cycle is maintained in these systems via the use of animal faeces containing *F. gigantica* eggs as fertilizer and by allowing animals to graze rice stubble and rice straw contaminated with infective metacercariae after harvest (Tum *et al.*, 2004; Mahato and Harrison, 2005; Suhardono *et al.*, 2006c, e; Nurhidayah *et al.*, 2020). Thus, exposure to and seasonality of *F. gigantica* infection in cattle and buffalo is intimately linked to the management and feeding of animals and the

production of aquatic rice in systems that are ruled by the fluctuation between wet and dry seasons (Fig. 13.3).

Wet and dry seasons vary across Asia according to latitude and coastal proximity and hence the seasonality of fasciolosis differs by region as a result. The mountain ranges of mainland Southeast Asia, such as those found in northern Vietnam, Lao PDR, Thailand, and in the eastern half of Myanmar, further contribute to the seasonality of *F. gigantica* infection due to the impact of the cooler climates on the *F. gigantica* life cycle (Xiao *et al.*, 2018). These regions also provide an environment conducive to the survival of *F. hepatica*, potentially enabling parasite translocations via livestock traded from areas where *F. hepatica* is endemic (Alba *et al.*, 2020; Calvani and Šlapeta, 2021).

Rice production tends to occur under one of two main systems, each of which affect the seasonality of fasciolosis due to the differing exposure of animals to metacercariae:

1. Low-input seasonal production reliant on rainfall to fill rice paddies with one to two crops planted per year.
2. Intensive irrigated rice production with several crops planted per year.

Fig. 13.3. The seasonal landscape of Southeast Asia and its role in the maintenance of the *Fasciola* spp. life cycle. Shifts between the wet (top) and dry (bottom) seasons in Southeast Asia and the ubiquity of aquatic rice production heavily influence the seasonality of fasciolosis in livestock.

Under the low-input system, animals remain tethered at home or are allowed to graze forested areas during the rice-growing season (Devendra and Sevilla, 2002; Nguyen *et al.*, 2011). When animals are brought home, livestock are fed a combination of opportunistically collected roadside grasses, rice straw harvested from the previous season, and other crop remnants (Devendra and Sevilla, 2002; Maxwell *et al.*, 2012). Rarely are forage crops planted for feed supplementation, with target-feeding for the fattening of animals prior to sale a relatively uncommon practice (Devendra and Sevilla, 2002; Bush *et al.*, 2014a,b; Baltenweck *et al.*, 2020). During the dry season, once rice has been harvested, animals are either allowed unlimited access to free-graze the remaining rice stubble or are tethered to allow access to specific plots (Devendra and Sevilla, 2002; Nguyen *et al.*, 2012). Animals maintained in conjunction with irrigated rice production systems on the other hand, remain penned, tethered, or allowed to free-graze in nearby forests year-round. These animals may be allowed to briefly graze rice stubble after harvest before fields are ploughed for the following rice crop (Devendra and Sevilla, 2002; Suon *et al.*, 2006b).

Both rice production systems result in exposure to infective metacercariae via the feeding of rice straw while penned or tethered and while being allowed to graze rice stubble after harvest. The number of exposures throughout the year differs, however, depending on the rice production system employed. Animals managed alongside irrigated rice are exposed to *F. gigantica* metacercariae repeatedly throughout the year, while infections in animals allowed to free-graze rice stubble in paddies throughout the dry season follow an annual seasonal pattern (Morel and Mahato, 1987; Tum *et al.*, 2004; Suon *et al.*, 2006a; ACIAR, 2008; Nguyen *et al.*, 2014). The epidemiology of fasciolosis and seasonality of infection associated with intensive and low-input rice production systems has been investigated in detail in Nepal, Indonesia and Vietnam, and to a lesser extent in Thailand, Cambodia, Lao PDR and the Philippines (Morel and Mahato, 1987; Widjajanti, 1989; Mahato, 1993; Holland *et al.*, 2000; Tum *et al.*, 2004, 2007; Molina *et al.*, 2005a; Suon *et al.*, 2006a,b). More recent (conducted in the past decade) evaluations of parasite prevalence and epidemiology in Asia and their association with animal husbandry practices are more limited in their depth and few have been conducted at the national level, reflecting a movement away from epidemiological investigations at the national scale (Dorny *et al.*, 2011; Nguyen *et al.*, 2011, 2012, 2014, 2017; Rast *et al.*, 2013; Phalee and Wongsawad, 2014; Gordon *et al.*, 2015; Rast *et al.*, 2015; Bui *et al.*, 2016; Quy, 2016; Sah *et al.*, 2018; Portugaliza *et al.*, 2019; Nurhidayah *et al.*, 2020; Sawitri *et al.*, 2020).

Both irrigated and seasonally flooded rice paddies are conducive to the maintenance of lymnaeid snail populations, with their abundance throughout the year related to the stability of available water levels (Morel and Mahato, 1987; Widjajanti, 1989). The conditions most favourable to the maintenance of snail populations differ by region but tend to occur towards the end of the wet season through to the start of the dry season, with snail populations declining prior to harvest (Morel and Mahato, 1987; Widjajanti, 1989; Nguyen *et al.*, 2012). Lymnaeid snail populations tend to be more stable in irrigated systems where conditions conducive to their survival are more constant (Kendall, 1954, 1965; Widjajanti, 1989). When paddies dry out, or water oxygenation drops below viable levels, snails enter a period of aestivation. In Nepal *L. auricularia rufenscens* and *L. viridis* were recorded surviving in dry mud for at least a month, while in Kenya *L. natalensis* was seen to survive in dry, hard mud for up to 24 weeks (Bitakaramire, 1968b; Mahato *et al.*, 1995). Snail eggs have been shown to maintain viability after rehydration when dried for up to 1 month (Widjajanti, 1989). Upon the return of favourable conditions, snails are known to recolonize rapidly and may be dispersed to new areas through irrigation channels or via flooding at the onset of the wet season (Morel and Mahato, 1987; Widjajanti, 1989). Environmental instability as a result of climate change, such as extended droughts and the increased occurrence of periods of high rainfall, may be quietly disrupting these seasonal patterns by altering the availability of environments suitable for the maintenance of snail populations (Mas-Coma *et al.*, 2009b; Ashfan *et al.*, 2014; Sri-aroon *et al.*, 2015; Hoang *et al.*, 2019). More recently, the altitudinal regions of mainland

Southeast Asia have been shown to provide the range of temperatures necessary for the maintenance of snail populations conducive to infection with both *F. hepatica* and *F. gigantica*, as demonstrated in Lao PDR, Pakistan, Nepal and southern China (Kendall, 1954; Morel and Mahato, 1987; Liu *et al.*, 2009; Sah *et al.*, 2018; Zhang *et al.*, 2019; Calvani *et al.*, 2020).

13.2.6 Impacts of the built environment on fasciolosis

One aspect of fasciolosis in Southeast Asia that has yet to be considered in great detail by the wider scientific community is the impact that large infrastructure projects, such as the construction of hydroelectric dams, have on the seasonality of infection and exposure of mammalian hosts – including people. There has been a rapid increase in the planning and construction of hydroelectric dams along several rivers in Southeast Asia, including the Mekong river and its tributaries, over the past decade (CGIAR, 2013; Hecht and Lacombe, 2014). The impacts of such projects on resource availability, biodiversity, the local economy, and some of the more overt and devastating parasitic diseases are relatively well known or are currently under investigation, yet the effects on chronic and neglected parasitic zoonoses, such as fasciolosis, remain to be determined (Ziv *et al.*, 2012; CGIAR, 2013; Hiscox *et al.*, 2013; Hecht and Lacombe, 2014; Sri-aroon *et al.*, 2015; Hoang *et al.*, 2019; Alamgir *et al.*, 2020). It is not unreasonable to assume that the redistribution of such large volumes of water at multiple locations would have a profound impact on the distribution of lymnaeid snail populations, given the impacts associated with far smaller infrastructure projects (Martínez-Valladares *et al.*, 2013; Sabourin *et al.*, 2018). As seen in Zimbabwe with the construction of Lake Kariba, the redistribution of water at such large scales may result in the increased risk of fasciolosis in areas previously considered safe (i.e. upstream of dams and around the edges of newly formed lakes) and a reduced risk in areas that no longer receive as much water (Carolus *et al.*, 2019). These dams are also known to influence farmers' ability to irrigate rice paddies, which would have clear follow-on effects on the seasonality of infection in affected areas. To properly assess the impacts of hydroelectric dams on the risk of fasciolosis in a given area, the prevalence of lymnaeid snails and *F. gigantica*-infected livestock in a region should be determined before and after dam construction, ideally at multiple sites both up- and downstream and with follow-up over consecutive years (Erlanger *et al.*, 2008; Martínez-Valladares *et al.*, 2013).

13.2.7 Prevalence and distribution of *F. gigantica* in Southeast Asia

It is worth noting that the vast majority of prevalence estimates available for *Fasciola* spp. infection do not provide an accurate reflection of the risk of infection at the national scale. Few take into account the variation of exposure across different climatic regions or production systems, which is likely to explain the difference in prevalence observed within individual countries (Table 13.3). Infection with either *Fasciola* spp. is highly seasonal, and thus epidemiological investigations should be conducted throughout the year in order to determine the peak of infections in a given area. Older animals are often over-represented in prevalence estimates as a result of the long life of the parasite and because treatment is rarely administered by farmers in less developed regions. It is also worth noting that each method of detection has its own inherent limitations, as is explained in the diagnosis section (section 13.5) and elsewhere in this volume.

Both *F. hepatica* and *F. gigantica*, along with 'hybrid' forms of the parasite, are distributed throughout Southeast Asia (Fig. 13.4) (Calvani and Šlapeta, 2021). *F. gigantica* is found at varying prevalences in all countries of mainland Southeast Asia, while *F. hepatica* is currently considered to be restricted to China and Lao PDR (Table 13.3) (Calvani *et al.*, 2020; Calvani and Šlapeta, 2021). Based on reports where infection has been detected in local animals at slaughter using a minimum of two genetic markers, 'hybrid' *Fasciola* spp. (discussed in section 13.6) appear to be more widespread in Southeast Asia than *F. hepatica* alone (Fig. 13.4) (Calvani and Šlapeta, 2021). These findings suggest that

Table 13.3. Prevalence of *Fasciola* spp. in cattle and buffalo in Southeast Asia.

Country	Prevalence	Month	Species	Animal age	Method of detection	References
Vietnam	5%	Year-round	Cattle	3–6 months	FEC	Holland *et al*. (2000)
	25%	Year-round	Cattle	> 2 years	FEC	Holland *et al*. (2000)
	28%	June–September	Cattle	3–24 months	Faecal exam	Geurden *et al*. (2008)
	39%	June–September	Cattle	Adults	Faecal exam	Geurden *et al*. (2008)
	72.2%	June–September	Cattle	Various	Serum ELISA	Nguyen *et al*. (2011)
	37.6%		Cattle	≤ 2 years	Serum ELISA	Nguyen *et al*. (2012)
	53.7%		Cattle	> 2 years	Serum ELISA	Nguyen *et al*. (2012)
	56.7–60.7%		Cattle	2–4 years	FEC	Nguyen *et al*. (2014)
	0.0–68.0%		Cattle	Various	FEC	Ngyen *et al*. (2017)
Cambodia	0.0–56.8%	December–January	Cattle & buffalo	N/A	FEC	Tum *et al*. (2004)
	24.7%		Cattle	N/A	FEC	Suon *et al*. (2006a)
	50.5–87.5%	April	Cattle	N/A	FEC in tracer animals	Suon *et al*. (2006b)
	5.0–29.4%	December–January	Cattle & buffalo	N/A	FEC	Tum *et al*. (2007)
	5–20%		Cattle	Adult	Faecal exam	Dorny *et al*. (2011)
Lao PDR	0–25%		Cattle	N/A	Abattoir surveillance	Guong Quang *et al*. (2008)
	75–100%		Buffalo	N/A	Abattoir surveillance	Guong Quang *et al*. (2008)
	14.3–50.0%	March–June	Cattle	Various	Abattoir surveillance and faecal exam	Rast (2014)
	27.3–53.8%	March–June	Buffalo	Various	Abattoir surveillance and faecal exam	Rast (2014)
Indonesia	61%		Cattle	N/A	Abattoir surveillance	Estuningsih *et al*. (2009)
	33%	February	Cattle	Adult	Faecal exam	Sawitri *et al*. (2020)
	16.03%		Buffalo	Various	FEC	Nurhidayah *et al*. (2020)
Thailand	52.94%		Cattle	N/A	Abattoir surveillance	Phalee and Wongsawad (2014)
	67.27%		Buffalo	N/A	Abattoir surveillance	Phalee and Wongsawad (2014)
Philippines	97.8%	July–September	Cattle	Various	FEC	Gordon *et al*. (2015)
	95.2%	July–September	Buffalo	Various	FEC	Gordon *et al*. (2015)
	63.58%		Buffalo	Various	FEC	Portugaliza *et al*. (2019)
Malaysia	7.46%	February–August	Cattle	N/A	Abattoir surveillance	Zainalabidin *et al*. (2015)
	7.69%	February–August	Buffalo	N/A	Abattoir surveillance	Zainalabidin *et al*. (2015)
	40–100%		Cattle & buffalo	N/A	FEC	Khadijah *et al.* (2017)
	41.0%		Cattle	Various	FEC	Rita *et al*. (2017)
	0.00–5.55%		Cattle	N/A	Faecal exam	Diyana *et al*. (2019)
	0.03–1.38%		Cattle & buffalo	N/A	Abattoir surveillance	Diyana *et al*. (2020)

Note: Prevalence ranges are reported across all animals in the study unless indicated otherwise and may include those in low- and high-risk areas over multiple seasons and years. Detail of when samples were collected has been provided where the prevalence was reported for a single season or when prevalence did not change year-round. In all other cases samples were collected throughout the year and prevalence differed by season, or information on seasonality was not included. When prevalence was reported over a range of ages, 'Various' indicates that more detail on prevalence according to age may be found in the reference indicated. N/A, no age data available. FEC, Fecal Egg Count.

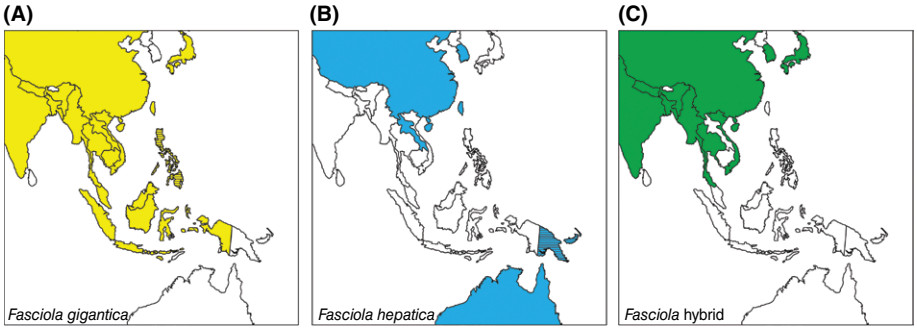

Fig. 13.4. The distribution of **(A)** *Fasciola gigantica,* **(B)** *Fasciola hepatica* and **(C)** *Fasciola* hybrids in Southeast Asia and neighbouring countries. The distribution of each *Fasciola* spp. was determined from available PubMed records where at least one instance of local infection was confirmed using genetic markers (blocked-out colours). Stripes indicate countries where no molecular confirmation of the species' presence has been conducted. Note that the distribution of each species is indicated at the country level, and regional and climatic variation are not accounted for. Figure adapted from Calvani and Šlapeta (2021).

F. hepatica may be more widespread in Southeast Asia than is currently known and warrants further investigation using appropriate molecular methods.

13.3 Production and Economic Impacts of Fasciolosis in Southeast Asia

It is difficult to quantify the impacts of this historically neglected disease on production and economic outcomes due to the range of animals and production systems affected and the geographical and climatic variation in regions where *F. gigantica* is prevalent. Along with animal species, breed, age, gender, production status, and nutrition level, production outcomes are affected by concurrent disease status, including co-infection with other production-limiting parasites, level of *F. gigantica* infection, and differences between and within the resilience of the various species and breeds while infected. Many, if not all, of these factors are difficult to standardize under field conditions. As a result, there is little or no work available that empirically demonstrates the impacts of *F. gigantica* on livestock production outcomes. This is in contrast to *F. hepatica*, the production impacts of which have been extensively studied and quantified across a range of farming systems and environmental conditions at both the local and national level. Unfortunately, the biological differences between these two parasites and the production systems under which they are found prevent the translation of these findings from one parasite species to the other. Some efforts have been made to understand the impacts of fasciolosis caused by infection with *F. gigantica* at the local level, and a comprehensive review of the work conducted in Southeast Asia involving the collaboration of many local government bodies and experts was compiled by the Australian Centre for International Agricultural Research (ACIAR, 2008). A summary of these findings and updates is presented below.

13.3.1 The smallholder farmer

To understand the impacts of fasciolosis on production outcomes in Southeast Asia it is important to first appreciate the main drivers and goals of the smallholder and subsistence farmers responsible for the management of livestock in these systems. For many, livestock ownership comes second to crop production and a livestock 'keeper' mentality predominates over that of being a livestock 'producer' (Dorward *et al.*, 2009; Bush *et al.*, 2014b; Garforth, 2015; Rast *et al.*, 2015). Different ethnic backgrounds and cultural activities heavily influence livestock management decisions and the production impacts of fasciolosis as a result. In some regions

cultural practices have led to the establishment of a strong dairy industry, while in others local milk production is almost non-existent and meat and offal are the primary production outcomes affected by fasciolosis. For many smallholder and subsistence farmers, livestock act as a 'living bank' and serve as a means of wealth accumulation. Animals are sold or slaughtered during times of need, such as to pay for schooling or medical bills, or to celebrate religious events and milestones including weddings and funerals. Animals are rarely fattened prior to sale and often the oldest or poorest performing animals are the ones sold (Suon *et al.*, 2006a; Carroll *et al.*, 2017). Additionally, animals inherited from relatives are rarely sold out of respect. Despite an increase in the uptake of mechanized ploughing methods, such as the adoption of hand-held tractors, cattle and buffalo are still routinely used for draught power (Pearson, 1993; O'Neill *et al.*, 1999; Rosilawati *et al.*, 2017). Thus, the impacts of fasciolosis on the energy availability of infected animals is an important production cost not usually considered in more developed regions. As a result, one of the most significant, yet difficult to quantify, impacts of fasciolosis on smallholder and subsistence farmers is the opportunity cost associated with the increased time spent ploughing fields due to the reduced draught power of infected animals. Most important of all is an understanding that farmers in these systems have limited access to resources to invest in the management of their livestock and hence inputs for disease prevention and control are minimal (Samkol *et al.*, 2015; Quy, 2016; Ashley *et al.*, 2018). In fact, the cost of disease control is rarely factored into impact assessments, which is in direct contrast to the systems in which *F. hepatica* is most prevalent. For these reasons it is unlikely that the economic impacts of *F. hepatica* on production outcomes will reflect those of *F. gigantica*.

