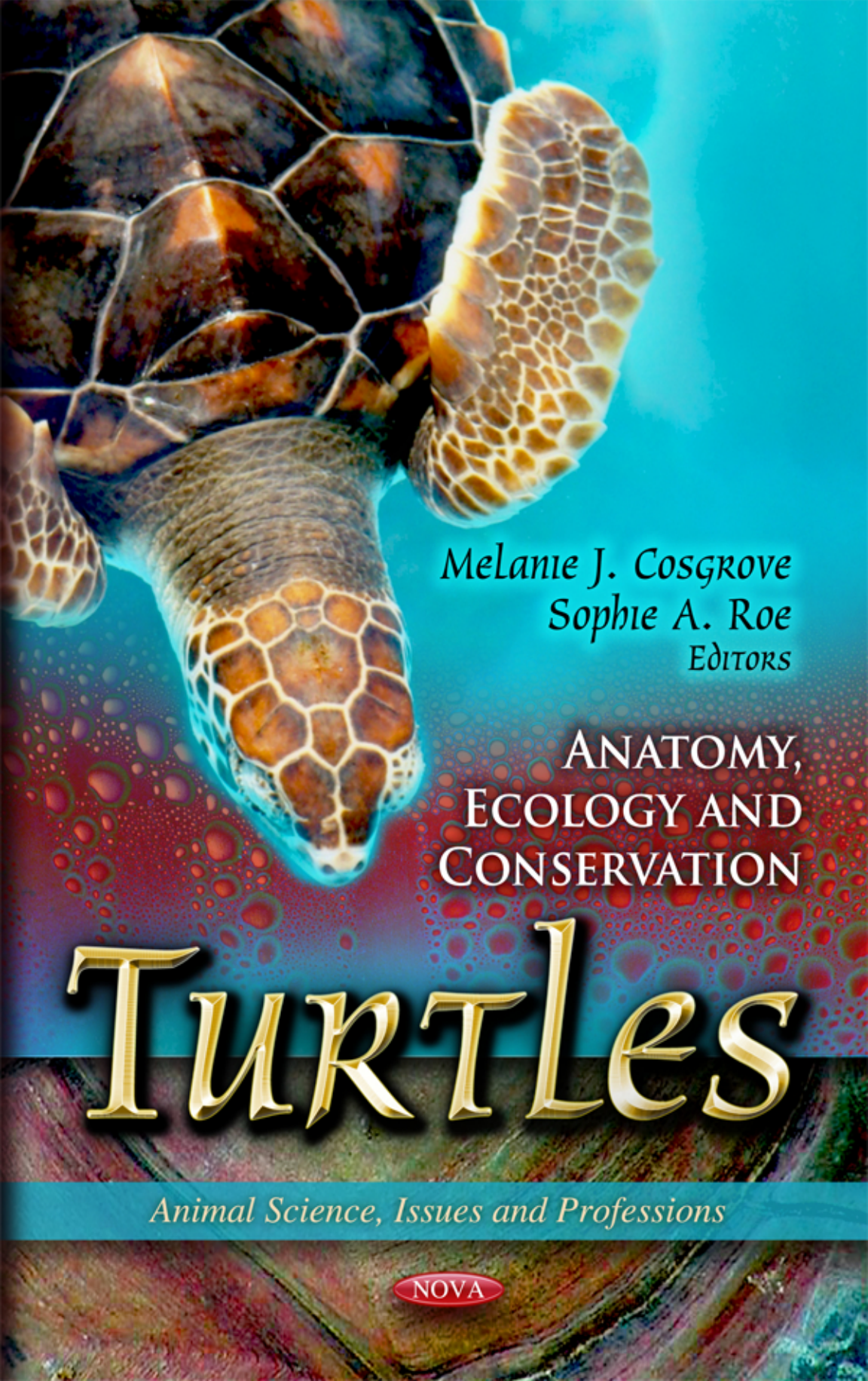
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Anatomy, Ecology and Conservation
Turtles
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TURTLES

ANATOMY, ECOLOGY AND CONSERVATION

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ANIMAL SCIENCE, ISSUES AND PROFESSIONS

TURTLES

ANATOMY, ECOLOGY AND CONSERVATION

MELANIE J. COSGROVE
AND
SOPHIE A. ROE
EDITORS

Nova Science Publishers, Inc.
New York

Library of Congress Cataloging-in-Publication Data

Turtles : anatomy, ecology, and conservation / editors, Melanie J. Cosgrove and Sophie A. Roe.
p. cm.
Includes bibliographical references and index.
ISBN: 978-1-61470-658-8 (eBook) 1. Turtles--Anatomy. 2. Turtles--Ecology. 3. Turtles--Conservation. I. Cosgrove, Melanie J. II. Roe, Sophie A.
QL666.C5T862 2011
597.92--dc23

2011024675

Published by Nova Science Publishers, Inc. + New York

CONTENTS

PREFACE

This book presents current research in the study of the anatomy, ecology and conservation of turtles. Topics discussed in this compilation include the accumulation and tissue distribution of metals and other elements in sea turtles across the globe as bioindicators of marine pollution; the pathological findings and causes of mortality of sea turtles in the Canary Islands; the painted turtle as a model of natural anoxia tolerance; the evolutionary history of turtles and responses of freshwater turtles to drought.

Chapter 1- All the sea turtle species have been classified as vulnerable, endangered or critically endangered by IUCN Red List 2010 (except for *Natator depressus*: data deficient). Among others, the greatest threats to the survival of marine turtles in the world include loss of nesting habitats, accidental capture by some types of fishing and marine pollution. For example, unnatural quantities of inorganic pollutants (metals and other toxic elements) have been released and continue to be released into the sea by contaminant human activities altering the natural biological equilibrium. Many of these inorganic elements are biologically essential, but they can be toxic to biota above certain exposure thresholds. The risk of toxicity is higher on those long-living species at the top of the marine food chains, such as sea turtles, which have the potential to accumulate them. Because of that, the scientific community has increased the interest in the assessment of metal exposure and accumulation in different species of marine turtles, mainly in the last decade. Stranded individuals of the seven existing species of sea turtles (*Caretta caretta*, *Chelonia mydas*, *Dermochelys coriacea*, *Eretmochelys imbricata*, *Lepidochelys kempii*, *Lepidochelys olivacea* and *Natator depressus*) have been analyzed in several studies for inorganic pollution monitoring in different areas around the world (America, Europe, Asia and Oceania). Concentrations of several metals and other elements of toxicological interest (Cd, Pb, Hg, As,

Cu, Zn, Fe, Mn, Ni, Se, Cr, etc.) have been measured in most of their organs and tissues (liver, kidney, muscle, lung, bone, fat, blood, carapace and eggs, among others). Elements related to biomagnification phenomena in marine environments, such as Hg and Cd, showed especially high levels in samples of liver and kidney, respectively, from areas with a great human presence (Japan, Mexico or Mediterranean Sea). The majority load of other elements was found in muscle (As) and hard tissues (Pb, Zn).

In spite of the difficulty to study these animals (due to their solitary nature or long pelagic phases), the information about metal levels in sea turtles has increased considerable in the last years. In this chapter, authors review the available scientific literature in order to provide a global view of the matter. This global view allow us to get a deeper knowledge of the presence of inorganic elements in the different species of sea turtles, to estimate the risk of metal exposure for them in different areas of the world, and to assess their role as a potential long-term bioindicators of inorganic marine pollution.