13.3.2 Weight gain and body condition score

Initially it appeared as though there was a linear relationship between fluke burden and weight gain, with each additional adult fluke expected to reduce annual weight gain by 200 g in Zebu cattle (Sewell, 1966; Hammond and Sewell, 1974). It is likely that the reality is far more complex and that adequate nutrition, particularly the inclusion of protein in the diet of growing ruminants, plays a key role in minimizing the impacts of *F. gigantica* infection on weight gain and body condition (Graber, 1971; Nour *et al.*, 1979). While increased nutrition may reduce the impacts of fasciolosis, it is important to note that the weight gain of livestock in low-input smallholder systems is typically depressed compared with animals in intensive production systems due to a lack of adequate nutrition year-round (Pen *et al.*, 2010; Bush *et al.*, 2014a,b; Napasirth and Napasirth, 2018). The impact of breed, sex, age, production status, and concurrent infections of livestock must also be taken into account when attempting to elucidate the relationship between *F. gigantica* infection and weight gain (Suon *et al.*, 2006a; Dorny *et al.*, 2011). If adequate nutrition is provided and when age and production status are standardized, it appears that buffalo are more resilient to *F. gigantica* infection than cattle when infected twice weekly with 15 metacercariae for 32 weeks, with little difference in the weight of control and *F. gigantica*-infected buffalo calves 36 weeks pi, compared with Bali and Ongole calves whose weight gain was depressed by 160 g/day and 98 g/day, respectively (Wiedosari *et al.*, 2006). The average daily liveweight gain of buffalo calves fed a single dose of 1000 metacercariae, on the other hand, was 328.8 g less than uninfected controls, suggesting a dose-dependent response to infection (Mehra *et al.*, 1999).

Scales suitable for weighing livestock are largely unavailable in less developed countries and are almost impossible to move between villages when conducting field work (Rast *et al.*, 2015). Instead, body condition score (BCS) and carcass weight during abattoir surveillance are used as alternative measures to estimate the impact of fasciolosis on weight gain, although both have their limitations (Molina *et al.*, 2005a; Dorny *et al.*, 2011). BCS is a subjective measure reliant on adequate training of field staff. When applied in the field, efforts to standardize BCS estimates should be made, either by using the same person to assess each animal enrolled in the study, or by using weight-tapes to provide an estimate of body weight based on the girth (cm) of animals (Lesosky *et al.*, 2013; Bush *et al.*,

2014a). Carcass weight is routinely used to estimate the impact of infection during abattoir surveillance (Da Costa *et al*., 2019). In this setting, background information on the nutritional and production status of affected animals is often non-existent and thus findings should be interpreted with caution (Kithuka *et al*., 2002; Molina *et al*., 2005a; Suon *et al*., 2006a). As a result of these limitations, there is a paucity of information outlining the impact of *F. gigantica* infection on carcass weight and BCS in Southeast Asia. Existing results, however, suggest that infection decreases BCS across a range of cattle breeds (Suon *et al*., 2006a; Dorny *et al*., 2011). Work conducted in Africa and India supports these findings, with decreased BCS considered to be the result of reduced dry matter intake and inappetence as opposed to a reduction in the digestibility of available nutrients (Wamae *et al*., 1998; Mehra *et al*., 1999).

13.3.3 Reproduction and lactation

Increased inter-calving intervals and time to first parturition, along with reduced milk output and quality, are expected to occur as a result of the impacts of *F. gigantica* infection on weight gain. Sexual maturity in livestock is a function of genetics and the environment, including adequate nutrition and body condition, hence impacts on weight gain as a result of infection with *F. gigantica* are likely to prolong the maturation of livestock and thus time to first parturition (Kenny *et al*., 2017). While there is little empirical evidence demonstrating the link between reduced fertility and infection with *F. gigantica*, one study in Indonesia noted significantly longer inter-calving intervals in infected Ongole cattle compared with those treated with triclabendazole over a 2-year period (Suhardono, 2001). The impacts of *F. hepatica* infection on reproduction and lactation in intensive production systems are well known, with a negative correlation frequently observed between infection status and milk yield, milk fat percentage, and inter-calving intervals (Charlier *et al*., 2007; Köstenberger *et al*., 2017). The impacts of *F. gigantica* infection on dairy parameters, however, remains to be quantified (Suzuki *et al*., 2006). One study conducted in Pakistan recorded an increase of 0.87 and 0.67 litres of milk per day in oxyclozanide-treated cattle and buffalo, respectively, compared with untreated controls (Khan *et al*., 2011). The financial loss of the reduced milk production was equivalent to US$0.33 and US$0.32 per animal per day for cattle and buffalo, respectively. Furthermore, uninfected cattle and buffalo produced 0.37% and 0.41% more milk fat per animal, suggesting that infection with *F. gigantica* impacts feed conversion and nutrient absorption (Khan *et al*., 2011). Further work to quantify the impacts of *F. gigantica* infection on reproduction and lactation is recommended, particularly in areas where there is a reliance on locally produced dairy produce as a source of protein.

13.3.4 Other production and economic impacts

One aspect of fasciolosis that is often overlooked as a significant cost to smallholder farmers is the impact of *F. gigantica* infection on draught performance. The primary purpose of large ruminant ownership in much of the developing world, aside from wealth accumulation, is for draught and ploughing activities (Pearson, 1993; O'Neill *et al*., 1999; Rosilawati *et al*., 2017; Paudel *et al*., 2019). Thus, any impact on draught performance represents either an opportunity cost to smallholder farmers as a result of increased time spent working their fields, or a financial cost if additional animals are hired to assist in manual labour (Pearson, 1989, 1993; ACIAR, 2008). Infection is expected to impact draught performance as a result of decreased body condition and weight along with anaemia-associated weakness (Roberts *et al*., 1991). Empirical data demonstrating the economic impact of *F. gigantica* infection on draught power is lacking, with existing work employing few animals or failing to account for concurrent production-limiting diseases. Nevertheless, one estimate suggests an opportunity cost of 7 days per year for infected buffalo in Indonesia, while another demonstrated that Ongole cattle treated with triclabendazole were capable of twice as many days' work than those that remained untreated (Suhardono, 2001; ACIAR, 2008).

Liver condemnation during abattoir surveillance is the most commonly employed method to determine the economic impact of *F. gigantica* infection, despite representing only a fraction of the true cost of fasciolosis. Regardless, several studies across both Asia and Africa have reported significant financial losses due to the condemnation of livers with visible signs of infection (Hyera, 1984; Okao, 1984; Morel and Mahato, 1987; Wamae *et al*., 1998; Kithuka *et al*., 2002; Mungube *et al*., 2006; Abunna *et al*., 2010; Nyirenda *et al*., 2019; Zewde *et al*., 2019). While liver condemnation is a largely inadequate measure of the economic impact of fasciolosis, these studies support the importance of this parasite in less developed regions by demonstrating the prevalence of *F. gigantica* infection and suggest that the true economic impact is far greater than is currently assumed.

Irrespective of the production outcome used to estimate the impact of fasciolosis on livestock in Southeast Asia and elsewhere, it is clear that significant research gaps still exist, prohibiting an accurate assessment of economic losses associated with *F. gigantica* infection. Existing estimates of total economic impacts are out of date and unlikely to be of much value, due to a lack of current prevalence data across the regions affected (Table 13.3). Despite the inherent difficulties associated with quantifying the economic impacts of fasciolosis in the less developed world, a baseline estimate is worth pursuing in order to aid in the allocation of funding for future prevalence, epidemiological, and control trials, particularly given the increasing competition for public and private investment. This is especially true in a post-COVID world where veterinary and public health research efforts are primarily directed towards the surveillance and control of diseases with pandemic potential, very few of which impact ruminants. In order to provide an overall assessment of the economic impacts of *F. gigantica* infection, an existing estimated loss per animal from 1997 has been updated to keep up with inflation (Suhardono, 2001; ACIAR, 2008) (Table 13.4). This estimate only takes into account the total annual cost of lost meat production, draught power, and reduced fertility in infected cattle and buffalo and thus the actual cost of fasciolosis on large ruminant production, including the cost incurred due to impacts on liver condemnation, milk yield, inter-calving intervals, and control, is likely to be far higher. Due to a lack of current prevalence data, an estimate of economic loss has been provided for three prevalence scenarios:

Table 13.4. Estimated annual economic impact of fasciolosis on large-ruminant production in Southeast Asia.

Country	No. of cattle and buffalo per country	Total large ruminants	Estimated economic losses (US$ million) by prevalence level[a]		
			Low (10%)	Medium (30%)	High (60%)
Vietnam	Cattle: 3,973,892 Buffalo: 2,469,682	6,443,574	$43.82	$131.46	$262.92
Cambodia	Cattle: 3,248,417 Buffalo: 519,083	3,767,500	$25.62	$76.86	$153.72
Lao PDR	Cattle: 1,586,200 Buffalo: 774,200	2,360,400	$16.05	$48.15	$96.30
Indonesia	Cattle: 12,831,303	12,831,303	$87.25	$261.75	$523.52
Thailand	Cattle: 3,648,738 Buffalo: 70,158	4,350,325	$29.58	$88.74	$177.48
Philippines	Cattle: 1,995,841 Buffalo: 1,790,681	3,786,522	$25.75	$77.25	$154.49
Myanmar	Cattle: 11,153,962 Buffalo: 1,504,836	12,658,798	$86.08	$258.24	$516.48
Total		46,198,422	$314.15	$942.45	$1,884.90

[a]Assumes a loss of US$68 per head (Suhardono, 2001; ACIAR, 2008). Livestock numbers as per most recent agricultural census conducted by respective governments and reported by FAO (2019). Animal census data collected over several years; no data on livestock numbers available for Malaysia, Timor Leste or Singapore.

low (10% prevalence), medium (30% prevalence), and high (60% prevalence) based on the estimates presented in Table 13.3.

13.4 Control of *Fasciola gigantica*

The control of *F. hepatica* in intensive production systems is relatively easily achieved via a combination of anthelmintics and livestock management decisions. Control of *F. gigantica* in the low-input systems prevalent throughout the less developed world is more difficult, where farmers are often unaware of the production impacts of fasciolosis and where access to anthelmintics is limited (Rast *et al.*, 2015). Existing recommendations for control of *F. gigantica* in tropical areas include the use of molluscicides, limiting access to sources of infection, decontaminating feed such as rice straw via exposure to sunlight, and relying on biological methods of control, such as ducks, to reduce environmental contamination (Roberts and Suhardono, 1996; Suhardono, 2001; Mahato and Harrison, 2005; Suhardono *et al.*, 2006c,d; ACIAR, 2008; Quy, 2016). While many of these recommendations have been proven to reduce infection levels in tropical farming systems, they require an understanding of the biology of the parasite alongside considerable time inputs by the smallholder farmers involved. Despite significant inputs from government and research agencies, attempts to educate smallholder and subsistence farmers on the importance of *Fasciola* spp. control and to incentivize them to invest in various control methods have been met with limited success over the long term (ACIAR, 2008; Quy, 2016). The primary goal of these programmes is often to equip farmers with cost-effective control strategies supplemented with training of local staff and engage farmers on routes of transmission and the impacts of disease on both human and animal health. This is difficult to achieve in an environment where very few farmers operate with a production mindset, particularly considering that the majority of *Fasciola* spp. infections are chronic and therefore do not cause obvious damage to their mammalian hosts (ACIAR, 2008).

13.4.1 Chemotherapy

A selection of anthelmintic drugs effective in the control of *F. hepatica* have been shown to have similar activity against *F. gigantica*, with only triclabendazole and clorsulon proven to have high efficacy against both adult and immature forms in cattle (Robin *et al.*, 1986; Suhardono *et al.*, 1991; Mahato *et al.*, 1994). Unfortunately, most flukicides are only registered for use in a limited number of species, namely cattle, goats, sheep and, occasionally, horses. Buffalo metabolize triclabendazole differently to other livestock and thus require a double dose compared with cattle to achieve equivalent parasite elimination (Estuningsih *et al.*, 1990; Sanyal and Gupta, 1996, 1998). For the most part, recommendations for the use of anthelmintics for the control of *F. gigantica* are based on those for *F. hepatica*, but very few drugs are registered for use against *F. gigantica*. Egaten, the form of triclabendazole from Novartis recommended by the World Health Organization, is registered for use against both *F. hepatica* and *F. gigantica* in humans and forms the basis of an international drug donation programme. Recommendations for the use of anthelmintics for the control of fasciolosis remain largely unchanged and thus readers are referred to existing reviews and the previous version of this chapter for further detail (Mas-Coma *et al.*, 2018; Fairweather *et al.*, 2020; Castro-Hermida *et al.*, 2021).

Two important factors to consider when attempting to eliminate *F. gigantica* infection via chemical means in less developed countries are the potential for limited access to the full range of anthelmintics available and a lack of animal restraint facilities necessary for drug delivery (Miller *et al.*, 2018). In an attempt to engage farmers in the benefits of parasite control, several studies have investigated the incorporation of anthelmintics into feed supplement blocks with the intention of meeting nutritional deficiencies while also combating infection with *F. gigantica* (Sanyal and Gupta, 1998; Windsor *et al.*, 2019). This method of anthelmintic administration solves issues surrounding medication delivery in regions where livestock handling facilities are limited and uptake by local farmers may be more likely as a result of the additional benefits associated with increased nutritional supplementation (FAO, 2007). There are issues, however, with guaranteeing accurate dose delivery to the range of infected animals requiring treatment, particularly when extensive livestock management systems limit the level of observation

possible. The use of medicated feed supplements should also be avoided in regions where withholding periods are not strictly monitored or adhered to and where treatment is not preceded by diagnosis, particularly as resistance to triclabendazole continues to develop as a result of its overuse.

13.4.2 Alternatives to anthelmintics

Alternative forms of control using biological methods have been trialled in Southeast Asia in an attempt to meet the time and financial constraints of smallholder farmers. These include the use of ducks in an attempt to exploit the antagonistic effect of the larval stages of *Echinostoma revolutum* on the establishment of *F. gigantica* in snails (Suhardono *et al.*, 2006d; ACIAR, 2008). While both methods decrease the level of environmental contamination with *F. gigantica*, neither has shown long-term uptake by farmers, possibly due to a need for continued extension and education programmes (ACIAR, 2008). The control of snails using molluscicides or salt is not recommended, due to the subsequent negative environmental impacts. The simplest methods of control, and thus the most likely to be maintained in the long term, are those that can be applied with minimal changes to existing agricultural practices. These include reducing environmental contamination by drying out manure containing eggs in the sun before applying it to fields and rice paddies as fertilizer, as well as limiting the exposure of livestock to infective metacercariae by feeding grass and rice straw cut 10 cm above the water surface level (Suhardono, 2001; Mahato and Harrison, 2005; Suhardono *et al.*, 2006a,c). Recently, investigations into the fasciolicidal effects of medicinal plants have been progressed in the hope that local remedies may be more readily employed by farmers and animal health workers (Koko *et al.*, 2000; Saowakon *et al.*, 2009; Anuracpreeda *et al.*, 2017; Ullah *et al.*, 2017; Hegazi *et al.*, 2018; Yamson *et al.*, 2019; Batiha *et al.*, 2020; Rehman *et al.*, 2020). While much of this work is still in its infancy, the characterization of the compounds found in local remedies may validate their use in smallholder systems while helping to reveal promising targets for the development of new anthelmintics.

Progress towards the identification of vaccine targets effective against *F. hepatica* has accelerated over the past two decades, yet similar work towards the development of a vaccine against *F. gigantica* is limited (Estuningsih *et al.*, 1997; Paykari *et al.*, 2002; De Bont *et al.*, 2003; Nambi *et al.*, 2005; Yadav *et al.*, 2005; McManus and Dalton, 2006; Jezek *et al.*, 2008; El-Ahwany *et al.*, 2012; Kumar *et al.*, 2012; Toet *et al.*, 2014). Differences in vaccine efficacy have been reported between cattle and sheep along with the *Fasciola* spp. targeted, and thus vaccines developed for use in *F. hepatica*-endemic regions are unlikely to confer similar levels of protection in areas where *F. gigantica* is more prevalent (Toet *et al.*, 2014; Molina-Hernández *et al.*, 2015). The main driver for the production of vaccines against *F. hepatica* is the need to replace chemical control in areas where resistance is rapidly developing (McManus and Dalton, 2006; Toet *et al.*, 2014). The need for vaccines in countries where chemical control is not routinely practised (as occurs throughout much of Southeast Asia), however, should not be overlooked. Although no vaccines are currently commercially available for the control of ruminant fasciolosis, they present perhaps the most promising solution in less developed countries based on the uptake of vaccines against other economically important pathogens (Roth *et al.*, 2003; Kansiime *et al.*, 2015; Bessell *et al.*, 2017; Hotez, 2018; Donadeu *et al.*, 2019). Thus, research into vaccines efficacious against *F. gigantica* and their use in the field is strongly encouraged.

13.5 Diagnosis in the Developing World

A variety of diagnostic methods, including those that are commercially available and those that have been developed for research purposes, have been advanced in recent years to assist in the diagnosis of *F. gigantica* in both human and animal populations. Many of these, particularly those that detect *F. gigantica* antibodies, antigens, or DNA, are unlikely to be applicable in Southeast Asia where laboratory infrastructure is limited and where the cost of routine diagnostics is often prohibitive. Thus, in this chapter we have chosen to focus on methods available to laboratories

and diagnosticians with limited diagnostic capacity. Of course, government and university laboratories, as well as those that are privately funded or supported by larger research institutions, may have access to the broader range of laboratory facilities necessary for more technical methods of *Fasciola* spp. diagnostics. As such, further detail on these methods and their application under a variety of diagnostic and epidemiological scenarios is available elsewhere in this volume.