Chapter 2- Because all species of sea turtles are included on the Red List of the World Conservation Union, the efforts to conserve sea turtles, the advances in their medical management, and the studies on diseases and causes of mortality and/or stranding of sea turtles must to be increased. A multidisciplinary approach to this focus carried out by veterinarians, biologists, and scientists, is necessary in order to compile data that prove the main threats for these endangered reptiles, and to design adequate strategies of conservation. This chapter lists the pathological findings and causes of mortality of 49 sea turtles (46 *Caretta caretta*, 2 *Chelonia mydas*, and 1 *Dermochelyscoriacea*) stranded on the coasts of the Canary Islands, Spain, between 2002 and 2009.Of these, 12 turtles (24.49%) had died of spontaneous diseases including different types of hepatitis, pneumonia, and septicemic processes. However, 37 turtles (75.51%) died from lesions associated with human activities: ingestion of hooks and monofilament lines (34.69%), entanglement in fishing nets (24.49%), and boat-strike injuries (16.33%). In addition, polychlorinated biphenyls (PCBs 28, 31, 52, 101, 138, 153, 180, and 209) and DDT and its metabolites (*o,p'*-DDT, *o,p'*-DDE, and *o,p'*-DDD) were measured in liver and fat from 32 sea turtles. Tissues from these turtles contained higher levels of PCBs and *o,p'*-DDT than those reported in turtles from other geographical regions. Statistically, a positive correlation was detected between ∑PCBs concentration and cachexia, and between ∑DDT concentrations and cachexia. No histological lesions exclusively attributed to the acute effects of PCBs and DDTs were described. However, chronic effects of organochlorines can not be discarded.

Chapter 3- Painted turtles range throughout the USA and Canada. The most northern member of this species is the western painted turtle *Chrysemys picta bellii;* which is also the most anoxia-tolerant vertebrate identified, surviving for up to 6 months buried in the mud of ice covered pond at 3˚C. *C. picta* provides a working model of an anoxia-tolerant vertebrate, and of particular interest - a brain that functions without oxygen. Authors have recently been exploring the role of GABA, the primary vertebrate inhibitory neurotransmitter, in anoxia tolerance. Increases in endogenous GABAergic signalling appear to mediate neuronal electrical depression in *C. picta* and in a number of other anoxia-tolerant species. Unlike most other vertebrate species that undergo severe neural injury within minutes of an anoxic episode, *C. picta* neurons reduce action potential frequency and suppress metabolism rapidly with the onset of anoxia, and avoid injury. Anoxia-sensitive mammals lack a hypoxia/anoxia-mediated increase in GABA concentrations and suffer electrical hyper-excitability and excitotoxic cell death following the onset of an anoxic/ischemic episode. Experimental interventions aimed at increasing inhibitory GABAergic signalling prevent over-excitation and rescue mammalian neurons from anoxic/ischemic cell death. Drawing upon evidence from a number of studies on a variety of naturally anoxia-tolerant species authors will explore in detail the role of GABA in neuroprotection. The anoxic response could be interpreted as a natural anaesthetic mechanism and is achieved by a reduction in excitatory neurotransmitter receptor activity (glutamate) and an increase in inhibitory receptor activity (GABA). An understanding of the complex regulation of GABAergic signaling in facultative anaerobes may provide insight into interventions that could prove efficacious against low oxygen situations in mammals, such as ischemic insult due to stroke.

Chapter 4- The history of turtle lineage began around 225 Mya. Since then, this group of reptiles developed diverse ecological strategies and colonized a wide range of environments, from marine to fully terrestrial habitats. In spite of their great ecological diversity, the bauplan of turtles is peculiar and little variable, with a body encased in a rigid shell consisting of a dorsal carapace and a ventral plastron. This fact has made the morphological comparison of turtles with other vertebrates very complicate. Thus, the understanding of the origins and phylogenetic relationships of turtles within the Amniota and the evolution of turtle biology are stimulating challenges for researchers. Several attempts in order to resolve these mysteries have been carried out from a multidisciplinary approach, but lacks consensus. The knowledge of the evolutionary history of turtle species and their inclusive

groups is relevant for their conservation management, especially taking into account that many species are among moderate and high risk of extinction. In this chapter, authors update and review the state of the art of the systematics of turtles from the point of view of several often conflicting disciplines, embryology, morphology, paleontology and molecular systematics. In addition, authors sound out the amount of threatened phylogenetic diversity in the turtle tree of life based on fossil evidence, recent phylogenetic hypotheses and data from the red list of the International Union for the Conservation of Nature.

Chapter 5- The study of the hematological characteristics of loggerhead turtles can be very helpful in the rescue and rehabilitation of this endangered species. Normal hemocellular and biochemical values are the basis for the evaluation of a subject's health status. The identification of the blood cells through cytochemical or immunocytochemical methods is the first step to obtain a correct classification of the cells. The following blood cell types were found: lymphocytes, monocytes, heterophils (the counterparts of mammalian neutrophils), eosinophils, basophils (rarely observed), erythrocytes along with some of their precursors, and thrombocytes. Lymphocytes and monocytes are easily distinguishable, as they are similar to the mammalian counterparts, whereas heterophils (which are the equivalent of mammalian neutrophils) and eosinophils both contain eosinophilic granules in their cytoplasm, making their identification difficult. The thrombocytes are nucleated cells, whose shape is variable in dry film smears of circulating blood, either round or oval; they take part in the hemostatic process, as suggested by their tendency to form aggregates. The erythrocytes are also nucleated cells, showing an ellipsoidal and flattened configuration. In all specimens of this marine reptile, both healthy and unhealthy, the mature erythrocytes contain one characteristic inclusion in their cytoplasm, which was recognized as a Heinz body, formed by the precipitation of hemoglobin. The loggerhead's hemoglobin was studied in depth and revealed some specific characteristics, one of which is that under physiologic conditions it precipitates due to its instability. While no data are available about the erythropoiesis in the primary organs, circulating erythropoiesis was revealed to occur in the loggerhead's blood, just as it also occurs at this site in other non-mammalian vertebrates. Loggerhead blood infestation by spirorchids was reported by Wolke *et al.* (1982), while Di Santi *et al.* (2010) found two specimens of loggerheads hemoparasitized by *Theileria.*

Chapter 6- Australia was not always arid, and the freshwater turtles that have survived to this day are either relicts residing in river systems that have

themselves had a history or continuity through the drying of the continent, or they are species that have adapted in some way to meet the challenges of drought. In this chapter, authors report on how Australian freshwater turtles cope with periodic loss of habitat through drought at a range of spatial and temporal scales, and consider how they might fare under changing climates predicted to occur through global warming. Global warming is not occurring in isolation of other environmental changes at a landscape scale, and authors look at what interactions there might be between climate change and the increasing demands of agriculture, industry and our cities for water, in presenting challenges for our unique freshwater turtle fauna.

In: Turtles: Anatomy, Ecology and Conservation ISBN: 978-1-61470-554-3
Eds: M. J. Cosgrove and S. A. Roe

Chapter 1

ACCUMULATION AND TISSUE DISTRIBUTION OF METALS AND OTHER ELEMENTS IN SEA TURTLES FROM ALL OVER THE WORLD

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ABSTRACT

All the sea turtle species have been classified as vulnerable, endangered or critically endangered by IUCN Red List 2010 (except for *Natator depressus*: data deficient). Among others, the greatest threats to the survival of marine turtles in the world include loss of nesting habitats, accidental capture by some types of fishing and marine pollution. For example, unnatural quantities of inorganic pollutants (metals and other toxic elements) have been released and continue to be released into the sea by contaminant human activities altering the natural biological equilibrium. Many of these inorganic elements are biologically essential, but they can be toxic to biota above certain exposure thresholds. The risk of toxicity is higher on those long-living species at the top of the marine food chains, such as sea turtles, which have the potential to accumulate them. Because of that, the scientific community has increased the interest

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in the assessment of metal exposure and accumulation in different species of marine turtles, mainly in the last decade.

Stranded individuals of the seven existing species of sea turtles (*Caretta caretta*, *Chelonia mydas*, *Dermochelys coriacea*, *Eretmochelys imbricata*, *Lepidochelys kempii*, *Lepidochelys olivacea* and *Natator depressus*) have been analyzed in several studies for inorganic pollution monitoring in different areas around the world (America, Europe, Asia and Oceania). Concentrations of several metals and other elements of toxicological interest (Cd, Pb, Hg, As, Cu, Zn, Fe, Mn, Ni, Se, Cr, etc.) have been measured in most of their organs and tissues (liver, kidney, muscle, lung, bone, fat, blood, carapace and eggs, among others). Elements related to biomagnification phenomena in marine environments, such as Hg and Cd, showed especially high levels in samples of liver and kidney, respectively, from areas with a great human presence (Japan, Mexico or Mediterranean Sea). The majority load of other elements was found in muscle (As) and hard tissues (Pb, Zn).

In spite of the difficulty to study these animals (due to their solitary nature or long pelagic phases), the information about metal levels in sea turtles has increased considerable in the last years. In this chapter, we review the available scientific literature in order to provide a global view of the matter. This global view allow us to get a deeper knowledge of the presence of inorganic elements in the different species of sea turtles, to estimate the risk of metal exposure for them in different areas of the world, and to assess their role as a potential long-term bioindicators of inorganic marine pollution.

INTRODUCTION

Six of the seven existing species of sea turtles have been included in the IUCN Red List 2010 [1]: *Caretta caretta* (endangered), *Chelonia mydas* (endangered and decreasing population trend), *Dermochelys coriacea* (critically endangered and decreasing population trend), *Eretmochelys imbricata* (critically endangered and decreasing population trend), *Lepidochelys kempii* (critically endangered) and *Lepidochelys olivacea* (vulnerable and decreasing population trend). About the other one, *Natator depressus*, the available data are deficient. Among others (fisheries impacts, direct take, coastal development or global warming), marine pollution is one of the five major hazards to sea turtles conservation [2]. Plastics, discarded fishing gear or light pollution pose a threat to sea turtles. In addition, chemical

pollution related to petroleum by-products, agricultural runoff or sewage could affect these animals weakening, for example, their immune system [2].

Several anthropogenic activities have been pointed out as sources of inorganic pollutants in marine environments, mainly related to growing population and industrialization: industrial plants (metallurgy, paints, tannery, etc.), chemical plants, mining, harbour activities, ship traffic, intensive farming, aquatic breeding, solid waste and sewage (urban and others), etc. [3, 4, 5]. It is quite clear that inorganic elements are natural components of rocks and soils, which find their way into the marine environment as a consequence of weathering and erosion [6]. However, unnatural quantities of several inorganic elements coming from human activities (previously cited) have been released, and continue to be released into the sea altering the natural biological equilibrium [7]. Many of these elements are biologically essential but they can be toxic to biota above certain exposure thresholds [8]. Mainly, some toxic elements may cause adverse effects in organisms located at the top of the marine food-chains [9] and long-living species such as sea turtles, which have the potential to accumulate them [10, 11], although the concentration of metals in the water column is often low due to their poor solubility [12].

With a view towards the preservation of these species, the interest in monitoring the presence of inorganic pollutants (metals and others) in sea turtles has increased in the last years [13]. Nowadays, a large number of studies on the matter have been carried out throughout the world: European Mediterranean area [14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25], European Atlantic area [10, 26, 27], American Atlantic area [28, 29, 30, 31, 32, 33, 34, 35, 36, 37], American Pacific area [38, 39, 40, 41, 42, 43, 44, 45, 46], Asia-Pacific area and Indian Ocean [11, 13, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62].

Nevertheless, the information is scarce and fragmented in comparison with other big marine organisms (marine mammals or seabirds), probably due to the solitary nature of the sea turtles, the length of their pelagic phase and long lasting apnoeas, so they are among the most difficult marine animals to study [63]. The purpose of this chapter is to put in order and unify all this information to provide a global view of the presence and distribution of eleven elements (key elements in the toxicological field, namely: cadmium, lead, mercury, arsenic, copper, zinc, iron, manganese, nickel, selenium and chromium) in several biological samples of marine sea turtles (liver, kidney, muscle, blood, fat, egg and stomach content). The majority of the data reported as wet weight in the original studies have been converted to dry weight, in

order to avoid errors related to different water contents of the samples (conversion ratios: 3.13 for liver, 4.17 for kidney, 4.76 for muscle, 5.10 for blood, 1.18 for fat tissue, 5.21 for egg content, 37.04 for albumen, 2.67 for yolk and 2.44 for eggshell) [22, 23, 35, 46].

This global view allows us to recognize areas, species or elements that need to be studied further. With this chapter we get a deeper knowledge of the presence of inorganic elements in the different species of sea turtles, in order to estimate the risk of metal exposure for them in different areas of the world. Besides, we assess the marine turtle role as potential long-term bioindicator of the inorganic marine pollution.

LOGGERHEAD SEA TURTLE (*CARETTA CARETTA*)

Referring to the information about levels of inorganic pollutants in marine turtles, *Caretta caretta* is the most studied species. The levels of heavy metals and essential elements (Cd, Pb, Hg, As, Cu, Zn, Fe, Mn, Ni, Se and Cr) that have been measured in this species (samples of liver, kidney, muscle, blood, fat and egg) are shown in Table 1. No data about levels of these elements in stomach contents of loggerhead sea turtles have been found. The metals most frequently analyzed are mercury, cadmium and lead (in that order). On one hand, probably mercury and cadmium are largely studied due to the greater relevance that these metals present in the marine environment (mercury and cadmium are often related to biomagnification and bioaccumulation phenomena in aquatic ecosystems). On the other hand, lead is largely studied because of this metal is related with several human contaminant activities, and it is one of the most suitable metals for anthropogenic pollution monitoring [64]. The tissues of this species mainly analyzed are liver, kidney and muscle (see Table 1). Generally, the highest mercury concentrations were found in liver [13, 14, 15, 18, 20, 21, 23, 31, 43, 51], whereas the highest cadmium levels were found in kidney [10, 13, 14, 18, 20, 23, 24, 25, 27, 42, 48, 51]. These patterns of metal accumulation are similar to those described for other marine vertebrates [65, 66]: mercury levels tend to be highest in hepatic tissue and cadmium levels tend to be highest in renal tissue. In the case of lead, soft tissues showed similar levels (see Table 1), although the studies that analyzed calcified tissues found the highest lead levels in bone and carapace [25, 51]. Arsenic showed the highest levels in muscle tissue [14, 16, 25, 49] and chromium in kidney [14].