13.5.1 Post-mortem diagnosis of fasciolosis in livestock

The gold standard of *Fasciola* spp. diagnosis in livestock occurs via post-mortem examination of the liver during abattoir surveillance by visualizing either the migratory tracks left behind by immature stages in livers or adults in the bile ducts and gallbladders. The inspection of livers and gallbladders at slaughter during abattoir surveillance provides valuable insight into local *Fasciola* spp. prevalence but has several limitations that are worthy of consideration. The primary limitation is a lack of animal traceability in many less developed countries, which prevents an accurate understanding of the local epidemiological situation and inhibits direct feedback to the primary producer. Additionally, the livestock population sent for slaughter is often biased towards older and sicker animals and thus may not provide an accurate representation of *Fasciola* spp. prevalence and production impact in a given region (Anderson *et al.*, 1999; Suon *et al.*, 2006a; Carroll *et al.*, 2017). Abattoir access in Southeast Asia is often controversial and it can be difficult to attain the necessary governmental approval required to undertake such surveillance. Furthermore, damage to the liver caused by cuts while inspecting for fluke tracks may reduce the retail value of the liver, a highly valued commodity in many regions of Asia. Similarly, opening the gallbladder and exposing the liver to bile may be unwanted, unless there is regulation surrounding liver inspection. Even with existing regulation around inspection and eventual downgrading or condemnation of infected livers, enforcing such practices remains problematic.

13.5.2 Ante-mortem diagnosis of fasciolosis

The detection of eggs in faeces is the most cost-effective and reliable method of ante-mortem *Fasciola* spp. diagnosis in regions with limited diagnostic capacity. In both human and animal cases, eggs of *Fasciola* spp. are traditionally concentrated via a variety of optimized sedimentation and/or flotation methods. Regardless of the method employed, the underlying concept is simple, often requiring only basic laboratory equipment, and can therefore be adopted by minimally equipped laboratories. *Fasciola* spp. eggs are heavier than many traditional flotation solutions such as saturated NaCl (with a specific gravity of 1.20) and therefore do not appear upon a standard faecal flotation for the detection of gastrointestinal nematodes. The size of *F. hepatica* and *F. gigantica* eggs varies according to the host species infected and the overlap in areas of parasite sympatry but, in general, eggs of *F. gigantica* are larger than those of *F. hepatica*: 130–200 µm × 60–120 µm versus 70–160 µm × 60–105 µm, respectively (Valero *et al.*, 2009) (Fig. 13.2). The essence of a traditional sedimentation is to exploit the weight of the large *Fasciola* spp. eggs in order to separate them from the surrounding ingesta and faecal matter to concentrate them and allow ease of visualization under a microscope. A variety of adaptations exist that principally differ by the limit of detection (Happich and Boray, 1969b; Anderson *et al.*, 1999; Suhardono *et al.*, 2006b; Charlier *et al.*, 2008; Brockwell *et al.*, 2013).

A protocol for a simple sedimentation utilizing only water and methylene blue to stain the remaining plant matter in the resultant sediment is included in Box 13.1. Besides *Fasciola* spp., the eggs of other trematodes will be similarly affected by gravity due to their size, and thus may need to be differentiated from *Fasciola* spp. eggs upon visualization under a light or stereo microscope. These primarily include paramphisome eggs (ruminant stomach flukes), which are of a similar size but uncoloured compared with *Fasciola* spp. eggs, which are dark brown to golden yellow in colour (Fig. 13.5). Other eggs difficult to differentiate from *Fasciola* spp. in human samples are those of *Fasciolopsis*

Box 13.1. Sedimentation method for the detection of *Fasciola* spp. eggs in faeces.

This technique was developed in Australia in the 1960s and relies on a series of sedimentations to separate *Fasciola* spp. eggs from ingesta using a sieve (270 µm) and a set of conical flasks (250 ml, 100 ml and 15 ml) (Happich and Boray, 1969b). The time for each sedimentation step is 3 min. While this technique is very robust and reproducible, 60% of the eggs are lost during the sieving and sedimentation process. The limit of detection for ruminants is 1 egg per gram (EPG) of faeces if the process is followed as outlined below.

Step 1 – Mix faecal samples (3 g or 6 g for small and large ruminants, respectively) with clean water to form a homogeneous solution.

Step 2 – Using pressure, hose the solution through a fine (270 µm) sieve (or at the very least, a tea strainer) into a 250 ml conical flask, top with water and allow to sediment for 3 min.

Step 3 – After 3 min, carefully pour off (or aspirate) the supernatant without disturbing the sediment.

Step 4 – Top the remaining sediment with water up to 100 ml and allow to sediment for a further 3 min.

Step 5 – Carefully pour off (or aspirate) the supernatant and pour the sediment into a 15 ml centrifuge tube (make sure to rinse any remaining sediment out of the conical flask into the 15 ml centrifuge tube), top with water and allow to sediment for a final 3 min.

Step 6 – Carefully aspirate the supernatant (being sure to not disturb the sediment) down to 2 ml.

Step 7 – To examine the sediment for fluke eggs, add 2 drops of methylene blue (1%), mix and rinse the entire sediment into a Petri dish (or a marked Perspex counting chamber if you wish to calculate EPG). Additional distilled water can be added to allow for ease of observation. Examine under a stereomicroscope at ~15× magnification. Methylene blue stains the residual plant matter blue, which contrasts against the eggs, allowing them to be seen more easily.

Step 8 – Observe at least ten fields under 15× magnification to evaluate the concentration of eggs semi-quantitatively (+, ++ and +++, based on the number of eggs seen per field of view). The use of a 5 × 15 × 1 cm Perspex counting chamber with 1 cm grids enables quantification of the total number of eggs. For small ruminants the final count is equivalent to the EPG, while for large ruminants the final count must first be divided by two to get the EPG. Both values account for the 60% loss of eggs during sieving. For a more detailed description of this method, see Calvani (2017).

buski, *Paragonimus* spp., *Echinostoma* spp. and *Gastrodicoides hominis*. Expert medical parasitologists should be consulted to differentiate fluke eggs in human samples in areas where these parasites co-exist.

Although methods relying on the visual confirmation of eggs in faeces are low-cost, they are often time-consuming and limited by their inability to diagnose infection inside the prepatent period. Additionally, the increased faecal volume produced by large ruminants compared with smaller animals such as sheep and goats has a tendency to dilute the number of eggs on offer for detection (Sewell, 1966; Happich and Boray, 1969a; Calvani *et al.*, 2017). Thus, it is important to know the analytical sensitivity of a given method in terms of eggs per gram of faeces and to maximize the starting volume used, particularly when examining the faeces of large ruminants.

It is also worth emphasizing that livestock livers are a highly prized commodity throughout much of Southeast Asia and are frequently consumed, either cooked or raw (Ale *et al.*, 2014). While consumption of infected livers does not lead to active infection, such practices can lead to spurious *Fasciola* spp. infections. Spurious infections are those where patients who have recently ingested cooked or uncooked liver from infected animals present with *Fasciola* spp. eggs in their faeces. Inquiry into the history of recent liver consumption is necessary when detecting *Fasciola* spp. eggs in humans in countries where the consumption of liver is common and where there are limited abattoir inspections and/or condemnation of infected livers. Furthermore, eggs will be absent in faeces before completion of the pre-patent period and during aberrant infections and thus a negative faecal result does not rule out infection in these cases. In human infections the stage of infection (early/incubation, acute, latent, and chronic) will ultimately influence the most appropriate method of detection

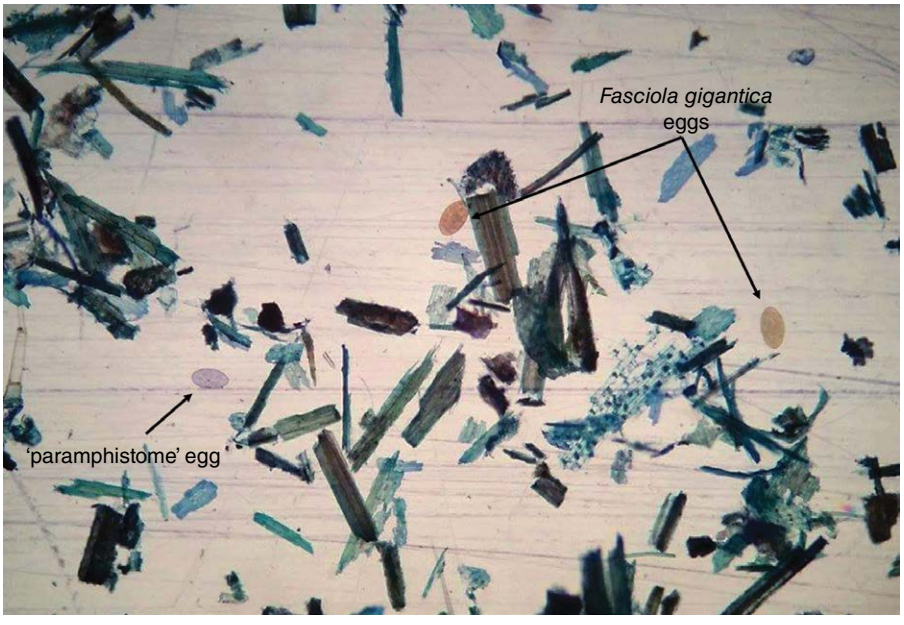

Fig. 13.5. Stained cattle faecal sediment containing both *Fasciola* spp. eggs and paramphistome eggs. *Fasciola* spp. eggs are dark brown to golden yellow in colour, while paramphistome eggs are colourless. The sediment is stained with 1% methylene blue to enhance the contrast of the *Fasciola* spp. eggs against the plant material and improve visualization of the eggs.

(Mas-Coma *et al*., 2009a, 2018). A combination of diagnostic imaging and the detection of circulating antibodies, however, is likely to provide the best indication of current infection in areas where these resources are available to local medical professionals. For widescale surveillance, serological methods are best suited to provide evidence of the areas at greatest risk of fasciolosis by indicating past exposure to both *F. hepatica* and *F. gigantica*. This information is desperately needed across most of Southeast Asia and elsewhere to help inform public health initiatives and provide an epidemiological background for future research investment.

Going forward it is hoped that low-cost point-of-care testing options will be advanced to commercialization in order to enable affordable and widespread diagnostics in resource-limited areas (Martínez-Sernández *et al*., 2011). Not only will this help farmers make informed decisions about managing fasciolosis in their animals, but it may also help by extending methods available for human diagnosis into regional areas where medical and diagnostic facilities are limited.

13.6 Genetic Characterization of *F. gigantica* and *Fasciola* Hybrids

In areas of parasite sympatry, *Fasciola* spp. confirmation is necessary to gain insights into the epidemiology of infection and species distribution in order to inform public health and parasite control decisions. Morphological methods to differentiate the two species are of limited use in areas where both species are present due to the influence of host species on the size of both adults and their eggs, resulting in overlaps between the two species (Valero *et al*., 2001; El-Rahimy *et al*., 2012). The occurrence of intermediate forms, as a result of hybridization and/or introgression between *F. hepatica* and *F. gigantica*, further complicates traditional morphology-based species identification (Agatsuma *et al*., 2000; Itagaki *et al*., 2011; Calvani and Šlapeta, 2021).

Despite the surprisingly high homology between the two species, identification of *F. gigantica* and differentiation from *F. hepatica* are relatively easily achieved using molecular methods based on polymerase chain reaction (PCR) (Mas-Coma

et al., 2009a). The characterization of DNA sequences for the purpose of *Fasciola* species identification began in 1993 with the sequencing of ITS2 rDNA (Adlard *et al*., 1993). ITS2 rDNA was shown to reliably differentiate several *F. gigantica* specimens from those of *F. hepatica* and the within-species variation was negligible (Adlard *et al*., 1993). Since then, sequence-based studies targeting rDNA and mtDNA have all demonstrated small but reproducible genetic differences between the two *Fasciola* species. Taking advantage of single nucleotide polymorphisms (SNPs) in 28S rDNA, Marcilla *et al*. (2002) developed a rapid PCR restriction fragment length polymorphism (RFLP) assay to identify *Fasciola* species. More recently, real-time PCR (qPCR) assays targeting ITS1 rDNA, ITS2 rDNA, and 28S rDNA have been used to differentiate the two distinct genetic signatures representing each species (Alasaad *et al*., 2011; Calvani *et al*., 2020). Newer methods targeting single-copy genes, *pepck* and *pold*, have also recently been developed (Shoriki *et al*., 2016; Hayashi *et al*., 2018).

This range of molecular methods is easily applied to individual specimens removed from the livers and gallbladders of infected hosts during post-mortem examination. The application of these tools to samples collected ante-mortem (i.e. the discrimination of DNA from eggs in faecal samples), however, has two limitations. First, in areas where species overlap of both *F. gigantica* and *F. hepatica* occurs, along with their potential 'hybrids', the above approaches are unable to differentiate co-infection from infection with *Fasciola* hybrids in samples containing DNA from multiple individuals (i.e. eggs) (Calvani *et al*., 2020; Calvani and Šlapeta, 2021). Secondly, amplification of single-copy genes using DNA isolated from faecal samples, and thus fluke eggs, suffers from limited analytical sensitivity due to low DNA concentration present in both individual and pooled eggs (Calvani *et al*., 2020). Molecular methods capable of discriminating the contribution of template DNA from each species, such as recently developed next-generation sequencing (NGS) and quantitative allelic discrimination assays, enable higher resolution of samples containing mixed DNA (Calvani *et al*., 2020). Unfortunately, the distinction between faecal samples containing DNA of species (in the case of co-infected animals) from those containing DNA from 'hybrid' flukes still evades us.

Itagaki and Tsutsumi (1998) were the first to suggest that anomalous *Fasciola* species in Japan were 'hybrids' between *F. hepatica* and *F. gigantica* (Oshima *et al*., 1968). Reports of *Fasciola* hybrids distributed across areas of parasite sympatry have been increasing since. Using individual specimens and PCR assays targeting the single-copy genes *pepck* and *pold*, Hayashi *et al*. (2018) unambiguously demonstrated the widespread distribution of *Fasciola* hybrids across Southeast Asia. The limitations of Sanger sequencing for the differentiation of *Fasciola* hybrids from either of the pure forms has only very recently been revealed (Calvani *et al*., 2020). Here it was seen that SNPs used to differentiate the two species across a variety of markers may mask the molecular identity of *Fasciola* hybrids due to preferential amplification of one species over the other (Calvani *et al*., 2020). Based on these results it appears that previous records reliant on Sanger sequencing for molecular species confirmation may require re-evaluation.

Hybridization between *F. hepatica* with *F. gigantica* has been experimentally demonstrated under laboratory conditions, yet the stability of these forms beyond the F2 generation remains in question (Itagaki *et al*., 2011). A more permanent consequence of these interspecific mating events is the possibility of backcrosses between *Fasciola* hybrids and either of the parental species and thus the potential for introgression of advantageous traits from one species into the other (Calvani and Šlapeta, 2021). In the context of Southeast Asia, with a historically dominant population of *F. gigantica*, introgressed traits from *F. hepatica* into *F. gigantica* may introduce virulence factors, host expansion factors or even drug resistance into the region. While introgression between *F. hepatica* and *F. gigantica* is yet to be confirmed, it is unlikely that the introduction of *F. hepatica* into Southeast Asia and elsewhere, and its impact on endemic *F. gigantica* populations, is negligible. It is clear that hybridization is continuously happening, as evidenced by the frequent detection of *Fasciola* hybrids in Southeast Asia and beyond (Hayashi *et al*., 2018).

Caution is advised when using existing molecular methods for the differentiation of *F. hepatica* and *F. gigantica* to detect *Fasciola* hybrids. Many are based on nuclear DNA and rely on either rDNA and/or single-copy genes (*pepck* and

pold) and thus are only capable of detecting recent mating and hybridization events (Mas-Coma and Bargues, 2009; Mas-Coma *et al.*, 2009a; Shoriki *et al.*, 2016; Hayashi *et al.*, 2018). Assuming that hybridization leads to introgression (for example, selected *F. hepatica* genes are integrated into the genome of *F. gigantica*), the above methods will report false results for all but the targeted sequences (Fig. 13.6). Furthermore, the use of mtDNA markers alone should be avoided, as they do not undergo recombination. At a minimum, the use of two markers is advised, one nuclear and one mitochondrial, as the detection of DNA from different *Fasciola* spp. between these markers will give some indication of interbreeding (Mas-Coma *et al.*, 2009a; Calvani and Šlapeta, 2021). It is important to recognize that the detection of DNA from only *F. hepatica* or only *F. gigantica* across both markers does not rule out the occurrence of introgression. In fact, where the two species are assumed to have coexisted in the same location for extended periods, it should be taken that all *F. gigantica* may carry some introgressed traits from *F. hepatica* and vice versa. The existence of draft reference genomes of both *F. gigantica* and *F. hepatica* is the first milestone to aid in the identification of introgression between these two species (Cwiklinski *et al.*, 2015; Pandey *et al.*, 2020). The application of whole-genome NGS methodologies to characterize both experimental and naturally occurring hybrids is essential to further our understanding of these genetic events and their functional implications. Computational analyses will be needed to overcome complications such as parthenogenesis and polyploidy (Itagaki and Tsutsumi, 1998; Terasaki *et al.*, 2000; Fletcher *et al.*, 2004; Itagaki *et al.*, 2009).

In summary, the identification of *F. gigantica* and *F. hepatica* is easily achieved using PCR-based methods, but in areas of current or prior parasite sympatry our ability to discriminate one species from the other is complicated by the potential for introgression between the two species. Existing reports of *Fasciola* hybrid or introgressed forms should be interpreted with caution, due to the limitations of currently available molecular methods. The potential epidemiological outcomes of hybridization and introgression between *F. hepatica* and *F. gigantica* remain unknown and the two terms should not be used interchangeably throughout the literature.

13.7 Zoonosis

Human fasciolosis, including aberrant forms of the disease, has been extensively reviewed, with more detailed information found elsewhere in this volume (Hammond, 1974; Mas-Coma *et al.*, 2014, 2018). Outside of known hyper-endemic areas, such as those that occur in Vietnam, information on the occurrence of human infection with *F. gigantica* remains sparse, particularly when compared with reports of infection with *F. hepatica* (Bui *et al.*, 2016). This has led to the assumption that human infection with *F. gigantica* occurs sporadically and thus the suggestion that humans are less susceptible to infection with *F. gigantica* than *F. hepatica* (Hammond, 1974; Thanh *et al.*, 2007). It is more likely, however, that cases are under-reported due to the predominance of *F. gigantica* in the less developed world, where access to medical resources and diagnostic facilities is limited. It has also been postulated that infection with *F. gigantica* results in a milder, less painful form of disease compared with infection with *F. hepatica* and thus may go unnoticed more often (Grange *et al.*, 1974; Hammond, 1974).

Human infection occurs via ingestion of metacercariae on aquatic and semi-aquatic plants, or by the use of contaminated water for cooking and drinking purposes (Mas-Coma *et al.*, 1999; Thanh *et al.*, 2008; Quy, 2016). Perhaps counter-intuitively, infection in livestock does not appear to correlate with a higher level of infection in people (Mas-Coma *et al.*, 1999). In areas where infection with *F. hepatica* and *F. gigantica* is endemic it has been shown that outdoor defecation and poor hygiene practices are sufficient to maintain the life cycle in areas where livestock are not infected at high levels (Mas-Coma *et al.*, 1999; Quy, 2016). Parasite elimination is readily achieved via administration of triclabendazole at a dose of 10 mg/kg with minimal side effects, and a treatment donation programme by the World Health Organization in collaboration with Novartis is currently in operation (WHO, 2021).

Human cases of *F. gigantica* infection have been reported throughout Africa, Asia and the Middle East, but rarely have the parasites in these reports been adequately identified to the species level. Instead, assumptions have been made based on recent travel history, the predominant *Fasciola* species found in the region where the case was

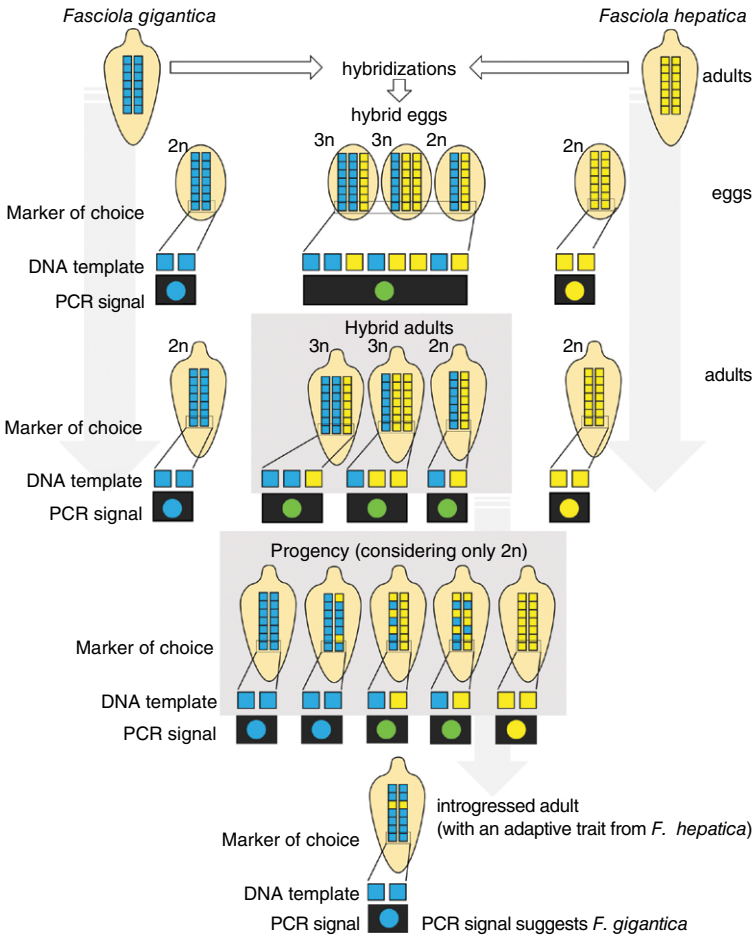

Fig. 13.6. Molecular identification of *Fasciola* spp. in areas of past and present parasite sympatry. *Fasciola* spp. adults and eggs are represented by set a of genes depicted as a series of squares (blue: *F. gigantica*; yellow: *F. hepatica*). Both *F. hepatica* and *F. gigantica* produce eggs and adult offspring, indicated by the arrows along each side, that can be moleculaly characterized by a variety of markers. In this case a single marker is represented by a pair of alleles (DNA template) that can be amplified by PCR. The 'net' PCR signal as a result of Sanger sequencing (either blue or yellow) is represented by the coloured circles. When hybridization between *F. gigantica* and *F. hepatica* occurs (as shown through the middle of the diagram), the eggs inherit a full set genes from both *F. hepatica* and *F. gigantica*. The 'net' PCR signal from pools of these eggs (as found in faecal samples) will be a mix of *F. hepatica* and *F. gigantica* (i.e. green). Note that the same 'net' green PCR signal can be achieved by manually mixing *F. hepatica* and *F. gigantica* DNA (not shown; see Calvani *et al*., 2020). To overcome the issue of analysing DNA from many invidviduals, single adult specimens are characterized and a mixed 'net' PCR signal (green) is obtained for hybrid individuals in the F1 generation because a full set of genes is present (middle grey box). The progeny of *Fasciola* hybrids may back-cross with either of the original parental species, creating a mixture of genomes over many generations that may not be detected via individual molecular markers. Ultimately, the seemingly unambigious molecular identification of *F. gigantica* using a single marker will mask the introgressed form of the fluke as shown by the bottom introgressed adult. Traditionally, *Fasciola* spp. are 2*n*, but under circumstances such as hybridization and possibly back-crossing, 3*n* individuals have been characterized, further complicating molecular identification.

diagnosed, non-specific serological results, and/or by using the morphology of eggs or adults collected from patients (Grange *et al.*, 1974; Hammond, 1974; Nguyen *et al.*, 2010; Salzer and Schmiedel, 2015; Sah *et al.*, 2018). Morphology of both eggs and adults should be avoided for species identification in human cases, due to the influence of host species on the size of both adults and eggs, particularly in areas of species overlap (Valero *et al.*, 2009; El-Rahimy *et al.*, 2012; Sah *et al.*, 2018). Where possible, and as described earlier in this chapter, molecular markers should be used to confirm the species present in order to provide information on distribution, epidemiology and populations at risk of infection (Inoue *et al.*, 2007; Thanh *et al.*, 2007, 2008b; Chen *et al.*, 2013; Rokni *et al.*, 2018; L'Ollivier *et al.*, 2020; Nguyen *et al.*, 2020b). While unable to differentiate between *Fasciola* species, serological surveys have provided valuable insight into areas at risk of infection and are recommended as a means of providing further evidence towards the importance of human fasciolosis in less developed countries (Nguyen *et al.*, 2010, 2016, 2020a; Bless *et al.*, 2015).

13.8 Future Directions for *F. gigantica* Research

While research interest in *F. gigantica* has increased over the past decade, our knowledge of affected populations, suitable control measures, and understanding of *F. gigantica* biology and epidemiology still lags significantly behind that of *F. hepatica*. This is largely due to the geographical distribution of these parasites throughout less developed countries and reflects a need for greater investment in research in human and animal health in these regions. In order to broaden our research focus to include these areas, large-scale human prevalence studies should be conducted to update the limited information available about the distribution and impacts of *F. gigantica*. Existing serological tests offer the best solution to gauge the extent of exposure across human populations, while molecular methods should be used for species confirmation in hospitalized patients. The identification of human endemic areas will help to inform mass drug administration programmes due to the precautions required where parasite burdens are high (WHO, 2006). The development of point-of-care diagnostic tests will aid in the rapid diagnosis of fasciolosis in both human patients and livestock by replacing highly laborious traditional microscopic methods and should help to determine the success of control programmes in these areas.

Alongside human and animal prevalence studies, control methods best suited for application by smallholder farmers should be progressed. Research has shown that there is limited uptake of anthelmintics against other parasites in low-input smallholder farm settings, and the same should be expected for *F. gigantica* (ACIAR, 2008; Rast *et al.*, 2014). The use of vaccines against other production-limiting diseases has proven successful with the aid of local government in collaboration with the World Health Organization and international investment and development programmes (Edwards, 2004; Roeder *et al.*, 2013; Nampanya *et al.*, 2018). Thus, the commercialization of a vaccine effective against both *F. hepatica* and *F. gigantica* is likely to be the best course of action for the control of fasciolosis in resource-limited settings. For this to have the greatest impact, increased awareness of both the human and animal health risks associated with these parasites should be communicated to local animal health workers and, ideally, smallholder farmers (Donadeu *et al.*, 2019).

Finally, continued globalization and a subsequent increase in the importation of animals from *F. hepatica*-endemic areas into Southeast Asia, Africa and the Middle East mean that the occurrence of hybridization and possible introgression events between *F. hepatica* and *F. gigantica* should be closely monitored (Calvani and Šlapeta, 2021). The biological, functional and epidemiological implications of these events remain unknown, yet may pose significant human and animal health risks. Empirical evidence should be generated to identify areas of the genome most likely to undergo introgression in order to select markers appropriate for widespread surveillance. New and emerging molecular methods, such as NGS, should be pursued to aid our understanding of parasite translocation events and the potential impacts on human and animal health and food security in affected areas.

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14 Global Impact of Human Fasciolosis

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14.1 Introduction

Fasciolosis, caused by parasites of the genus *Fasciola*, has afflicted humans since prehistoric times (Dittmar and Teegen, 2003). While primarily a disease of herbivorous mammals, notably domestic livestock such as cattle, sheep and goats, fasciolosis has a significant global health impact on humans. The World Health Organization (WHO) categorizes fasciolosis as a neglected tropical disease (WHO, 2017) and the US Food and Drug Administration (FDA) describes fasciolosis as a tropical disease for which development of new drugs and biological products for prevention and treatment is to be encouraged (FDA, 2020). Fasciolosis has been described as the most geographically widespread parasitic infection (Cabada and White, 2012; Nyindo and Lukambagire, 2015), with an estimated 180 million people at risk of infection (Cwiklinski *et al.*, 2020). As human infection occurs primarily by ingestion of contaminated food, fasciolosis is classified as a food-borne trematodiasis (Cwiklinski *et al.*, 2016). Human fasciolosis was historically regarded as a secondary zoonosis, but recently it has become apparent that its epidemiology is highly varied in different regions and that in some areas humans are the main reservoir of infection (Mas-Coma *et al.*, 2019).

14.2 Epidemiology

14.2.1 Geography

Human fasciolosis is a global disease, reported on all continents apart from Antarctica (Fig. 14.1), with over 70 countries affected (Fürst *et al.*, 2012; CDC, 2019). The prevalence and patterns of infection vary widely between countries and regions, due to a complex interplay of demographics, dietary habits, agricultural practices, sanitation systems, vector and animal host distribution, climate, landscape and parasite species (*F. hepatica* or *F. gigantica*). The most affected regions are the Andean Altiplano, particularly the area around Lake Titicaca in Bolivia and Peru, the Nile valley, the Caspian Sea basin, China and parts of south-east Asia. Fasciolosis is also common in some areas in sub-Saharan Africa (WHO, 2017). *Fasciola hepatica*, the more widespread of the two *Fasciola* species, is believed to be of European origin – it appears to have been present in Germany in approximately 3000 BCE (Dittmar and Teegen, 2003) – but has spread to all inhabited continents through global colonization by Europeans and the export of livestock both during and after the colonial period (Robinson and Dalton, 2009).

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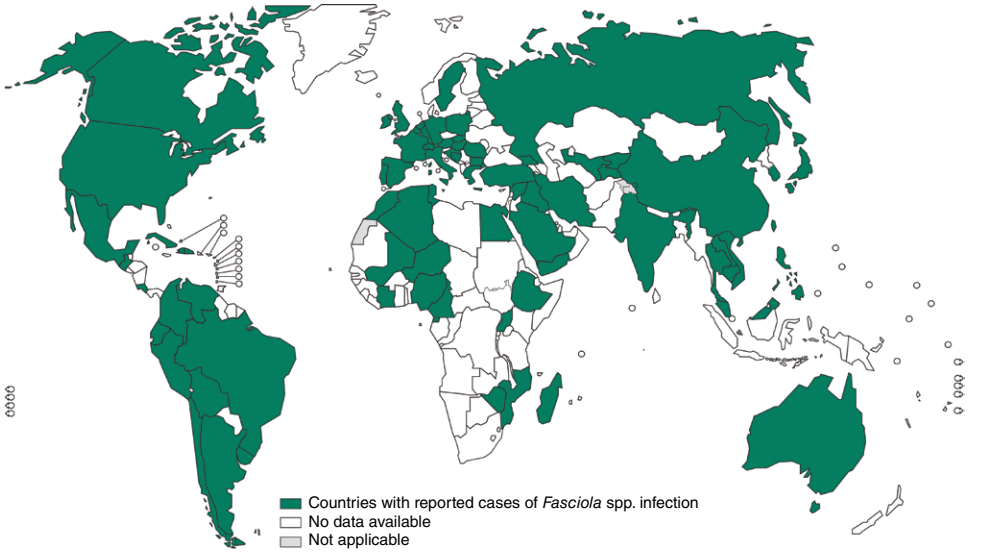

Fig. 14.1. Global distribution of human fasciolosis. Worldwide distribution of fasciolosis according to WHO, based on data for the latest year available (WHO, 2014).

14.2.2 Scale of infection

In 2005, there were an estimated 2.6 million cases of human fasciolosis globally; of these 1.3 million were in Latin America, 1.1 million in North Africa and the Middle East, approximately 144,000 in China and 4000 in Europe (Fürst *et al*., 2012). In North America, cases are rare and mainly observed in travellers returning from other countries (Fried and Abruzzi, 2010). In many areas, cases occur sporadically (for example, in many countries in Europe), but in some regions in developing countries a constant high prevalence of infection occurs, or epidemics may occur in regions in which infections are generally less common (Mas-Coma *et al*., 2018). It is difficult to assess the true incidence of fasciolosis and the published figures likely underestimate the number of cases.

The region with the highest prevalence of fasciolosis is the Andean Altiplano, particularly the region around Lake Titicaca, in Bolivia and Peru. In Bolivia, coprological screening (mainly in children) revealed prevalence in some areas to be over 70% in the 1980s and 1990s (Mas-Coma *et al*., 1999). Similar surveys in Peru found prevalence rates as high as 48% (González *et al*., 2011). Control programmes have subsequently greatly reduced prevalence in some of these areas (Mollinedo *et al*., 2019) (see section 14.5.1).

In Southeast Asia, Vietnam is recognized as an endemic country for human fasciolosis, although relatively little information from the country was published in international journals until recently (Bui *et al*., 2016). *F. gigantica* is the main species involved. Central areas have hyperendemic levels of infection: a study in one hospital in Hanoi found that 17% of patients seeking medical attention had *Fasciola* infection (Bui *et al*., 2016). A seroprevalence study in Ho Chi Minh City found an overall prevalence of 5.9%, with a higher infection risk in people aged > 60 years (Nguyen *et al*., 2016).

The Nile Delta in Egypt is another region where fasciolosis is very common, coprological surveys having shown prevalences of up to 19%, with a mean of 12% (Esteban *et al*., 2003). Another study found a prevalence of 6% (Curtale *et al*., 2005) and a review by Soliman (2008) reported prevalence ranging from 0.8% to 11% in different areas. Successful control programmes have been implemented in parts of Egypt (Curtale *et al*., 2005) (see section 14.5.1). Sporadic cases of human fasciolosis have been reported in many African countries, such as Cameroon, Chad, Senegal, South Africa and Zimbabwe, but despite widespread prevalence

of fasciolosis in livestock, and environmental conditions that appear to favour transmission to humans, there are few publications about human fasciolosis in sub-Saharan Africa (Nyindo and Lukambagire, 2015; Lukambagire *et al.*, 2018).

In other endemic regions, such as Iran (Yadegari *et al.*, 1999; Sarkari *et al.*, 2012; Ashrafi, 2015) and China (Qian *et al.*, 2019), the prevalence appears to be much lower, although outbreaks of disease occur periodically.

14.2.3 Climate, weather and seasonal effects

Human fasciolosis occurs across geographical areas that differ greatly in terms of climate. *Fasciola* species depend on fresh water in order to complete their life cycle, and rainfall, together with temperature, has a major impact on infection rates in many regions, related to the availability of lymnaeid snails as vectors (Mas-Coma *et al.*, 2019). In Europe, for example, infection is most likely in the spring and especially the autumn, when lymnaeid snails are most active, in response to patterns of rainfall (Mas-Coma *et al.*, 2018). In parts of South Asia, for example the Punjab province of Pakistan, transmission occurs primarily during the monsoon season (Afshan *et al.*, 2014). Year-round transmission may occur in areas where temperatures remain within the range suitable for larval development throughout the year, and where lymnaeid snails live in permanent water bodies. Examples include parts of Cambodia, Southern Europe and the Andean Altiplano (Mas-Coma *et al.*, 2018). Flooding and periods of heavy rain have been reported to be associated with large outbreaks of human fasciolosis (Ashton *et al.*, 1970; Millán *et al.*, 2000). Seasonal patterns of transmission in particular regions may be disrupted by human activities. For example, construction of dams and irrigation systems may lead to greater availability of water at times of year other than those that would be expected on the basis of rainfall patterns (Afshan *et al.*, 2014). Any effects of climate change on human fasciolosis remain to be determined but are likely to depend on changes to temperature and rainfall patterns in specific areas.