Table 1. Heavy metal and essential element levels (μg g^{-1} dry weight) in tissues and other samples of *Caretta caretta* from different locations

Tissue - Location	Cadmium		Lead		Mercury		Arsenic		References
	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	
Liver									
Mediterranean Sea: Italy	7,60 ± 6,05	3,06 - 20,23	1,23 ± 1,01	BDL - 3,38	1,68 ± 1,04	0,35 - 3,72	21,67 ± 17,22	0,83 - 56,55	[14]
Mediterranean Sea: Italy					2,19 ± 1	1,16 - 3,44			[15]
Mediterranean Sea: Cyprus	8,64	5,14 - 12,97	BDL	BDL - 4,90	2,41	0,82 - 7,50			[20]
Mediterranean Sea: Italy							20,94 ± 14,03	8,38 - 40,84	[16]
Mediterranean Sea: Turkey	1,26 ± 0,43	(Em)	2,48 ± 0,46	(Em)	0,51 ± 0,05	(Em)			[21]
Mediterranean Sea: Italy	2,84 ± 0,72								[22]
Mediterranean Sea: Italy	19,30 ± 34,20	1,60 - 114,00			1,10 ± 1,70	0,42 - 8,76			[23]
Mediterranean Sea: Italy	10,50 ± 6,06	3,44 - 20,47	0,50 ± 0,16	BDL - 0,91	1,34 ± 0,91	0,41 - 3,94			[18]
Mediterranean Sea: Italy	2,40 ± 0,40		0,10 ± 0,08						[24]
Mediterranean Sea: Spain	0,81 ± 0,48	0,05 - 1,88	0,20 ± 0,11	0,08 - 0,51	0,39 ± 0,41	0,08 - 1,57	12,73 ± 13,03	3,50 - 43,43	[25]
Atlantic Ocean: France	8,06 ± 12,88	0,94 - 36,88							[10]
Atlantic Ocean: Canary Is.	7,91 ± 1,41	0,13 - 68,69	9,19 ± 1,84	0,16 - 103,41	0,13 ± 0,03	0,003 - 1,47	53,34 ± 9,25	0,25 - 412,06	[27]
Atlantic Ocean: US					1,84 ± 0,50	1,09 - 4,19			[31]
Pacific Ocean: Mexico	1,75	BDL - 30,62	BDL						[42]
Pacific Ocean: Mexico					0,15	0,12 - 0,18			[43]
Pacific Ocean: Japan	29,03 ± 10,31	17,69 - 45,63			4,72 ± 9,16	0,78 - 25,47			[13]

Table 1 (Continued)

Tissue - Location	Cadmium		Lead		Mercury		Arsenic		References
	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	
Pacific Ocean: Australia**	51,25 ± 10,31	22,81 - 109,69			0,05 ± 0,02	0,00 - 0,09	1,44 ± 0,75	0,00 - 4,88	[48]
Pacific Ocean: Japan							6,32 ± 1,56	4,24 - 9,43	[49]
Pacific Ocean: Japan	30,44 ± 10,53	(F)	0,25 ± 0,09	(F)	1,25 ± 0,50	(F)			[51]
	20,63	(M)	0,66	(M)	25,47	(M)			[51]
Pacific Ocean: Japan							11,20 ± 3,00		[54]
Kidney									
Mediterranean Sea: Italy	24,23 ± 21,40	0,39 - 64,00	0,70 ± 0,35	BDL - 1,35	0,65 ± 0,34	0,30 - 1,53	29,91 ± 39,48	6,09 - 139,60	[14]
Mediterranean Sea: Cyprus	30,50	18,80 - 42,20	2,45	BDL - 4,90	0,47	0,13 - 0,80			[20]
Mediterranean Sea: Italy	57,20 ± 34,60	10,90 - 158,00			0,90 ± 0,70	0,37 - 3,41			[23]
Mediterranean Sea: Italy	34,79 ± 20,13	5,25 - 68,34	0,50 ± 0,29	BDL - 0,88	0,67 ± 0,29	0,25 - 1,29			[18]
Mediterranean Sea: Italy	5,80 ± 1,10		0,10 ± 0,07						[24]
Mediterranean Sea: Spain	6,88 ± 6,26	0,02 - 16,16	1,22 ± 0,86	0,08 - 2,26	0,44 ± 0,51	0,07 - 1,32	34,72 ± 31,79	6,84 - 97,75	[25]
Atlantic Ocean: France	55,42 ± 56,67	7,00 - 148,76							[10]
Atlantic Ocean: Canary Is.	22,88 ± 4,25	0,04 - 254,52	10,17 ± 2,00	0,08 - 72,05	0,17 ± 0,04	0,04 - 1,38	57,50 ± 10,00	4,83 - 508,87	[27]
Atlantic Ocean: US					0,88 ± 0,21	0,54 - 1,83			[31]

Pacific Ocean: Mexico	73,11	13,72 - 140,00	0,03	BDL - 69,89					[42]
Pacific Ocean: Mexico					0,10	0,06 - 0,14			[43]
Pacific Ocean: Japan	164,18 ± 67,51	75,42 - 235,44			1,04 ± 0,54	0,17 - 1,83			[13]
Pacific Ocean: Australia**	117,93 ± 23,75	47,50 - 164,18			0,21 ± 0,04	0,13 - 0,29	2,96 ± 1,08	1,00 - 4,79	[48]
Pacific Ocean: Japan							9,47 ± 5,37	4,01 - 20,20	[49]
Pacific Ocean: Japan	159,60 ± 72,92	(F)	0,67 ± 0,21	(F)	1,00 ± 0,58	(F)			[51]
	189,60	(M)	BDL	(M)	1,25	(M)			[51]
Muscle									
Mediterranean Sea: Italy	0,55 ± 0,63	0,09 - 2,21	0,54 ± 0,17	BDL - 0,74	0,69 ± 0,46	0,17 - 1,81	68,94 ± 45,80	11,21 - 139,60	[14]
Mediterranean Sea: Italy					1,00 ± 0,62	0,33 - 2,05			[15]
Mediterranean Sea: Cyprus	0,57	0,30 - 1,43	2,46	BDL - 5,53	0,48	BDL - 1,78			[20]
Mediterranean Sea: Italy							73,67 ± 56,72	12,57 - 154,24	[16]
Mediterranean Sea: Italy	0,36 ± 0,11								[22]
Mediterranean Sea: Italy	0,20 ± 0,20	0,06 - 0,78			0,40 ± 0,30	0,14 - 1,92			[23]
Mediterranena Sea: Italy	0,33 ± 0,14	BDL - 0,62	0,19 ± 0,14	BDL - 0,43	0,86 ± 1,00	0,14 - 3,14			[18]
Mediterranean Sea: Italy	0,81 ± 0,04		BDL						[24]
Mediterranean Sea: Spain	0,08 ± 0,06	0,02 - 0,24	0,20 ± 0,25	0,01 - 0,62	0,14 ± 0,22	0,01 - 0,82	40,95 ± 37,32	6,36 - 133,48	[25]
Atlantic Ocean: France	0,38 ± 0,24	0,02 - 0,86							[10]
Atlantic Ocean: Canary Is.	5,43 ± 1,33	0,71 - 59,43	10,76 ± 2,43	1,05 - 100,34	BDL		35,00 ± 6,52	7,38 - 320,10	[27]
Atlantic Ocean: US					0,76 ± 0,33	0,24 - 2,38			[31]