14.2.4 Populations at risk

Human fasciolosis is primarily a rural disease. The key determinant of infection risk is contact with freshwater bodies colonized by lymnaeid snails, as demonstrated in an epidemiological study in the Bolivian Altiplano where the prevalence of infection was found to be related to proximity to water sources harbouring lymnaeid snails (Mas-Coma *et al.*, 1999). As might be expected, areas where livestock graze have a higher risk of fasciolosis (Mas-Coma *et al.*, 2018). The range of hosts for *Fasciola* flukes is not restricted to domestic livestock: wild mammal hosts are also more likely to be present in rural than urban areas. Infections in urban settings are generally linked to contaminated vegetables transported to market, or are acquired when urban dwellers visit rural areas (Mas-Coma *et al.*, 2018). Even within the poor rural settings in which fasciolosis most commonly occurs, greater poverty is associated with a higher rate of infection (Cabada *et al.*, 2014, 2018).

Outbreaks of fasciolosis frequently occur within families, in all settings from hyperendemic areas (e.g. Peru: Marcos *et al.*, 2005) to those with sporadic cases (an example in Germany is reported in a German-language publication by Bechtel *et al.*, 1992), due to consumption of foods and water from common sources (Mas-Coma *et al.*, 2018). While infection can occur irrespective of sex and age (Esteban *et al.*, 2003), in hyperendemic areas such as the Nile Delta or the Andean Altiplano, school-age children are reported to be at heightened risk (Esteban *et al.*, 1997, 2003; Mas-Coma *et al.*, 1999; Lopez *et al.*, 2012). In patients in this age group, infection may lead to serious illness (Mas-Coma *et al.*, 2014). Fasciolosis has been reported in very young children: De *et al.* (2020) described five cases in children ranging from 10 months to 4 years old in Vietnam, and a review of the literature identified a further 38 reported cases in children 1–4 years of age. In hyperendemic areas, women and girls are more likely to be infected, or to have a higher intensity of infection (in terms of faecal ova counts) than men or boys (Esteban *et al.*, 2002, 2003). This may be due to the activities typically performed by women in these areas; for example, in Egypt, such activities include washing clothes and kitchen equipment in canals where metacercariae may be present, and the gathering and preparation of vegetables.

14.3 Pathology in the Human Host

14.3.1 Pathogens

The two *Fasciola* species, *F. hepatica* and *F gigantica*, both infect humans. The former has a worldwide distribution and the latter is primarily found in Asia and parts of Africa (Mas-Coma *et al*., 2018), although the two species overlap in some regions, for example the Nile Valley and Rift Valley in Africa (Nyindo *et al*., 2015). The two species have different lymnaeid snail species as vectors, with *F. hepatica* mainly transmitted via *Lymnaea*/*Galba* species, and *F. gigantica* by *Radix* species. The distribution of snail vector species is thought to be an important determinant of the distribution of infection (Mas-Coma *et al*., 2018). One lymnaeid species, *Pseudosuccinea columella*, an invasive species from North America that has been introduced into many countries worldwide (Bargues *et al*., 2011), can transmit both *Fasciola* species (Grabner *et al*., 2014; Dar *et al*., 2015). Hybrids of the two *Fasciola* species also occur and have been reported in several countries where both species are endemic, including Vietnam, Bangladesh, China, India, Iran, Japan, Korea, Myanmar, Nepal, Pakistan, Thailand and Egypt (Nguyen *et al*., 2018).

14.3.2 Mechanisms of human infection

Human infection with *Fasciola* species requires ingestion of the cercariae, or metacercariae, stages of the parasite. Metacercariae are typically found attached to aquatic plants, although they may also float freely in water (Fig. 14.2).

Routes of infection (Fig. 14.3) vary widely between countries and regions and have been reviewed extensively by Mas-Coma *et al.* (2018). The consumption of uncooked aquatic plants is the most common route. In many countries, watercress (*Nasturtium officinale*), collected from the wild in areas with livestock nearby, is the most common vehicle for human infection. This is certainly the most common pattern of infection in Europe (Facey and Marsden, 1960; Ashton *et al*., 1970; Hardman *et al*., 1970; Anonymous, 1988; Bechtel *et al*., 1992; Arjona *et al*., 1995; Sampaio Silva *et al*., 1996). Globally, however, a wide range of wild and cultivated aquatic and terrestrial plants have been reported to be involved in human fasciolosis. Cultivated crops may be contaminated with metacercariae due to flooding (Anonymous, 1988; Milas *et al*., 2020), suboptimal farming practices (Mailles *et al*., 2006) and irrigation or washing using water in which lymnaeid snails are present, with livestock nearby (Soliman, 2008). Some sources of infection are highly culturally specific. Examples include the chewing of khat (leaves of *Catha edulis*) in the horn of Africa (Cats *et al*., 2000; De Bree *et al*., 2013), or the consumption of alfalfa (*Medicago sativa*) juice in Peru and Mexico (Marcos, 2006; Zumaquero-Ríos *et al*., 2013). In both cases, irrigation practices can lead to contamination with *Fasciola* metacercariae. Table 14.1 provides a non-exhaustive list of plant species known or suspected to be involved in human fasciolosis.

While consumption of contaminated uncooked plants appears to be the most common route of infection, it is not the only one. As noted previously, drinking-water contaminated with unattached metacercariae is also associated with infection (Mas-Coma *et al*., 2018). Infection linked to contaminated water has been reported in Spain (Arjona *et al*., 1995), Bolivia (Mas-Coma *et al*., 2018), Argentina (Mera y Sierra *et al*., 2011), Egypt (Curtale *et al*., 2003), Ethiopia (Fentie *et al*., 2013) and Peru (Esteban *et al*., 2002; Cabada *et al*., 2014). On the western side of Lake Titicaca, in Peru, drinking contaminated water appears to be the main source of fasciolosis (Mas-Coma *et al*., 2018). While transmission via drinking-water appears to be a local phenomenon, and less common than transmission via raw vegetables (Mas-Coma *et al*., 2018), interventions to provide clean water can have a significant impact on the prevalence of fasciolosis (Esteban *et al*., 2003; Mas-Coma, 2004). In some hyperendemic areas, local customs may determine the main route of transmission.

The potential for human infection following consumption of raw or undercooked infected animal liver remains controversial (Mas-Coma *et al*., 2018). This has been demonstrated experimentally in animals but the relevance to humans is unclear. A syndrome known as halzoun, an acute allergic oedematous reaction involving the upper respiratory tract and nasopharyngeal mucosa, has been reported to occur following consumption of raw goat or sheep livers and

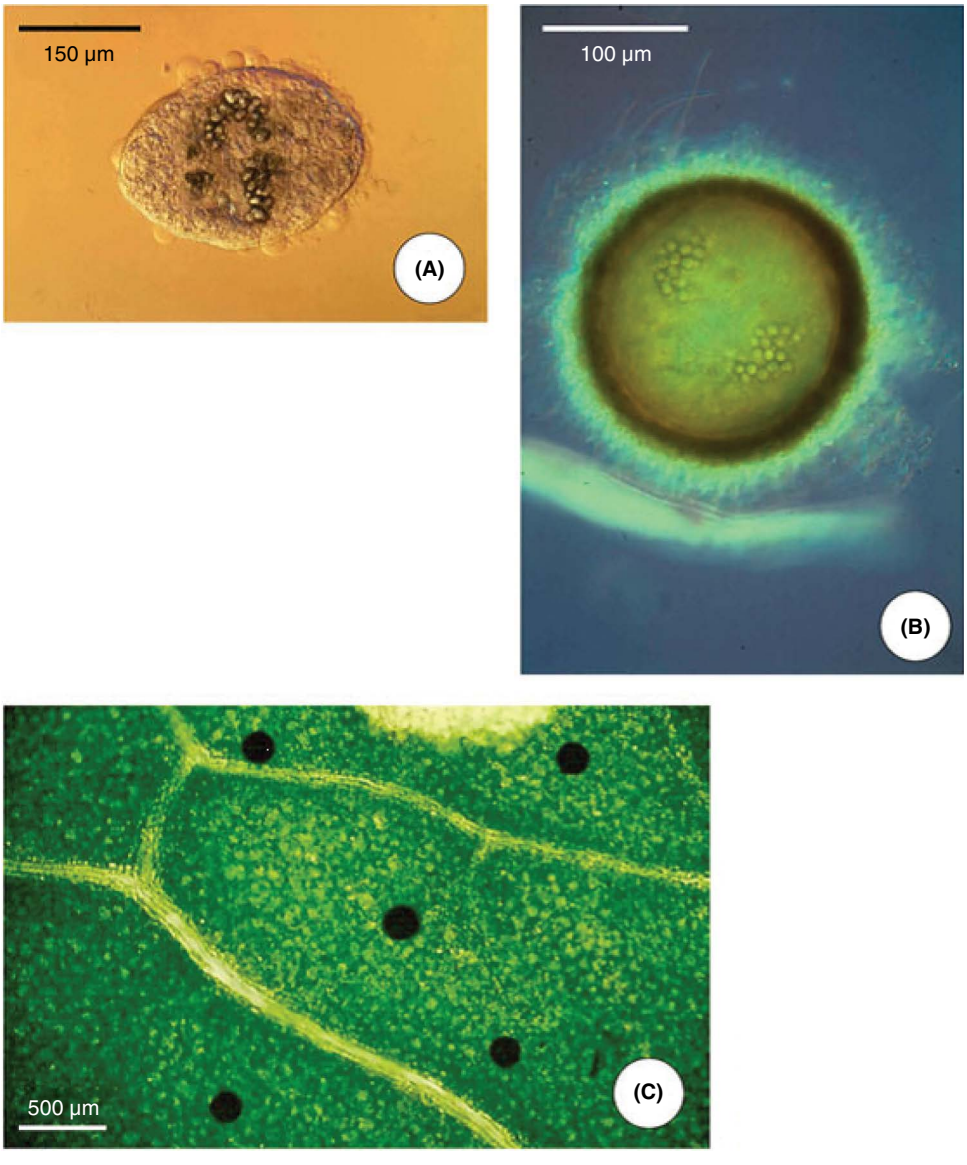

Fig. 14.2. *Fasciola hepatica* life cycle stages involved in the infection of humans. **(A)** Cercarial body beginning the encystment process. **(B)** Metacercarial attached cyst. **(C)** Metacercariae attached to a green plant leaf. After Mas-Coma *et al*. (2018)

there is some evidence to suggest that this may be due to attachment of adult *Fasciola* to the pharyngeal mucosa. However, the role of *Fasciola* in halzoun is controversial and this syndrome does not appear to have any relationship with other forms of fasciolosis (Mas-Coma *et al*., 2018).

14.3.3 Role of domestic livestock in transmission

The classic view of the *Fasciola* life cycle (Chen and Mott, 1990) includes domestic livestock, typically sheep or cattle, as the definitive hosts for the fluke, with human fasciolosis regarded as

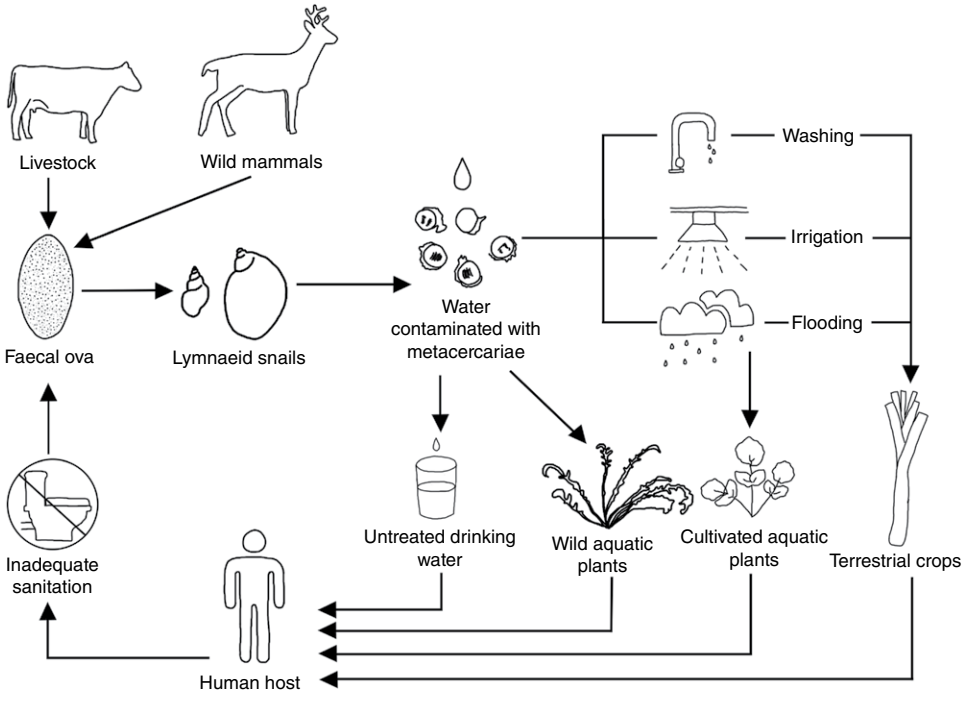

Fig. 14.3. Reported or potential routes of human infection with *Fasciola* species.

a secondary zoonosis. Only relatively recently has fasciolosis been categorized as an important parasitic disease in humans, as research over the past 30 years has identified the scale of the problem (Mas-Coma *et al*., 2019). The consensus that human fasciolosis is always linked to local livestock infection, with animals as the main reservoir of parasites, also no longer appears to be true in all settings (Mas-Coma *et al*., 1999). There seems to be no clear relationship between high local prevalence in livestock and the prevalence of human infection, and in some regions humans may contribute significantly to the transmission of infection. This occurs primarily in areas with a high prevalence of human infection and poor sanitation, where *Fasciola* ova in human faeces may come into contact with water bodies in which lymnaeid snails are present and from which food plants are collected (Mas-Coma *et al*., 2019).

While sheep and cattle have been regarded as the main livestock hosts for *Fasciola* species, several other species are known to be important, including pigs, donkeys (Mas-Coma *et al*., 1999), goats (Shafiei *et al*., 2014) and buffalo (Bui *et al*., 2016). Wild animals can also host *Fasciola* species but their importance in human infection remains unclear. Species that have been found to harbour *Fasciola* include deer (French *et al*., 2019; Matsuda *et al*., 2020), black rat (Mas-Coma *et al*., 1999) and in South America the semi-aquatic rodents, nutria or coypu (Gayo *et al*., 2011) and capybara (Dracz *et al*., 2016).

14.3.4 Clinical presentation

14.3.4.1 Hepatobiliary fasciolosis

Fasciolosis may be an infection of very long duration, with flukes being present in the human host for over 10 years in some cases (Marcos *et al*., 2008). Human infection can be broadly divided into four stages, although only two of these are usually encountered in clinical settings. The first stage is termed the incubation period. This is the period between ingestion of metacercariae and development of symptoms. During this period, larvae excyst from the metacercariae and migrate to the liver. Occasionally,

Table 14.1. Plant species known to be involved in transmission of fasciolosis to humans.

Plant species	Country or region	Notes	References
Aquatic plants			
Nasturtium officinale (watercress)	UK, France, Germany, Portugal, Spain, Iran, Turkey, Argentina, Mexico, Peru, Bolivia, Rwanda, Burundi, South Africa, Cuba, Dominican Republic, Hawaii	Mainly related to wild-collected plants, but some reports related to cultivated watercress, contaminated due to mismanagement of production	Facey and Marsden (1960); Ashton *et al*. (1970); Hardman *et al*. (1970); Anonymous (1988); Bechtel *et al.* (1992); Arjona *et al.* (1995); Mailles *et al*. (2006); Karahocagil *et al*. (2011); Mera y Sierra *et al*. (2011); Black *et al*. (2013); Zumaquero-Ríos *et al*. (2013); Cabada *et al.* (2016); Mas-Coma *et al*. (2018)
Nasturtium species (wild watercress)	Iran		Mas-Coma *et al*. (2018)
Rorippa species (wild watercress)	Mexico, Venezuela, Bolivia		Abdul-Hadi *et al*. (1996); Zumaquero-Ríos *et al*. (2013); Mas-Coma *et al*. (2018)
Erythranthe glabrata (monkey flower)	Bolivia		Mas-Coma *et al*. (2018)
Juncus species (rushes)	Bolivia		Mas-Coma *et al*. (2018)
Porphyra purpurea (chlorophyte alga)	Bolivia		Mas-Coma *et al*. (2018)
Nostoc species (cyanobacteria)	Bolivia		Mas-Coma *et al*. (2018)
Hydrocotyle ranunculoides (floating pennywort)	Bolivia		Mas-Coma *et al*. (2018)
Eleocharis species (hairgrass)	Bolivia		Mas-Coma *et al.* (2018)
Trapa species (water chestnuts)	China, Taiwan, Thailand, Bangladesh	Also known as water calthrop	Mas-Coma *et al*. (2018)
Eleocharis dulcis – formerly *E. tuberosa* (water chestnuts)	China	Formerly known as *E. tuberosa*	Mas-Coma *et al*. (2018)
Ipomoea aquatica (water morning glory)	Thailand	Also known as water spinach	Mas-Coma *et al*. (2018)
Nymphaea lotus (lotus)	Thailand		Mas-Coma *et al*. (2018)
Eichhornia crassipes (water hyacinth)	Thailand		Mas-Coma *et al*. (2018)
Neptunia oleracea (water mimosa)	Thailand		Mas-Coma *et al*. (2018)
Terrestrial plants			
Lactuca sativa (lettuce)	Egypt, Mexico, Peru	Contaminated by washing or irrigation	Soliman (2008); Zumaquero-Ríos *et al*. (2013); Cabada *et al*. (2014)

Continued

Table 14.1. Continued.