Table 1 (Continued)

Tissue - Location	Cadmium		Lead		Mercury		Arsenic		References
	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	
Pacific Ocean: Mexico	0,01	BDL - 1,45	0,01	BDL - 1,57					[42]
Pacific Ocean: Mexico					0,03	0,02 - 0,04			[43]
Pacific Ocean: Japan	0,29 ± 0,14	0,19 - 0,57			0,52 ± 0,24	0,24 - 0,90			[13]
Pacific Ocean: Japan							20,60 ± 13,10	5,19 - 45,50	[49]
Pacific Ocean: Japan	0,29 ± 0,14	(F)	0,10 ± 0,14	(F)	0,43 ± 0,19	(F)			[51]
	0,24	(M)	BDL	(M)	0,90	(M)			[51]
Blood									
Mediterranean Sea: Spain	0,12 ± 0,21	0,01 - 0,5	0,31 ± 0,31	0,02 - 0,64	0,02 ± 0,01	0,01 - 0,03	6,99 ± 9,28	2,07 - 23,53	[25]
Atlantic Ocean: US					Dead: 0,51 ± 0,20	0,20 - 1,58			[31]
					Alive: 0,15 ± 0,05	0,03 - 0,97			[31]
Atlantic Ocean: US					0,15 ± 0,01	0,03 - 0,41			[32]
Atlantic Ocean: US*					Red blood cell: 0,07 ± 0,02				[33]
Altantic Ocean: US					0,03 ± 0,04				[33]
Fat									
Mediterranean Sea: Italy	2,33 ± 0,52								[22]

			0,11 ±		0,05 ±				
Mediterranean Sea: Italy	0,09 ± 0,07	BDL - 0,21	0,04	0,07 - 0,17	0,04	BDL - 0,11			[18]
Pacific Ocean: Mexico	0,50	0,20 - 1,37	BDL						[42]
Pacific Ocean: Mexico					0,01	BDL - 0,03			[43]
					0,007 ±				
Pacific Ocean: Japan	0,08 ± 0,05	(F)	BDL	(F)	0,004	(F)			[51]
	0,11	(M)	BDL	(M)	0,05	(M)			[51]
Egg									
Mediterranean Sea:	Yolk: 0,36 ±		Yolk: 1,31		Yolk:				
Turkey	0,14		± 0,23		BDL				[21]
	Shell: 0,65 ±				Shell:				
	0,13		Shell: 0,63 ± 0,16		BDL				[21]
	Yolk: 0,10 ±		Yolk: 1,58		Yolk: 0,95				
Atlantic Ocean: US	0,05		± 1,06		± 0,04				[28]
			Egg		Egg				
			content:		content:		Egg content:	BDL -	
Atlantic Ocean: US			0,69	BDL - 2,74	0,12	BDL - 0,22	1,70	14,01	[29]
					Egg:				
	Egg: 0,01 ±				0,006 ±	0,004 -			
Pacific Ocean: Japan*	0,004	0,008 - 0,02			0,002	0,007			[13]
	Yolk: 0,08 ±				Yolk: 0,03				
Pacific Ocean: Japan	0,03	0,05 - 0,11			± 0,01	0,02 - 0,04			[13]
					Albumen:				
	Albumen:				0,02 ±	0,004 -			
	BDL				0,01	0,03			[13]
					Shell: 0,01	0,005 -			
	Shell: BDL				± 0,002	0,01			[13]
Pacific Ocean: Japan*			Egg: BDL						[51]
			Yolk:						
Pacific Ocean: Japan			BDL						[51]
			Albumen:						
			BDL						[51]
			Shell:						
			BDL						[51]

Table 1 (Continued)

Tissue - Location	Cadmium		Lead		Mercury		Arsenic		References
	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	
Liver									
Mediterranean Sea: Turkey	21,21 ± 2,62	(Em)	23,84 ± 3,11	(Em)	35,83 ± 9,98	(Em)			[21]
Mediterranean Sea: Italy	7,40 ± 3,90		27,90 ± 6,50		377,40 ± 211,20		6,23 ± 2,80		[22]
Mediterranean Sea: Italy	37,30 ± 8,70	9,40 - 41,80	66,00 ± 42,70	23,80 - 178,00					[23]
Mediterranean Sea: Italy	24,03 ± 14,47	4,47 - 55,63	91,56 ± 24,09	58,75 - 145,31	1425,00 ± 686,56	656,25 - 2465,63			[18]
Mediterranean Sea: Italy	17,50 ± 2,44		103,00 ± 14,00		1232,00 ± 404,00		7,48 ± 1,04		[24]
Mediterranean Sea: Spain			30,23 ± 12,24	17,03 - 51,96					[25]
Atlantic Ocean: France	25,78 ± 20,59	7,25 - 65,31	78,13 ± 26,69	45,31 - 120,00					[10]
Atlantic Ocean: Canary Is.	46,94 ± 6,47	0,03 - 204,91	42,13 ± 5,31	0,28 - 285,56	1071,03 ± 133,75	1,09 - 6813,63			[27]
Atlantic Ocean: US									[31]
Pacific Ocean: Mexico	33,94	16,60 - 58,98	69,14	42,45 - 91,87	301,00	71,88 - 2042,00	1,29	0,11 - 8,60	[42]
Pacific Ocean: Mexico									[43]
Pacific Ocean: Japan	55,94 ± 25,53	20,22 - 105,94	87,19 ± 13,59	72,50 - 109,69	2028,13 ± 1203,13	706,25 - 3937,50	6,47 ± 1,44	4,50 - 9,19	[13]
Pacific Ocean: Australia**			71,25 ± 9,38	42,81 - 101,88					[48]
Pacific Ocean: Japan									[49]

	55,31 ±		87,81 ±		1887,50 ±				
Pacific Ocean: Japan	27,91	(F)	14,78	(F)	1253,13	(F)	6,81 ± 1,25	(F)	[51]
	60,63	(M)	83,13	(M)	2865,63	(M)	4,50	(M)	[51]
Kidney									
			97,00 ±	62,40 -					
Mediterranean Sea: Italy	2,60 ± 0,70	1,70 - 4,70	31,70	206,00					[23]
			96,26 ±	69,17 -	200,85 ±	72,51 -			
Mediterranean Sea: Italy	5,04 ± 2,25	1,50 - 8,83	18,88	116,26	157,51	508,37			[18]
			119,00 ±		764,00 ±				
Mediterranean Sea: Italy	5,56 ± 0,96		8,00		160,00		7,01 ± 1,32		[24]
Mediterranean Sea:			32,64 ±	19,73 -					
Spain			12,25	55,16					[25]
			98,34 ±	68,76 -					
Atlantic Ocean: France	9,21 ± 1,92	7,33 - 11,79	28,75	140,84					[10]
Atlantic Ocean: Canary			37,88 ±	0,29 -	158,18 ±	1,21 -			
Is.	19,17 ± 4,04	0,54 - 204,43	4,46	160,55	23,92	1138,17			[27]
				2,68 -		94,59 -		2,37 -	
Pacific Ocean: Mexico	4,35	1,39 - 8,23	32,47	130,00	237,00	660,00	6,00	9,97	[42]
			107,51 ±	80,01 -	149,60 ±	47,50 -		3,38 -	
Pacific Ocean: Japan	5,42 ± 0,83	4,13 - 6,50	17,38	126,68	146,26	458,37	6,54 ± 2,04	8,21	[13]
Pacific Ocean:			76,67 ±	69,59 -					
Australia**			3,75	88,76					[48]
			105,84 ±		125,01 ±				
Pacific Ocean: Japan	5,42 ± 0,92	(F)	18,29	(F)	152,51	(F)	6,25 ± 2,13	(F)	[51]
	5,38	(M)	118,34	(M)	47,50	(M)	8,21	(M)	[51]
Muscle									
			30,90 ±		60,90 ±				
Mediterranean Sea: Italy	1,50 ± 0,40		8,00		38,30		2,70 ± 1,20		[22]
			107,00 ±	76,40 -					
Mediterranean Sea: Italy	2,70 ± 1,40	0,80 - 7,00	26,10	177,00					[23]
			132,86 ±	94,29 -	151,91 ±	102,86 -			
Mediterranena Sea: Italy	2,81 ± 1,95	0,90 - 6,43	23,10	167,15	50,00	246,67			[18]