Plant species	Country or region	Notes	References
Eruca sativa (rocket, arugula)	Egypt	Contaminated by washing or irrigation	Soliman (2008)
Allium kurrat (Egyptian leek)	Egypt	Contaminated by washing or irrigation	Soliman (2008)
Falcaria vulgaris (sickleweed)	Iran		Mas-Coma *et al*. (2018)
Valerianella locusta (lamb's lettuce)	France	Related to flooding	Anonymous (1988)
Taraxacum officionale (dandelion)	France, Argentina		Anonymous (1988); Mera y Sierra *et al*. (2011)
Mentha spicata (spearmint)	North Africa (treated in France)		Anonymous (1988)
Mentha pulegium (pennyroyal)	Iran		Mas-Coma *et al*. (2018)
Mentha longifolia (horse mint)	Iran		Mas-Coma *et al*. (2018)
Mentha piperita (peppermint)	Iran		Mas-Coma *et al*. (2018)
Eryngium species	Iran		Mas-Coma *et al*. (2018)
Catha edulis (khat)	Horn of Africa, Netherlands	Cases in Netherlands from imported material	Cats *et al*. (2000); De Bree *et al*. (2013)
Medicago sativa (alfalfa)	Peru, Mexico	Infections result from consumption of plant or juice	Marcos (2006); Zumaquero-Ríos *et al*. (2013)
Spinacia oleracea (spinach)	Peru, Mexico	Contaminated by washing or irrigation	Zumaquero-Ríos *et al*. (2013); Cabada *et al*. (2014)
Petroselinum sativum (parsley)	Egypt	Contaminated by washing or irrigation	Mas-Coma *et al*. (2018)
Portulaca oleracea (purslane)	Egypt	Contaminated by washing or irrigation	Mas-Coma *et al*. (2018)
Raphanus sativus (radish)	Mexico	Contaminated by washing or irrigation	Zumaquero-Ríos *et al*. (2013)
Oryza species (rice)	Many countries	Wet rice fields provide a habitat for lymnaeid snails; if fertilized with animal manure, or livestock graze nearby, may become reservoirs of *Fasciola*. Infection may result from sucking or chewing on rice stems	Mas-Coma *et al*. (2018)

they migrate to other parts of the body and cause ectopic fasciolosis (see below). In the vast majority of infections, however, immature flukes invade the liver. The duration of the incubation period in humans is unclear, but appears, on the basis of patients' reporting of the time between ingestion of the likely sources of infection and the onset of symptoms, to be somewhere between a few days and 3 months or more (Mas-Coma *et al*., 2019).

Invasion of the liver by immature flukes signals the beginning of the acute phase of infection, sometimes referred to as the invasive or hepatic stage. During this phase, which typically

lasts for 2–4 months, the flukes penetrate the peritoneum and the liver capsule and tunnel through the parenchyma of the liver. The mechanical damage and inflammation caused by the migrating larvae lead to signs and symptoms of infection becoming apparent (Chen and Mott, 1990).

Fever, abdominal pain, gastrointestinal disturbances and urticaria are the most common acute-phase symptoms. Abdominal pain may be the only symptom and can range from mild to excruciating; the pain is often initially generalized but localizes to the right hypochondrium. Gastrointestinal disturbances typically include anorexia, flatulence, nausea and diarrhoea. Fever is often the first symptom and can follow a variety of patterns. Urticaria, potentially lasting for months, is frequent and may be accompanied by asthma. A dry cough may also occur at this stage. Patients with acute fasciolosis may have hepatomegaly and more rarely splenomegaly. Ascites also occurs in some patients. Anaemia may be present. Eosinophilia, often at very high levels, is a frequent laboratory manifestation.

The larval flukes then migrate into the biliary tract, where they mature into adults. During this process of maturation, referred to as the latent phase, infection may be asymptomatic. This phase may last for several months or even years (Chen and Mott, 1990). Asymptomatic cases may be detected by epidemiological screening for faecal ova.

The chronic, biliary, or obstructive phase occurs when, in response to the presence of adult *Fasciola*, the epithelium of the biliary system becomes inflamed and hyperplasia occurs. Cholangitis and cholecystitis may result and obstruction of the bile duct by flukes or gallstones can occur, potentially leading to severe symptoms. In chronic fasciolosis, the signs and symptoms are similar to those of cholangitis, cholelithiasis and cholecystitis resulting from other causes. Biliary colic (epigastric or right upper-quadrant abdominal pain) resulting from blockage of the bile duct by flukes or gallstones is common, together with intolerance of fatty food, nausea, pruritus and jaundice. Hepatomegaly, splenomegaly and ascites may occur. In endemic and hyperendemic areas, patients may suffer repeated infections and can present with an acute-phase infection superimposed on existing chronic fasciolosis.

14.3.4.2 Ectopic fasciolosis

While most cases of human fasciolosis affect the hepatobiliary system, *Fasciola* flukes occasionally invade other parts of the body, leading to so-called ectopic fasciolosis. A systematic review of ectopic fasciolosis has been published by Taghipour *et al.* (2019) and revealed that cases have been reported across a wide range of countries. Using rigorous criteria for reporting, 25 publications were identified; among these, the most common sites of infection were the abdomen (intestines), skin and subcutaneous tissues, the eye and brain. In most cases, *Fasciola* ova were not present in faeces, although many of the patients also appeared to have hepatobiliary infection. Ectopic infection may be preceded by symptoms of acute fasciolosis, but signs and symptoms vary widely, according to the site of infection; inflammation and fibrosis due to the lesions caused by the migrating flukes are common.

Mas-Coma *et al.* (2014) reviewed ocular and neurological fasciolosis and noted worldwide occurrence of cases, although with a greater proportion of reports from Europe rather than hyperendemic countries such as Bolivia, Peru and Egypt, which probably results from reporting issues related to the availability of healthcare services. Both species of *Fasciola* have been reported as causative organisms. In a small number of cases, intracranial fasciolosis has been reported, with signs and symptoms resembling those of strokes, tumours or meningitis, sometimes together with ocular symptoms. In some cases, live *Fasciola* could be observed within the eye. The authors note that a wide range of neurological signs and symptoms have also been reported in both hepatobiliary and ectopic fasciolosis, even without direct infection of the brain.

In the skin and subcutaneous tissues, abscesses and granulomatous lesions occur and *Fasciola* may be observed or recovered from the lesions; in some cases, flukes were also found in lymph nodes. In intestinal disease, lesions, sometimes granulomatous, are found in the intestinal wall and symptoms include abdominal pain, malaise, nausea and vomiting. Symptoms of pancreatic infection were similar and flukes or ova were found within lesions in the pancreas. Abdominal pain was also observed in infections of the peritoneum. Other sites reported to be

infected include the thoracic spine, manifested as symptoms of spinal cord compression, and the lung, with symptoms of a lung abscess, from which *Fasciola* ova were recovered (Taghipour *et al*., 2019).

14.3.5 Other impacts of infection

The symptoms of fasciolosis in both hepatobiliary disease and the rarer ectopic forms can be severe and distressing, and in rare cases fatalities have been reported (Millán *et al*., 2000; Mas-Coma *et al*., 2014). *Fasciola* infection may also have effects on the human host beyond the immediate symptomatic impact. Children in hyperendemic areas are at the highest risk of infection, and while fasciolosis in these patients may be asymptomatic in many cases, it can be associated with anaemia and malnutrition (Curtale *et al*., 1998; Webb and Cabada, 2018). Mild to moderate anaemia has been observed in approximately 50% of fasciolosis patients (Chen and Mott, 1990). In a review of fasciolosis in Egypt, Soliman (2008) noted that many children infected with *Fasciola* were severely anaemic, although this contrasted with the early study by Curtale *et al*. (1998), which found severe anaemia to be rare. Rodent models suggest that anaemia is correlated with the intensity of infection (Valero *et al*., 2008) and that reinfection of an already infected host, via effects on the host immune system, may be associated with the development of severe anaemia (Valero *et al*., 2017). In hyperendemic regions, secondary infection of patients who have chronic or biliary phase fasciolosis is thought to be common (Mas-Coma *et al*., 2019).

Fasciola flukes appear to be able to suppress the immune systems of both human and animal hosts, although this phenomenon is better characterized in the latter. In animals, suppression of Type 1 immune responses (protective pro-inflammatory responses involving cytotoxic T cells) appears to be involved in host tolerance of *Fasciola* spp. (Cwiklinski *et al*., 2016). One important aspect of immunomodulation by *Fasciola* spp. is that infected hosts do not appear to develop immune resistance to the parasite and re-infection of patients after or during the primary infection can occur, especially in hyperendemic areas (Mas-Coma *et al*., 2019). In addition, immune responses directed against concurrent or secondary infections involving other pathogens may be suppressed. Particularly in hyperendemic regions, many other pathogens may be present. For example, in the northern Bolivian Altiplano, common childhood illnesses include tuberculosis and whooping cough (pertussis) and children may be co-infected with a range of other parasites, including waterborne protozoans such as *Giardia lamblia*, and a range of helminths, in addition to *Fasciola* (Cwiklinski *et al*., 2016). The impact of immunosuppression resulting from fasciolosis on such children can be considerable, given their potential burden of other infections.

14.3.6 Diagnosis

The diagnosis of fasciolosis is challenging. Many clinical features of fasciolosis are non-specific, for example abdominal pain, nausea, anorexia, urticaria and eosinophilia, among others. Even when signs and symptoms suggest hepatobiliary disease, clinical features are not specific to fasciolosis. Diagnosis is especially difficult in countries where cases are rare and clinicians are unfamiliar with the disease, and more common conditions will generally be suspected (Micic *et al*., 2020). Even in hyperendemic areas, where physicians are aware of the possibility of fasciolosis, clinical features do not provide sufficient basis for definitive diagnosis. In combination with symptomatology, a number of diagnostic techniques can be used to identify fasciolosis with greater confidence. It should be noted that in endemic regions with constrained resources, access to imaging equipment and even immunodiagnostic kits may be limited.

Chronic fasciolosis is considered easier to diagnose than the acute stage of the disease, as the detection of *Fasciola* ova in faeces provides definitive evidence of infection. Faecal ova counts are of no value in identification of acute fasciolosis, and even in the chronic phase may be negative, as ova production is believed to be intermittent, may cease in older flukes and depends to some degree on the number of flukes present (Mas-Coma, 2019). Also, ova will not be detected unless faecal samples are specifically examined with a possible diagnosis of fasciolosis

or other parasitosis in mind. Other techniques are useful in the diagnosis of both chronic and acute forms of the disease.

Immunological techniques, whether serological or designed to detect coproantigens, can provide highly sensitive and specific detection of *Fasciola* infection, using either ELISA or lateral flow immunochromatographic techniques. A number of immunodiagnostic kits are commercially available, their development probably driven to some degree by the importance of *Fasciola* infections in livestock (Mas-Coma, 2019) (see Chapter 10, this volume).

Imaging may also be useful (Fig. 14.4), using ultrasound, computed tomography (CT) scanning, or magnetic resonance imaging (MRI). Ultrasonography may show focal hypoechoic or hyperechoic lesions in the acute phase of the disease, and in the chronic phase ductal ectasia and wall thickening may be seen. Occasionally, flukes can be visualized moving in the bile ducts or gallbladder. CT scanning can show a similar appearance, with small, round or oval, clustered hypodense lesions with peripheral contrast enhancement, or subcapsular, low-attenuation hepatic lesions. In chronic disease, dilated biliary

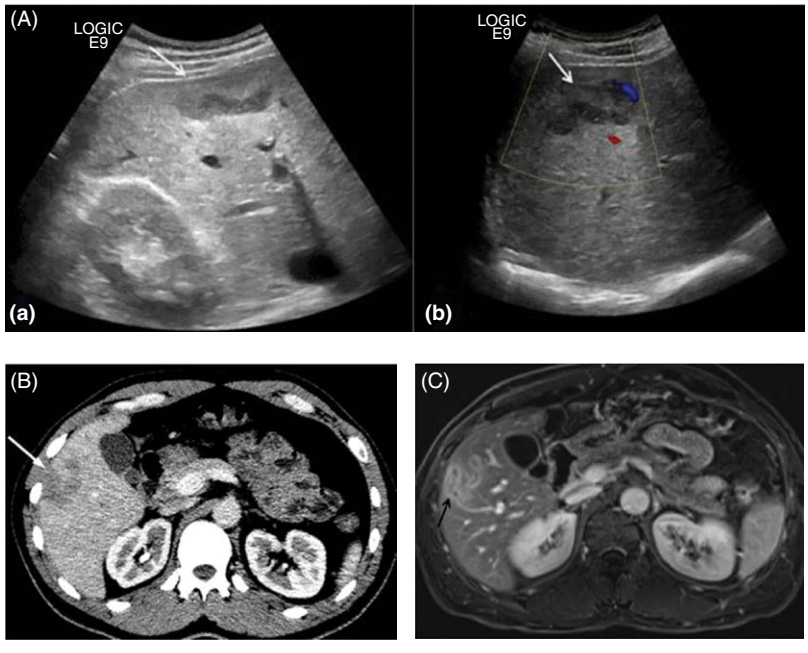

Fig. 14.4. Imaging techniques used in the diagnosis of human fasciolosis; different techniques applied to the same patient, a 39-year-old male with fasciolosis. **(A)** Technique: ultrasound performed on GE LOGIC E9 with 4C-RS transducer at a frequency of 4.0 MHz. Findings: (a) a sagittal grey scale ultrasound demonstrates a 4.3 cm ill-defined hypoechoic lesion in the periphery of the liver; (b) a sagittal Doppler ultrasound demonstrates the lesion without vascularity. **(B)** Technique: (Philips. Briliance 64) abdomen CT scan with intravenous injection of contrast material 95 ml Xenetix™ (iobitridol) (120 Kvp, 17 mAs, 3 mm slice thickness). Findings: an axial abdomen CT scan with intravenous injection of contrast material (portal venous phase) demonstrates a 4.2 × 3 cm ill-defined hypodense lesion (arrow) in the periphery of the liver with mild enhancement. Normal enhancing liver tissue is seen within the lesion. **(C)** Technique: (Siemens Magnetom Skyra 3 Tesla) MRI T1W fat suppressed axial with intravenous injection of contrast material 20 ml Dotarem™ (gadoteric acid) (TE = 1900 ms, TR = 3900 ms, 5.1 mm slice thickness). Findings: an axial fat suppressed T1 weighted image after contrast administration reveals clustered lesions with a maximum diameter of 2.7 cm and peripheral enhancement (arrow). From Preza, O., Klapa, I., Tsiakalos, A., Cokkinos, D.D. and Chatziioannou, A (2019) Fasciolosis: a challenging differential diagnosis for radiologists. *Journal of Radiology Case Reports* 13, 11–16. DOI: 10.3941/jrcr.v13i1.3451.

ducts with periportal tracking may be visualized and, rarely, flukes in the bile ducts. MRI, although less widely available, can also be used to visualize hepatic lesions, as can positron emission tomography, although this technology is seldom used (Preza *et al*., 2019). Imaging is considered to be more useful in the diagnosis of acute rather than chronic fasciolosis (Marcos *et al*., 2008).

While the diagnostic experience may vary between countries, depending on local medical practice and available facilities, case reports can be revealing as to the challenging process of diagnosis. A case series from Switzerland (Perrodin *et al*., 2019) illustrated the multiple investigations, in some cases over a period of years, that patients may undergo prior to the identification of fasciolosis. These most commonly involve multiple types of imaging, including in some cases endoscopic retrograde cholangiopancreatography. Because some of the clinical features of fasciolosis are also suggestive of hepatobiliary malignancies, some patients underwent unnecessary surgery.

Temido *et al*. (2017), describing a case of fasciolosis in Portugal, provided a good example detailing the diagnostic process for a single patient. A 47-year-old man sought medical attention due to abdominal pain that had been present for over a month, together with profuse night sweats, weakness, anorexia, diarrhoea and weight loss (over 30% of body weight). He had initially been treated with omeprazole, in the belief that he was suffering from dyspepsia. On admission, the patient was given metronidazole, as it was suspected that his diarrhoea was the result of infection. He underwent a physical examination, which was unremarkable except for slight tenderness over the right iliac fossa. Blood tests revealed eosinophilia, slight cholestasis and elevation of C reactive protein. He next underwent both upper GI endoscopy and colonoscopy, which showed no lesions. An abdominal CT scan followed, showing enlargement of the liver and spleen, and nodular hypodense liver lesions, with signs of portal hypertension. Abdominal doppler ultrasound did not confirm the latter. An MRI scan suggested that the nodular lesions were compatible with infected metastases from an unknown primary tumour. Biopsy of the liver lesions was then planned, using an ultrasound-guided technique. Imaging prior to biopsy showed that the lesions had diminished in size and some had disappeared, so the biopsy did not go ahead. The diminution of the lesions and improvement in diarrhoea, anorexia and night sweats were attributed to the metronidazole treatment. Serological testing for anti-*Fasciola* antibodies established the diagnosis of fasciolosis and the patient was successfully treated using a single dose of triclabendazole.

The case described above illustrates the degree of invasive testing that patients often undergo before a diagnosis can be established, and the serious differential diagnoses that must be considered (listed in this case as hepatic metastasis of unknown primary tumour and hepatocellular carcinoma). This lengthy, complex, invasive, and to many patients presumably frightening, process of diagnosis is in stark contrast to the simplicity of treatment.

Ectopic fasciolosis may pose even more of a diagnostic challenge, given the rarity of the condition, the very wide range of sites that may be infected and the resulting diversity of potential signs and symptoms. The diagnosis is generally established via a combination of serology, imaging and histopathology (Taghipour *et al*., 2019).

14.4 Treatment of Human Fasciolosis

14.4.1 The challenge of treating human fasciolosis

Human fasciolosis, in addition to being difficult to diagnose, was, until the beginning of the current millennium, challenging to treat. As discussed below, common anthelminthics do not appear to be reliably effective; some earlier treatments that had good efficacy were associated with significant safety issues, required lengthy treatment courses and are no longer commercially available. Attempts to repurpose other antimicrobial or antiprotozoal therapies have met with limited success. The treatment of fasciolosis in humans was transformed by development for human use of the veterinary fasciolicide triclabendazole; this is described in detail below. Triclabendazole is the only treatment for fasciolosis currently recommended by WHO and the Pan

American Health Organization (PAHO, 2020) and is the drug of choice recommended by the US Centers for Disease Control and Prevention (CDC, 2020).

14.4.2 Early treatments

While it is possible that herbal treatments may have been used to treat fasciolosis in ancient times (Harrison and Turfa, 2010), little is known about therapies prior to the 20th century. Early reports of human fasciolosis describe the use of a variety of different therapies. Facey and Marsden (1960) described an outbreak in Hampshire, UK, possibly related to watercress consumption. The treatment of six patients with chloroquine/hydroxychloroquine was described; all patients had relief of symptoms but not parasitological cure. The authors also reviewed the early literature (stretching back to 1936) on the treatment of human fasciolosis, including the establishment of emetine hydrochloride as an effective therapy (administered subcutaneously or intramuscularly, first used in 1932 and widely adopted by 1944). Hardman *et al.* (1970) described the use of emetine in the treatment of adult patients in another outbreak in the UK; 38 of 41 (93%) patients were cured after intramuscular administration of 30 mg/day for 18 days. While emetine was in use, and providing effective treatment, as late as 1999 in some countries (del Risco Barrios *et al.*, 2001), the potential cardiotoxicity of this compound, requiring inpatient treatment and ECG monitoring (Chen and Mott, 1990), led to its withdrawal from use; therefore, it is not currently commercially available in most countries.