Table 1 (Continued)

Tissue - Location	Cadmium		Lead		Mercury		Arsenic		References
	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	
Mediterranean Sea: Italy	2,40 ± 0,24		105,00 ± 14,00		85,40 ± 9,90		1,35 ± 0,24		[24]
Mediterranean Sea: Spain			113,29 ± 267,33	25,42 - 1002,40					[25]
Atlantic Ocean: France	3,48 ± 2,14	1,62 - 10,62	93,34 ± 27,14	58,10 - 172,86					[10]
Atlantic Ocean: Canary Is.	13,57 ± 2,48	0,05 - 129,76	31,91 ± 4,57	0,24 - 154,15	41,19 ± 6,67	0,76 - 468,44			[27]
Pacific Ocean: Mexico	0,41	BDL - 3,44	31,11	0,63 - 100,00	77,44	52,48 - 97,12	0,84	BDL - 5,40	[42]
Pacific Ocean: Japan	3,95 ± 1,24	2,52 - 6,10	115,24 ± 18,10	92,86 - 147,62	95,72 ± 38,10	53,81 - 167,62	1,43 ± 0,57	0,62 - 2,14	[13]
Pacific Ocean: Japan	3,86 ± 1,33 4,52	(F) (M)	119,05 ± 16,62 92,86	(F) (M)	94,29 ± 41,48 105,72	(F) (M)	1,33 ± 0,52 2,05	(F) (M)	[51] [51]
Blood									
Mediterranean Sea: Spain			7,07 ± 2,85	3,60 - 11,95					[25]
Fat									
Mediterranean Sea: Italy	3,40 ± 1,70		68,20 ± 34,70		132,30 ± 121,00		8,40 ± 2,50		[22]
Mediterranean Sea: Italy	0,22 ± 0,17	0,12 - 0,60	76,48 ± 26,56	44,44 - 112,17	13,47 ± 5,80	8,87 - 26,36			[18]
Pacific Ocean: Mexico	0,69	0,53 - 1,15	12,66	0,53 - 44,76	1,33	BDL - 13,61	1,82	0,80 - 3,20	[42]

Pacific Ocean: Japan	0,13 ± 0,04 0,28	(F) (M)	113,59 ± 22,22 101,18	(F) (M)	11,73 ± 6,63 22,34	(F) (M)	0,14 ± 0,11 0,21	(F) (M)	[51] [51]
Egg									
Mediterranean Sea: Turkey	Yolk: 0,93 ± 0,10		Yolk: 57,21 ± 2,23		Yolk: 15,79 ± 3,62				[21]
	Shell: 5,29 ± 0,48		Shell: 5,00 ± 2,20		Shell: 17,75 ± 7,00				[21]
Atlantic Ocean: US	Yolk: 5,75 ± 1,08		Yolk: 77,41 ± 6,30		Yolk: 72,65 ± 12,38				[28]
Atlantic Ocean: US	Egg content: 5,75	2,36 - 51,10	Egg content: 92,83	60,62 - 139,30	Egg content: 77,66	44,56 - 210,80	Egg content: 1,96	0,82 - 17,62	[29]
Pacific Ocean: Japan*	Egg: 1,05 ± 0,20	0,77 - 1,31	Egg: 14,70 ± 1,44	13,20 - 16,50	Egg: 11,50 ± 1,29	10,50 - 13,70	Egg: 0,52 ± 0,26	0,30 - 0,90	[13]
Pacific Ocean: Japan	Yolk: 4,19 ± 0,19	3,95 - 4,49	Yolk: 91,85 ± 8,49	81,44 - 101,46	Yolk: 67,02 ± 5,82	61,14 - 75,56	Yolk: 2,43 ± 1,12	1,39 - 3,74	[13]
	Albumen: 4,82 ± 2,96	1,11 - 8,89	Albumen: 21,85 ± 21,48	2,22 - 57,04	Albumen: 32,22 ± 14,08	18,52 - 48,15	Albumen: 6,30 ± 11,11	BDL - 26,30	[13]
	Shell: 13,59 ± 1,88	10,37 - 15,08	Shell: 5,29 ± 1,44	4,05 - 7,00	Shell: 25,86 ± 5,37	17,64 - 32,45	Shell: 1,66 ± 1,17	BDL - 2,93	[13]

Table 1 (Continued)

Tissue - Location	Nickel		Selenium		Chromium		References
	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	
Liver							
Mediterranean Sea: Italy			15,88 ± 7,40	2,12 - 27,44	1,05 ± 0,58	0,20 - 2,07	[14]
Mediterranean Sea: Italy			15,19 ± 2,66	12,50 - 19,09			[15]
Mediterranean Sea: Italy	4,38 ± 1,43						[22]
Mediterranean Sea: Italy			9,80 ± 5,30	1,00 - 24,90			[23]
Mediterranean Sea: Italy			11,06 ± 4,66	3,16 - 17,13			[18]
Mediterranean Sea: Spain			3,10 ± 1,45	1,46 - 6,59			[25]
Atlantic Ocean: Canary Is.	9,00 ± 1,09	0,03 - 43,16					[27]
Pacific Ocean: Mexico	0,35	BDL - 3,26					[42]
Pacific Ocean: Australia**			6,91 ± 0,63	4,44 - 8,44			[48]
Pacific Ocean: Japan	BDL	(F)					[51]
	BDL	(M)					
Kidney							
Mediterranean Sea: Italy			10,33 ± 3,25	5,73 - 15,57	1,57 ± 2,05	0,20 - 6,80	[14]
Mediterranean Sea: Italy			15,50 ± 9,10	4,50 - 41,80			[23]
Mediterranean Sea: Italy			9,17 ± 2,75	2,75 - 11,58			[18]
Mediterranean Sea: Spain			5,66 ± 5,77	0,56 - 17,72			[25]
Atlantic Ocean: Canary Is.	24,21 ± 4,58	0,17 - 200,56					[27]
Pacific Ocean: Mexico	0,04	BDL - 3,38					[42]
Pacific Ocean: Australia**			6,33 ± 0,58	5,33 - 7,42			[48]
Pacific Ocean: Japan	0,92 ± 0,38	(F)					[51]
	0,21	(M)					
Muscle							
Mediterranean Sea: Italy			10,81 ± 2,89	6,51 - 15,45	1,43 ± 0,87	0,30 - 2,89	[14]
Mediterranean Sea: Italy			11,10 ± 3,19	5,67 - 15,43			[15]
Mediterranean Sea: Italy	2,76 ± 0,60						[22]
Mediterranean Sea: Italy			11,20 ± 4,90	4,00 - 24,10			[23]