Another treatment that was commonly used was bithionol. In another early case report from the UK, Ashton *et al.* (1970) described successful treatment of four patients with bithionol and noted that it had been reported as an effective treatment for fasciolosis in 1967. Bithionol gradually emerged as the treatment of choice for fasciolosis, mainly on the basis of case reports and case series rather than prospective studies, although some small uncontrolled studies did suggest good efficacy (Farag *et al.*, 1988; Bassiouny *et al.*, 1991). Bithionol required administration as two to three doses per day on alternate days for 10 or 15 days (Chen and Mott, 1990). The compound is no longer commercially available for therapeutic use and the most recent reports of its use in the treatment of fasciolosis was in Turkey in 2006 (Aksoy *et al.*, 2006).

14.4.3 Anthelminthics

Benzimidazole anthelminthics, such as albendazole and mebendazole, are commonly used to treat fasciolosis in livestock, but (although little studied) are considered to be ineffective in the treatment of human fasciolosis. While there are case reports of successful treatment (Dugernier *et al.*, 1986; Saba *et al.*, 2004), in general the small number of available publications suggests that these compounds are not effective (Markwalder *et al.*, 1988; Laird and Boray, 1992; Yilmaz *et al.*, 1998; Millán *et al.*, 2000; Cabada *et al.*, 2014; Fang *et al.*, 2014).

Praziquantel is a standard therapy for schistosomiasis and is used in numerous other parasitoses. It does not, however, appear to have consistent efficacy in human fasciolosis. Some case reports describe successful treatment (Holm and Kristofferson, 2002; Cosme *et al.*, 2003; Adachi *et al.*, 2005), but a number of case series and small experimental studies document treatment failure (Knobloch *et al.*, 1985; Farid *et al.*, 1986; Farid *et al.*, 1988, 1989; Patrick and Isaac-Renton, 1992; Laird and Boray, 1992; El-Karaksy *et al.*, 1999).

Mirazid, a patented extract of myrrh (resin extracted from the tree *Commiphora molmol*), is commercially available in Egypt and has been evaluated for the treatment of schistosomiasis and fasciolosis in both livestock and humans. While some studies have reported high cure rates in fasciolosis (Hegab and Hassan, 2003; Abo-Madyan *et al.*, 2004; Hassan *et al.*, 2004; Soliman *et al.*, 2004; Massoud *et al.*, 2010), another study found no effect (Osman *et al.*, 2010). Mirazid does not appear to have been investigated outside Egypt.

14.4.4 Repurposed antiprotozoal treatments

A number of compounds that are known to be active against protozoan parasites have been

investigated in the treatment of human fasciolosis. Metronidazole has activity against a range of anaerobic bacteria and protozoan parasites and is commonly used to treat giardiasis, amoebiasis and trichomoniasis. One report (Mansour-Ghanaei *et al*., 2003) described successful treatment of fasciolosis with metronidazole during a large outbreak in Iran in 1999, in patients who had apparently failed treatment with triclabendazole. In this open-labelled, uncontrolled study, a 3-week course of 1.5 g/day was used. Patients had to have faecal samples that were positive for *Fasciola* ova and serum that was positive for anti-*Fasciola* antibodies, at least 3 months after treatment with triclabendazole. Two months after metronidazole treatment, 76% (35/46) of patients were negative for faecal *Fasciola* ova and 67% (31/46) were also seronegative. Although this trial suggested that metronidazole may be effective, no further development appears to have been undertaken and treatment with this compound was reported to have failed in patients who were subsequently treated successfully with triclabendazole (Millán *et al*., 2000).

Nitazoxanide is used to treat giardiasis and cryptosporidiosis and has been studied in the treatment of fasciolosis. Trials suggest that nitazoxanide has some efficacy in human fasciolosis, although the response rate appears to be rather inconsistent. Rossignol *et al*. (1998), in a non-comparative study in Egypt, treated 137 patients, with fasciolosis confirmed by faecal ova counts, with a 6-day course of 500 mg nitazoxanide given twice daily. At Day 30, 113 patients (82.4%) were cured and treatment was reported to be well-tolerated. Favennec *et al*. (2003) described the only placebo-controlled study in human fasciolosis. In northern Peru, 100 patients with faecal samples positive for *Fasciola* ova were randomized to receive nitazoxanide (500 mg tablet in adults, 10 ml or 5 ml of 20 mg/ml suspension, according to age, in children) or matching placebo twice a day (b.i.d). The cure rate was 49% (32/65) for nitazoxanide and 6% (1/16) for placebo and treatment was well tolerated. In a further study, performed in Mexico (Zumaquero-Ríos *et al*., 2013), 50 children with chronic fasciolosis, in many cases co-infected with other parasites, were treated with nitazoxanide at 7.5 mg/kg b.i.d for 7 days. After a single course, 94% of patients had parasitological cure and the remaining patients were cured after a second course. Lukambagire *et al*. (2015) reported nitazoxanide treatment of 302 patients with fasciolosis (identified by faecal sample screening) in rural Tanzania. Of these, 36 patients were reported to have discontinued treatment due to apparent severe reactions to the drug (mainly abdominal pain, diarrhoea, pharyngitis and flu-like syndrome) and only 134 had post-treatment faecal ova counts (of these, 122 were cured and 12 continued to have *Fasciola* ova).

In a study by Ramadan *et al*. (2019), conducted in Egypt, patients with acute fasciolosis (diagnosed on the basis of signs and symptoms, eosinophilia, serology and imaging) were initially treated with triclabendazole (two doses of 10 mg/kg, 12 h apart). Patients who had not responded after 1 month were then treated with nitazoxanide (500 mg b.i.d. for 7 days). Non-responders to nitazoxanide were re-treated with triclabendazole. Response was defined as resolution of clinical symptoms and signs, normalization of eosinophil counts, and improvement of hepatic lesions on ultrasonography. Those patients who were treated with nitazoxanide were reassessed for response 1 month after treatment. Of 67 patients treated with triclabendazole, 37 were responders according to the study criteria. The remaining 30 patients received nitazoxanide and 11 (37%) of these were responders. The 19 patients who did not respond to nitazoxanide were re-treated with triclabendazole and all responded. The observation that the majority of patients who did not have a response to initial triclabendazole treatment subsequently responded to re-treatment suggests that the flukes were not resistant to triclabendazole, although in most cases they appeared not to be susceptible to nitazoxanide.

Artemisinin derivatives are highly effective and widely used in the treatment of malaria, usually as combination therapies. *In vitro* and in animal models, artemisinins exhibit fasciocidal activity, including against triclabendazole-resistant strains of *F. hepatica* (Keiser *et al*., 2007; Keiser and Morson, 2008). Natural infections in sheep have also been successfully treated with intramuscular and intravenous artesunate (Keiser *et al*., 2010). Artesunate and artemether have been investigated in the treatment of human fasciolosis, but efficacy was not optimal in two published studies. In a study of acute fasciolosis performed by Hien *et al*. (2008) in

Vietnam (discussed in more detail under clinical trials in section 14.4.5, below), a complete response to therapy at 3 months was reported in only 10% of patients treated with artesunate (4 mg/kg once daily for 10 days), despite 100% of patients having resolution of abdominal pain at 10 days (the primary endpoint). Keiser *et al.* (2011) reported two studies in chronic fasciolosis performed in Egypt. In one study, 20 patients received 6 × 80 mg artemether over 3 consecutive days, with a 28-day parasitological cure rate of 35%. In the second study, where 17 patients received 3 × 200 mg artemether within 24 h, the 28-day parasitological cure rate was 6%.

14.4.5 Triclabendazole

14.4.5.1 A repurposed veterinary fasciolicide

The ampholytic benzimidazole derivative triclabendazole (6-chloro-5-(2,3-dichlorophenoxy)-2-(methylthio)-1H-benzimidazole) has been used to treat fasciolosis in domestic livestock since 1983 (McCarthy and Moore, 2015). It was marketed for veterinary use as Fasinex®. When commercial production of bithionol ceased, development of triclabendazole for human use was initiated by CIBA (now Novartis) in collaboration with WHO.

Triclabendazole and its active sulfoxide and sulfone metabolites have a narrow spectrum of activity, restricted to *Fasciola* and *Paragonimus* species. The mechanism of action is unclear but may involve multiple targets, including tegumental disruption via inhibition of microtubule-based processes or adenylate cyclase activity (Gandhi *et al.*, 2019).

Clinical trials, case series and case reports have subsequently demonstrated that triclabendazole is highly effective and well tolerated in the treatment of all forms of fasciolosis, in both *F. hepatica* and *F. gigantica* infections, and in adults and children. Regulatory approvals for the use of triclabendazole in the treatment of human fasciolosis were obtained in Egypt (in 1997) and France (in 2002), and led to the WHO treatment recommendation of a 10 mg/kg single dose, followed, in case of treatment failure, by two 10 mg/kg doses 12–24 h apart (WHO, 2007). National guidelines for the detection of fasciolosis and treatment with triclabendazole were also developed in some endemic countries. In 2019, the US FDA approved triclabendazole for human use, at two doses of 10 mg/kg given 12 h apart (FDA, 2019). Following the initial regulatory approvals in Egypt and France, a donation programme for triclabendazole for the treatment of fasciolosis in endemic countries was established, under the guidance of the WHO's Department of Control of Neglected Tropical Diseases. Since 2006, the manufacturer has donated triclabendazole to WHO as treatment for fasciolosis patients (Access to Medicine Foundation, 2018), with approximately 4 million doses donated to date (Novartis, 2020).

14.4.5.2 Clinical trials with triclabendazole in human fasciolosis

The treatment of human fasciolosis with triclabendazole has been evaluated in 15 clinical trials involving over 900 patients (not including those in which triclabendazole was essentially used as rescue therapy or in a similar context, for example the studies by Keiser *et al.* (2011) and Ramadan *et al.* (2019), described earlier). The studies were performed in a range of different countries over a period of around 14 years and employed a variety of designs. For ethical reasons, no placebo-controlled studies were conducted. Most studies were in chronic fasciolosis, but patients with acute fasciolosis were included in two studies. Triclabendazole was found to be highly effective and well tolerated in all of the studies.

Between 1989 and 1992, a clinical trials programme in the treatment of human fasciolosis and paragonimiasis (lung fluke infection) was conducted by WHO in collaboration with CIBA. Six trials were performed in human fasciolosis (two in Bolivia, one each in Chile, Peru, Cuba and Iran) (Laburte *et al.*, 1998). All of the studies had open-label designs and were uncontrolled (no active control treatments were available and it was considered unethical to use a placebo control). Three of the studies assessed different triclabendazole dose regimens, and across the studies postprandial and fasting dosing were evaluated. All of the studies recruited only patients with chronic fasciolosis, as demonstrated by the presence of *Fasciola* spp. ova in faecal samples. Each of the studies used the same

efficacy endpoint: parasitological cure, based on absence of *Fasciola* spp. ova in faecal samples at Day 60. These studies established that a triclabendazole dose of 10 mg/kg (10 mg/kg single dose or two 5 mg/kg doses) was effective (with cure rates ranging from 14/20 (70%) to 22/22 (100%) (Table 14.2) and well tolerated in adult and paediatric patients. A total dose of 20 mg/kg (10 mg/kg doses given on Days 1 and 3) also showed very high cure rates and was well tolerated.

In addition, three studies in patients with fasciolosis were performed in Egypt in 1996 (Gandhi *et al*., 2019). Analysis and reporting were done by the investigators under the supervision of the Egyptian Ministry of Health. These were also uncontrolled studies, performed in patients with chronic fasciolosis who had faecal samples positive for *Fasciola* spp. ova at baseline. The triclabendazole doses used were 10 mg/kg, administered either as a single dose or as two 5 mg/kg doses given on the same day. Doses were given under postprandial conditions in two studies; in the other, the relationship of dosing to food intake was not reported. Across the studies, rates of cure (defined as absence of eggs in faeces at 60 days, Table 14.2) ranged from 24/30 (80%) to 24/25 (96%).

Subsequent studies, performed in a wide range of countries, have also been published. The efficacy results of these studies are summarized in Table 14.3.

Most of the published studies enrolled patients with chronic fasciolosis. Two studies, however, reported the treatment of patients with acute infection. One of these, conducted in Vietnam, is the only reported randomized trial comparing triclabendazole with another therapy (Hien *et al*., 2008). Patients with acute fasciolosis were identified on the basis of serology, imaging, clinical signs and symptoms, and eosinophilia; faecal ova counts were not used for either diagnosis or efficacy assessment. Patients were randomized, on an open-label basis, to receive either triclabendazole (two doses of 10 mg/kg, 12 h apart) or artesunate (4 mg/kg once daily for 10 days). The primary efficacy parameter was resolution of abdominal pain at Day 10, and the secondary endpoint was complete response at 3 months (defined as resolution of symptoms,

Table 14.2. Cure rates by dose regimen in studies conducted by WHO/CIBA and Egyptian government.

Study location/date	Triclabendazole dose regimen	Cure rate[a] n/N (%)
CIBA/WHO studies		
Bolivia (paediatric) 1991–1992	5 mg/kg single dose postprandial	10/20 (50%)
	10 mg/kg single dose postprandial	17/20 (85%)
	2 × 5 mg/kg postprandial, 1 day	20/20 (100%)
Iran 1989–1991	5 mg/kg daily for 3 days fasting	16/17 (94%)
	10 mg/kg single dose postprandial	14/20 (70%)
	10 mg/kg single dose fasting	13/17 (76%)
	2 × 5 mg/kg 1 day fasting	9/14 (64%)
Bolivia 1990–1992	10 mg/kg single dose postprandial	22/22 (100%)
Cuba 1990–1992	10 mg/kg single dose fasting	12/14 (86%)
Chile 1990–1992	10 mg/kg single dose fasting	19/24 (79%)
Peru 1991	10 mg/kg fasting on Day 1, then 10 mg/kg postprandial Day 3	9/10 (90%)
	10 mg/kg postprandial on Day 1, then 10 mg/kg fasting Day 3	12/12 (100%)
Egyptian government studies		
Egypt	10 mg/kg single dose postprandial	23/25 (92%)
	2 × 5 mg/kg postprandial, 1 day (6 hours apart)	24/25 (96%)
Egypt	10 mg/kg single dose[b]	24/30 (80%)
	2 × 5 mg/kg, 1 day[b]	26/30 (87%)
Egypt	10 mg/kg single dose postprandial	22/25 (88%)

[a]Cure rate: percentage of patients with absence of eggs in faeces (duodenal drainage in the paediatric study in Bolivia) at 60 days after start of treatment (90 days in Study B2212). [b]Relationship to food not specified. After Gandhi *et al*. (2019).

Table 14.3. Efficacy outcomes in clinical studies with triclabendazole in human fasciolosis.

Study	Design	Disease stage[a]	Location/date/population	Triclabendazole regimen	Efficacy endpoint	Efficacy outcome
Maco *et al*., 2015	R, OL, PG	Chronic	Peru/2001–2006/Children 2–16 years	Two 7.5 mg/kg doses 12 hours apart	Cure: faecal ova clearance at 60 days	44/44 (100%)
				10 mg/kg single dose		38/40 (95%)
Millán *et al*., 2000	OL, NC	Chronic	Cuba/1996/Adults & adolescents 15–81 years	Two 10 mg/kg doses 12 hours apart	Cure: faecal ova clearance at 60 days	71/77 (92%)
El-Morshedy*et al*., 1999	R, OL, PG	Chronic	Egypt/NS/adults and children 2–60 years	10 mg/kg single dose	Cure: faecal ova clearance at 35 days	54/68 (79.4%)
				Two 10 mg/kg doses 1 day apart		62/66 (93.9%)
Villegas *et al*., 2012	OL, NC	Chronic[b]	Bolivia/2008/children 5–14 years	10 mg/kg single dose	Cure: faecal ova clearance at 3 months	70/90 (77.8%)
				Two 10 mg/kg doses 3 months apart[c]	Cure: faecal ova clearance at 2 months	18/20 (90.0%)
Talaie *et al*., 2004[d]	R, OL, PG	Chronic	Iran/NS/adults and children 10–65 years	10 mg/kg single dose	Cure: faecal ova clearance at 60 days	7/7 (100%)
				Two 10 mg/kg doses 1 day apart		9/9 (100%)
				Three 10 mg/kg doses 1 day apart		9/9 (100%)[e]
		Acute		10 mg/kg single dose	Cure: absence of faecal ova, decrease or disappearance of signs/ symptoms, and anti-*Fasciola* IgG levels ≤ 128 at 60 days	23/36 (63.9%)
				Two 10 mg/kg doses 1 day apart		24/35 (68.6%)
				Three 10 mg/kg doses 1 day apart		23/36 (63.9%)

Continued

Table 14.3. Continued.

Study	Design	Disease stage[a]	Location/date/population	Triclabendazole regimen	Efficacy endpoint	Efficacy outcome
Hien *et al*., 2008	R, AC, OL	Acute	Vietnam/2004–2005/ adults & children 9–65 years	Two 10 mg/kg doses 12 hours apart	Primary: resolution of abdominal pain at Day 10Secondary: complete response at 3 months[f]	Primary: 44/50 (88%)* Secondary: 18/50 (36%)**
				Control group: artesunate 4 mg/kg once daily for 10 days		Primary: 50/50 (100%) Secondary: 5/50 (10%)

[a]Chronic fasciolosis: patients were faecal egg positive at baseline. Acute fasciolosis: faecal egg negative but with characteristic signs/symptoms/laboratory data/imaging.
[b]Patients identified as faecal egg positive in a community screening programme.
[c]Patients who were egg-positive at 3 months were re-treated with a second 10 mg/kg dose; cure rate 2 months after second dose.
[d]Study included patients who were faecal egg positive ('cases', i.e. chronic infection) or faecal egg negative but with characteristic signs or symptoms and laboratory data ('suggestive cases', i.e. acute infection).
[e]Includes two patients who were egg-positive at Days 14 and 30; these patients received a further 10 mg/kg dose of triclabendazole on Day 30, and were egg-free at Day 60.
[f]Complete response defined as resolution of symptoms, normalization of eosinophilia, and improvement in ultrasound appearance.
*$P < 0.05$ vs control group,
**$P < 0.01$ vs control group
AC, active controlled; NC, non-comparative; NS, not specified; OL, open-label; PG, parallel group; R, randomized

normalization of eosinophilia and improvement in ultrasound appearance). The individual components of complete response were also summarized separately at 3 months.