Mediterranena Sea: Italy			7,86 ± 2,76	3,24 - 10,95			[18]
Mediterranean Sea: Spain			3,27 ± 1,65	0,87 - 6,67			[25]
Atlantic Ocean: Canary Is.	8,29 ± 1,38	0,14 - 62,62					[27]
Pacific Ocean: Mexico	0,01	BDL - 0,65					[42]
Pacific Ocean: Japan	0,38 ± 0,14	(F)					[51]
	0,29	(M)					[51]
Blood							
Mediterranean Sea: Spain			2,80 ± 1,29	0,92 - 4,48			[25]
Fat							
Mediterranean Sea: Italy	18,77 ± 4,40						[22]
Mediterranean Sea: Italy			2,17 ± 0,74	1,42 - 3,58			[18]
Pacific Ocean: Mexico	0,17	BDL - 1,63					[42]
Pacific Ocean: Japan	BDL	(F)					[51]
	BDL	(M)					[51]
Egg							
Atlantic Ocean: US	Yolk: 0,63 ± 0,24				Yolk: 1,28 ± 0,19		[28]
Atlantic Ocean: US	Egg content: 0,56	BDL - 1,07	Egg content: 5,46	1,10 - 15,84	Egg content: 1,20	BDL - 11,53	[29]
Pacific Ocean: Japan*	Egg: BDL						[51]
Pacific Ocean: Japan	Yolk: BDL						[51]
	Albumen: BDL						[51]
	Shell: BDL						[51]

BDL = bellow detection limit; * = µg g^{-1} wet weight; ** = Mean ± SEM; F = female; M = male; Egg = whole egg.

Concerning essential metals, a large variety of these have been measured in *Caretta caretta*. The most frequently analyzed are zinc, copper and iron (in that order). The tissues mainly analyzed are liver, kidney and muscle (see Table 1). The highest copper and iron levels were found in liver [10, 13, 18, 21, 22, 23, 24, 27, 42, 48, 49, 51]. Muscle and fat tissues contained considerable zinc levels, although zinc also showed a high affinity for calcified tissues such as bone and carapace [18, 22, 25, 27, 42, 43, 51]. Regarding fat tissue, Sakai et al. [51] suggested that the accumulation of zinc in this tissue could be associated with pigment proteins, which probably play an important role in zinc storage in the body. Among the analyzed metals in the turtle eggs, zinc and iron exhibited the highest levels in whole egg, while mercury, cadmium, lead, arsenic, copper, manganese, nickel, selenium and chromium showed the lowest ones [13, 21, 28, 29, 51]. Concerning the excretion of metals via eggs, Sakai et al. [13] reported that essential metals can be freely transferred from mother to eggs, whereas toxic metals, such as mercury and cadmium, are poorly transferred.

Metals in eggs were mainly present in the yolk: higher levels of cadmium, mercury, zinc and iron were found in yolk than in albumen [13], whereas eggshell contained the highest levels of iron and copper [13, 21].

GREEN SEA TURTLE (*CHELONIA MYDAS*)

Chelonia mydas is the second most studied marine turtle with respect to levels of heavy metals and essential elements. These levels (Cd, Pb, Hg, As, Cu, Zn, Fe, Mn, Ni, Se and Cr) measured in samples of this species (liver, kidney, muscle, blood, fat, stomach content and egg) are shown in Table 2.

A large variety of metals have been measured in green turtles, but the most frequently analyzed are again cadmium, lead and mercury (in that order), probably due to the importance of these heavy metals in the marine ecosystems because of the features previously described (see section “Loggerhead Sea Turtle”). The tissues mainly analyzed are liver, kidney and muscle (see Table 2). Generally, kidney showed the highest concentrations of cadmium [11, 19, 24, 38, 42, 43, 44, 48, 49, 50, 51, 56, 58, 59, 61] and lead [11, 20, 42, 44, 50, 56, 61]. But when calcified tissues were included in the studies, normally the highest lead levels were found in bones and carapaces (2.35 and 2.70 μg g^{-1} wet weight, respectively) [51]. The highest mercury levels were found in liver [11, 20, 50, 51, 56, 61] or kidney [43, 48], while arsenic was mainly retained in muscle [49, 56, 58, 59, 61] and blood [61], and chromium in kidney [11] and muscle [56].

Table 2. Heavy metal and essential element levels (µg g^{-1} dry weight) in tissues and other samples of *Chelonia mydas* from different locations

Tissue - Location	Cadmium		Lead		Mercury		Arsenic		References
	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	
Liver									
Mediterranean Sea: Cyprus	5,89	2,53 - 10,73	BDL	BDL - 1,84	0,55	0,27 - 1,37			[20]
Mediterranean Sea: Italy	13,31 ± 9,44	6,94 - 28,84							[19]
Atlantic Ocean: Costa Rica	10,60 ± 1,10		0,07 ± 0,01						[24]
Pacific Ocean: Hawaiian Is.	27,06 ± 26,69	1,22 - 81,25						BDL - 20,00	[38]
Pacific Ocean: Mexico	3,30	BDL - 102,00	BDL						[42]
Pacific Ocean: Mexico					0,09	0,03 - 0,17			[43]
Pacific Ocean: Mexico	16,92	BDL - 72,57	0,00	BDL - 0,07					[44]
Pacific Ocean: Australia**	39,06 ± 6,25	7,81 - 177,81			0,06 ± 0,01	0,00 - 0,16	0,81 ± 0,13	0,13 - 2,31	[48]
Pacific Ocean: Japan							1,76 ± 0,95	0,44 - 5,34	[49]
Pacific Ocean: Japan	17,44 ± 12,66	0,94 - 58,13	BDL		0,91 ± 0,50	0,16 - 2,00			[50]
Pacific Ocean: Japan	25,00			BDL - 0,38	0,59				[51]
Pacific Ocean: Japan	18,20 ± 9,70	3,58 - 38,30	0,51 ± 0,41	0,06 - 1,55	0,42 ± 019	0,23 - 0,88			[11]
Pacific Ocean: Japan	28,00	10,03 - 67,50	0,44	0,09 - 1,13					[52]
Pacific Ocean: Japan							3,65 ± 2,03		[54]

Table 2 (Continued)