At Day 10, the proportion of patients with resolution of abdominal pain was higher with artesunate than triclabendazole (50/50 (100%) vs 44/50 (88%), $P < 0.05$). However, at 3 months the complete response rate (18/50, 36% vs 5/50, 10%, $P < 0.01$) and the rate of resolution of eosinophilia (21/50 (42%) vs 8/50 (16%), $P < 0.01$) were significantly higher in the triclabendazole group. The rate of resolution of symptoms was also higher with triclabendazole (46/50 (92%) vs 38/50 (76%), $P = 0.05$) and rates of improvement of ultrasound appearance were similar in the two groups (35/50 (70%) and 33/50 (66%)). The authors considered it likely that the low rate of complete response was due to the low rate of resolution of eosinophilia. High rates of co-infection with other parasites in the study population could have led to eosinophilia being maintained in some patients despite resolution of fasciolosis.

Acute fasciolosis was also assessed in a study by Talaie *et al.* (2004), although patients with chronic fasciolosis were also included in this randomized open-label trial in Iran, in which one, two and three daily 10 mg/kg doses of triclabendazole were compared. Those patients with acute disease, defined on the basis of symptoms, serology and eosinophilia in the absence of faecal ova, were termed 'suggestive cases' and those with chronic disease (with faecal ova) were termed 'cases'. For acute disease, cure was defined as absence of faecal *Fasciola* spp. ova, decrease or complete disappearance of clinical signs and symptoms, and IgG levels ≤ 128. Cure rates at 60 days were 23/36 (63.9%) for one dose, 24/35 (68.6%) for two doses and 23/36 (63.9%) for three doses. As symptom resolution was reported in almost all patients, these relatively low cure rates appear to be due to the proportions of patients with IgG levels remaining > 128 at Day 60. For patients with chronic disease, cure rates (absence of faecal ova) at Day 60 were 100% for all doses.

One clinical study reported in the literature (Villegas *et al.*, 2012) is of particular interest with respect to potential control measures for human fasciolosis in highly endemic areas. The endemicity of fasciolosis in the Bolivian northern Altiplano region is very high, particularly in children (Parkinson *et al.*, 2007). In 2008, the Ministry of Health in Bolivia, after consultation with experts from WHO, decided to implement a control programme for fasciolosis by mass administration of triclabendazole, without individual diagnosis. Villegas *et al.* (2012) conducted a pilot study performed prior to implementation of this policy. Asymptomatic schoolchildren (5–14 years of age) were tested for *Fasciola* spp. carriage by faecal ova counts and those with positive tests were treated with triclabendazole, given as a single 10 mg/kg dose. Those children with 300 or more ova per gram were hospitalized for treatment; those with a lower parasite burden were treated as outpatients. A total of 90 children were identified as infected and were treated. Three months post-treatment, 70/90 (77.8%) patients had parasitological cure; the remaining 20 patients received a second dose of triclabendazole and 18/20 were cured 2 months later. The parasitological cure rate from baseline was therefore 88/90 (97.8%).

14.4.5.3 Case series and case reports with triclabendazole

In addition to the studies described above, numerous case series and case reports describing the use of triclabendazole in the treatment of human fasciolosis have been published and successful treatment of all forms and stages of fasciolosis has been described (Gandhi *et al.*, 2019), including in children as young as 10 months (De *et al.*, 2020). Case series hail from a very wide range of geographical areas, notably from Turkey (for example, case series by Saba *et al.*, 2004; Koç *et al.*, 2009; Karahocagil *et al.*, 2011; Kaya *et al.*, 2011; Ulger *et al.*, 2014; Taş Cengiz *et al.*, 2015; Boşnak *et al.*, 2016) and Egypt, with case series reported by El-Karaksy *et al.* (1999); Shehab *et al.*, (1999); Tawfeek and Hussein (2000); Rehim *et al.* (2003); Curtale *et al.* (2005); Osman *et al.* (2011) and Mekky *et al.* (2015). Case series from Latin America have also been described, including from Peru (Marcos *et al.*, 2008), Argentina (Mera y Sierra *et al.*, 2011), Brazil (Luz *et al.*, 1999) and Chile (Apt *et al.*, 1995). One case series from China is of particular interest, as it describes an outbreak of *F. gigantica* infection in Yunnan province; most other reports either do not identify flukes to species level, or describe

treatment of *F. hepatica*. Triclabendazole treatment of *F. gigantica* infection was highly effective in this series (Chen *et al.*, 2013).

In addition to the acute and chronic stages of infection (as described in the studies and case series discussed previously), case reports describe triclabendazole treatment of asymptomatic chronic fasciolosis (Apt *et al.*, 1995; Incani *et al.*, 2003; Dauchy *et al.*, 2006) and ectopic fasciolosis (Dauchy *et al.*, 2006; Marcos *et al.*, 2009; Tanir *et al.*, 2011; Musa *et al.*, 2013; De *et al.*, 2020).

14.4.5.4 Safety and tolerability

Triclabendazole is generally well tolerated in the treatment of human fasciolosis. The most common adverse events (AEs) associated with treatment are probably related to the expulsion of dead or dying flukes from the hepatobiliary system. Characterized as biliary colic, such AEs include abdominal, hypochondrial and epigastric pain, often accompanied by sweating, in some cases with associated obstructive jaundice and elevations in serum levels of hepatic enzymes, most commonly alkaline phosphatase (el-Morshedy *et al.*, 1999; Millán *et al.*, 2000; Talaie *et al.*, 2004; Keiser *et al.*, 2011; Villegas *et al.*, 2012; Maco *et al.*, 2015). These events tend to occur 3–7 days post-therapy, with elevations of hepatic enzymes, where present, generally occurring around 7 days post-treatment. This indicates that parasite expulsion is the most likely cause, rather than a direct relationship to triclabendazole. Triclabendazole C_{max} occurs 4–10 h post-ingestion, whereas expulsion of parasites typically happens 48 h after dosing (Millán *et al.*, 2000). Also commonly reported during the studies were urticaria, pruritus and rash, which may be related to fasciolosis as they commonly occur in infected patients. Other relatively common AEs were headache, dizziness and vertigo. These AEs are noted in the US prescribing information (FDA, 2019), together with warnings of potential drug interactions with compounds metabolized by cytochrome P450 2C19, and of potential QT interval prolongation.

14.4.6 Drug resistance in human fasciolosis

Currently, triclabendazole is the only available effective treatment for human fasciolosis. Given that triclabendazole has been in widespread veterinary use for several decades, it is perhaps unsurprising that resistance has been widely reported in fasciolosis affecting livestock. Drug resistance in *Fasciola* species is explored in detail in Chapter 7, this volume. Fairweather *et al.* (2020) reviewed drug resistance in liver flukes, including *Fasciola* species. Triclabendazole resistance was first identified in *F. hepatica* in sheep in Australia in 1995 and since then has been reported in Europe, South America and Africa. The mechanism of resistance is unclear, but may involve altered binding to β-tubulin, or changes in drug uptake or metabolism. Resistance to other anthelminthics used in livestock but not human fasciolosis, such as albendazole, clorsulon and closantel, has also been reported.

The situation in humans is less clear. It appears that in most cases where initial treatment is unsuccessful, repeat treatment with higher doses or longer courses of treatment with triclabendazole clears the infection. A few cases in which patients failed to respond to multiple treatment courses with recommended doses of triclabendazole have been reported (Winkelhagen *et al.*, 2012; Gil *et al.*, 2014; Gülhan *et al.*, 2015; Cabada *et al.*, 2016; Ramadan *et al.*, 2019; Branco *et al.*, 2020). The chronology with regard to evolution of symptoms, treatment details and evaluation of response to treatment suggest that some of these cases are reinfections rather than treatment failures, but treatment failure due to resistance could not be ruled out. Also, assessment of efficacy in acute fasciolosis may be difficult (as seen in the efficacy study by Hien *et al.*, 2008; see section 14.4.5). For example, in one study (Ramadan *et al.*, 2019) (see section 14.4.4), patients with acute fasciolosis who did not initially respond to triclabendazole were treated with nitazoxanide. A minority of the patients responded to nitazoxanide, while the remainder were retreated with triclabendazole and showed complete resolution of clinical symptoms, laboratory parameters and imaging. On the basis of this response on re-treatment, it is unlikely that the *Fasciola* flukes involved were resistant to triclabendazole. In another case report of acute fasciolosis (Gulhan *et al.*, 2015), a partial hepatectomy was performed in a child in whom triclabendazole treatment had apparently failed, and the remains of a dead *F. hepatica* were found within a necrotic tunnel in the excised

portion of the liver. This suggests that while the patient continued to be symptomatic, the drug had been effective in killing the parasite.

In summary, although resistance to triclabendazole in livestock infections is well established, to date it appears to be rare and sporadic in human fasciolosis. In most reported cases, the possibility of resistance is hard to assess, due to the potential for reinfection and the difficulty of assessing response to treatment in acute disease. However, as triclabendazole is currently the only reliably effective treatment for human fasciolosis, the development of widespread resistance, which has not occurred to date, would clearly be of great concern.

14.5 Future Prospects

14.5.1 Control of fasciolosis

Fasciolosis, as a zoonotic disease with a significant impact on both human and animal health, is a good example of a global issue requiring a 'One Health' approach, in which multiple sectors communicate and work together to achieve better public health outcomes (Cwiklinski *et al.*, 2016). Measures to control fasciolosis vary between countries and regions, driven by endemicity and patterns of infection. WHO (2007) recommended that, where sporadic cases occur, treatment of infected individuals by local hospitals is sufficient and that triclabendazole should be made available in local health centres to ensure that treatment is widely accessible. In communities where clusters of infection occur, WHO suggested that large-scale distribution of triclabendazole treatment, and potentially preventive chemotherapy, should be considered.

As noted in section 14.4.5, in 2008 the Ministry of Health in Bolivia initiated a mass drug administration (MDA) programme in the Altiplano region bordering Lake Titicaca, a hyperendemic area. A pilot study by Villegas *et al.* (2012) demonstrated that treating school-age children (the most at-risk group) with triclabendazole was effective and well tolerated, and that at baseline 21.7% of the children surveyed were asymptomatically infected with *F. hepatica*. Mollinedo *et al.* (2018) compared the prevalence of *Fasciola* spp. (as determined via ova in faecal samples) in four regions in the Altiplano in 1999 (prior to the MDA programme) and in 2017 (after 9 years of the programme). Under the MDA programme, a single dose of 250 mg triclabendazole was administered annually to all residents aged 5–65 years (although no administration took place in 2015); the proportions of the eligible population treated ranged from 55% to 94%. In 2016, 516,000 people were treated across four hyperendemic regions. Across the four regions, decreases in ova carriage prevalence were striking: the largest decrease observed (in Huaculiani/Tiahuanaco) was from 26.9% to 0.7%. Other decreases were from 12.6% to 1.05% (Batalias), from 2.7% to 0.6% (Pucarani) and from 1.3% to zero (Viacha). No intervention programmes were in place over this period to treat domestic livestock in these areas for fasciolosis, or to control the intermediate hosts (freshwater snails), and the authors concluded that the MDA initiative was responsible for the observed near-eradication of fasciolosis.

Another programme of fasciolosis control was undertaken in Egypt, beginning in 2000 (Curtale *et al.*, 2005). In Behera governorate in the Nile Delta, faecal sampling of children in schools in villages and towns was used to identify areas of high prevalence. In such areas, all children in primary schools (the age group previously identified as at greatest risk of infection) were screened using faecal ova counts and those who were positive for *Fasciola* species were treated with triclabendazole. The prevalence of *Fasciola* species declined from pre-intervention levels; across five schools within three endemic foci, 18 months after screening and treatment of infected children, the total prevalence had decreased from 6.3% (95% CI 5.5, 7.3%) to 1.9% (95% CI 1.5, 2.4%), a statistically significant decrease ($P < 0.001$). In addition, among the children found to be infected, the intensity of infection (in terms of the numbers of *Fasciola* spp. ova per gram of faeces) was also found to have decreased relative to pre-intervention levels. The authors also assessed the costs and feasibility of their approach, noting that the local health infrastructure was capable of delivering the programme and that the selective treatment approach used was more affordable than using an MDA strategy (at the time of publication, cost estimates were US$0.45 and US$1.26, respectively).

Control programmes using either MDA (Mollinedo *et al*., 2018) or targeted intervention (Curtale *et al*., 2005) are clearly effective in themselves. However, other anthelminthic control programmes (particularly those for schistosomiasis), including those using MDA, have identified some issues with such measures. An MDA programme targeting schistosomiasis in Kenya found that 4 years of annual mass administration of praziquantel reduced the prevalence and infection intensity of *Schistosoma mansoni* (Abudho *et al*., 2018). However, within areas undergoing schistosomiasis control programmes, locations occur where the prevalence of disease does not decrease significantly, known as persistent hotspots; multiple, and sometimes unknown, factors are responsible (Kittur *et al*., 2020). In addition, compliance with treatment may not be optimal; Inobaya *et al*. (2018) found a non-compliance rate of 27% with praziquantel in an MDA schistosomiasis control programme in the Philippines. Reinfection rates after successful treatment can also be high: for one school included in the Kenyan MDA programme no decrease in infection rates was observed after 4 years, which was suggested by the authors to be due to frequent reinfection (Lelo *et al*., 2014).

In view of these various challenges, it has been suggested that other strategies should be used to complement MDA in order to achieve optimal and sustained disease control (Ross *et al*., 2013; Tchuem Tchuenté *et al*., 2017; Mollinedo *et al*., 2018). In addition to maintaining and improving supplies of effective drugs, measures that could be taken using this approach include provision of sanitation and clean drinking-water, and targeted snail control (Tchuem Tchuenté *et al*., 2017). Such measures would be as applicable to fasciolosis as to schistosomiasis. In addition, control of livestock infections could be of importance in reducing the impact of fasciolosis in human populations (Mollinedo *et al*., 2018). While appropriate livestock management could have several beneficial effects on human infection with *Fasciola* species, one specific benefit, where treatment options are essentially limited to triclabendazole, may be the containment of resistance (Fairweather, 2020).

Rinaldi *et al*. (2012) described a proposed project using a 'One Health' approach intended to control fasciolosis in the Cajamarca valley, a hyperendemic area in Peru. Set up in 2011, the project uses an integrated approach to controlling infection in both people and livestock, with epidemiological monitoring in both populations and with surveillance and treatment of infections. A range of measures aimed at controlling infection in livestock was also included, for example animal husbandry control measures, and geospatial techniques to predict foci of infection.

14.5.2 Potential new therapies for human fasciolosis

While triclabendazole remains the only current therapy with reliably high efficacy in the treatment of human fasciolosis, research efforts continue to identify other compounds, either new or repurposed, that might be potential therapies. While attempts to repurpose other anti-parasitic compounds have met with little success to date (see section 14.4), a wide range of compounds remains to be explored. One interesting approach to looking for new therapies was the 'Pathogen Box', a project that was administered by Medicines for Malaria Venture (MMV) in collaboration with WHO. This project provided researchers around the world with free access to around 400 compounds with activity against a range of parasitic organisms. The intention was to use innovative ways to develop medicines and other health technologies for diseases that disproportionately affect developing countries and where market failures have led to gaps in research and development.

Machicado *et al*. (2019) used the Pathogen Box to search for compounds with anti-*Fasciola* activity, initially against metacercariae, then against adult flukes. Compounds were selected on the basis of their predicted non-toxic properties and having IC_{50} values for adult flukes of $< 10\ \mu M$. Using this approach, three compounds were identified as potential candidates for further development. Other approaches to identifying potential new therapies include searching for small molecules that inhibit a variety of *Fasciola* enzymes (Ferraro *et al*., 2020a); candidate molecules have been identified using this approach (Ferraro *et al*., 2020b).

In livestock fasciolosis, attempts to develop vaccines are in progress (Toet *et al*., 2014; Maggioli *et al*., 2020) (see Chapter 12, this

volume). While it is unlikely that human vaccination would be practical, even assuming a successful vaccine is developed in the veterinary setting, vaccination of livestock could have a beneficial effect on the prevalence of human infection, given the close interrelationship between livestock and human fasciolosis in many endemic areas.

14.5.3 Future possibilities

Human fasciolosis was poorly recognized and little studied in the past, but in the past 30 years awareness of the condition and its significance as a global health problem has grown tremendously. Increased research efforts in many countries, and the designation by WHO of fasciolosis as a neglected tropical disease, have transformed the understanding of the disease and raised its profile. The development of triclabendazole as a safe and effective treatment, coupled with programmes to make it available where needed, have transformed the outlook for patients.

The natural history of fasciolosis, with a complex life cycle involving multiple hosts and requiring specific environmental conditions, provides a unique mix of potential opportunities to control the disease. While new therapies and veterinary vaccines may be developed in future, perhaps through public–private partnerships, control programmes using single measures, for example the triclabendazole MDA programme in Bolivia (Mollinedo *et al.*, 2018), have shown that large reductions in prevalence are possible in hyperendemic areas with available resources. Making such efforts part of a One Health approach, which could potentially also include measures ranging from education, through improving sanitation and control of snail vectors, through to changes in livestock husbandry, could yield even greater dividends in terms of reducing the global burden of fasciolosis.

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FASCIOLOSIS

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Fasciolosis is a major global infection of livestock causing both huge losses to the agricultural community and affecting human health as a food-borne disease. Fully updated throughout, this new edition continues to cover the life cycle, biology, and development of the parasite; clinical pathology, immunology, diagnosis and vaccine development; and emergence, cause and mechanisms of drug resistance. It reviews the temperate liver fluke *Fasciola hepatica*, together with molecular, biochemical, control, and epidemiologial aspects of the tropical liver fluke *F. gigantica*.

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