Tissue - Location	Cadmium		Lead		Mercury		Arsenic		References
	Mean ± SD	Min – Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	
Pacific Ocean: Japan							3,75 ± 1,88	2,50 - 7,19	[55]
Pacific Ocean: China	1,45 ± 0,61	(A)	0,83 ± 0,09	(A)	0,13 ± 0,08	(A)	19,57 ± 1,98	(A)	[56]
	1,10 ± 0,10	(J)	0,15 ± 0,04	(J)	0,78 ± 0,19	(J)	4,65 ± 3,96	(J)	[56]
Pacific Ocean: Japan							5,30	0,90 - 9,70	[58]
Pacific Ocean: Japan							4,90 ± 3,30		[59]
Pacific Ocean: Australia	42,31 ± 7,50	13,53 - 101,44	0,28 ± 0,06	BDL - 0,88	0,59 ± 0,13	BDL - 1,69	9,97 ± 2,47	1,13 - 30,34	[61]
Kidney									
Mediterranean Sea: Cyprus	3,46		1,81		BDL				[20]
Mediterranean Sea: Italy	21,09 ± 9,29	9,00 - 31,21							[19]
Atlantic Ocean: Costa Rica	39,20 ± 3,10		0,04 ± 0,002						[24]
Pacific Ocean: Hawaiian Is.	108,32 ± 87,89	19,67 - 292,52						BDL - 28,34	[38]
Pacific Ocean: Mexico	121,00	6,09 - 653,00	0,01	BDL - 0,36					[42]
Pacific Ocean: Mexico					0,09	0,003 - 0,31			[43]
Pacific Ocean: Mexico	110,00	65,08 - 653,00	0,05	BDL - 1,74					[44]
Pacific Ocean: Australia**	63,76 ± 10,42	7,08 - 316,28			0,08 ± 0,02	0,00 - 0,21	0,79 ± 0,21	0,00 - 2,88	[48]
Pacific Ocean: Japan							5,72 ± 2,99	0,15 - 9,99	[49]

Pacific Ocean: Japan	160,43 ± 88,76	30,46 - 336,28	0,75 ± 0,29	0,21 - 1,17	0,54 ± 0,33	0,13 - 1,04			[50]
Pacific Ocean: Japan	171,89			BDL - 0,58	0,21				[51]
Pacific Ocean: Japan	142,00 ± 64,00	20,30 - 285,00	0,81 ± 0,56	0,16 - 2,38	0,30 ± 0,14	0,11 - 0,55			[11]
Pacific Ocean: China	2,49 ± 1,75	(J)	0,31 ± 0,19	(J)	0,34 ± 0,04	(J)	6,97 ± 0,05	(J)	[56]
Pacific Ocean: Japan							16,50	4,60 - 44,30	[58]
Pacific Ocean: Japan							17,00 ± 14,00		[59]
Pacific Ocean: Australia	191,60 ± 37,75	52,92 - 421,91	0,38 ± 0,04	BDL - 0,50	0,25 ± 0,08	BDL - 0,83	10,29 ± 3,71	0,50 - 38,63	[61]
Muscle									
Mediterranean Sea: Cyprus	0,37	0,12 - 0,78	BDL	BDL - 2,45	0,09	BDL - 0,37			[20]
Pacific Ocean: Mexico	0,01	BDL - 39,24	0,01	BDL - 1,23					[42]
Pacific Ocean: Mexico					0,02	0,003 - 0,06			[43]
Pacific Ocean: Japan							24,1 ± 13,1	2,58 - 74,9	[49]
Pacific Ocean: Japan	0,24 ± 0,38	0,05 - 2,57	BDL		0,10 ± 0,14	0,005 - 0,57			[50]
Pacific Ocean: Japan	0,10		BDL		0,02				[51]
Pacific Ocean: Japan	0,24 ± 0,17	0,07 - 0,64	0,09 ± 0,05	0,03 - 0,21	0,04 ± 0,07	BDL - 0,23			[11]
Pacific Ocean: China	0,17 ± 0,06	(A)	0,26 ± 0,11	(A)	0,05 ± 0,04	(A)	14,61 ± 7,47	(A)	[56]
		BDL - 0,10 (J)	0,08 ± 0,10	(J)	0,43 ± 0,22	(J)	14,45 ± 4,88	(J)	[56]
Pacific Ocean: Japan							61,60	11,20 - 165,00	[58]

Table 2 (Continued)

Tissue - Location	Cadmium		Lead		Mercury		Arsenic		References
	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	
Pacific Ocean: Japan							69,00 ± 52,00		[59]
Pacific Ocean: Australia*	0,08 ± 0,01	0,05 - 0,19	BDL		0,03 ± 0,004	BDL - 0,05	5,25 ± 1,47	0,32 - 14,01	[61]
Pacific Ocean: Australia	0,38 ± 0,05	0,24 - 0,90	BDL		0,14 ± 0,02	BDL - 0,24	25,00 ± 7,00	1,52 - 66,72	[61]
Blood									
Pacific Ocean: Australia	0,20 ± 0,05	0,005 - 0,61	0,10 ± 0,03	0,03 - 0,31	0,01 ± 0,003	0,001 - 0,04	22,24 ± 7,19	0,46 - 101,93	[61]
Fat									
Atlantic Ocean: Costa Rica	0,11 ± 0,02		0,06 ± 0,03						[24]
Pacific Ocean: Mexico	0,002	BDL - 1,47	0,03	BDL - 1,11					[42]
Pacific Ocean: Mexico					0,005	BDL - 0,01			[43]
Pacific Ocean: Japan	0,07		BDL		0,004				[51]
Pacific Ocean: China	BDL	(J)	0,09 ± 0,03	(J)	0,005 ± 0,003	(J)	1,27 ± 0,20	(J)	[56]
Stomach content									
Pacific Ocean: Mexico		2,22 - 11,11		BDL - 0,12					[44]
Pacific Ocean: Japan	0,37 ± 0,40	0,05 - 1,03	0,20 ± 0,05	0,13 - 0,28	0,03 ± 0,01	BDL - 0,05			[11]
Pacific Ocean: China	175,50 ± 248,00	(J)	0,60 ± 0,70	(J)	0,3 ± 0,06	(J)	4,06 ± 2,08	(J)	[56]
Pacific Ocean: Japan							6,10		[59]

Egg									
Pacific Ocean: Hawaiian Is.	Shell: 0,49						Shell: BDL		[38]
Pacific Ocean: Japan*	Egg: BDL		Egg: BDL		Egg: 0,001				[51]
Pacific Ocean: Japan	Yolk: BDL		Yolk: BDL		Yolk: 0,01				[51]
	Albumen: BDL		Albumen: BDL		Albumen: 0,002				[51]
	Shell: BDL		Shell: BDL		Shell: 0,002				[51]
Pacific Ocean: Hong Kong	Yolk: BDL		Yolk: 0,13 ± 0,03	0,08 - 0,37	Yolk: 0,005 ± 0,0003	0,003 - 0,005	Yolk: 6,68 ± 0,99	3,74 - 13,35	[57]
	Albumen: BDL		Albumen: 0,19 ± 0,04	BDL - 0,37	Albumen: 0,004 ± 0,001	0,004 - 0,07	Albumen: 6,30 ± 0,74	3,70 - 12,59	[57]
	Shell: 0,05 ± 0,02	BDL - 0,39	Shell: 0,27 ± 0,05	0,07 - 0,68	Shell: 0,002 ± 0,0005	0,0002 - 0,005	Shell: 0,54 ± 0,05	0,32 - 0,90	[57]
Pacific and Indian Ocean: Malaysia*	Egg:	BDL - 0,03	Egg:	BDL - 0,12	Egg: BDL		Egg:	BDL - 0,35	[60]
Liver									
Mediterranean Sea: Italy	102,34 ± 51,31	57,69 - 184,31	107,91 ± 41,75	60,22 - 168,38					[19]
Atlantic Ocean: Costa Rica	100,00 ± 11,00		82,50 ± 4,90		2482,00 ± 286,00		8,92 ± 0,93		[24]
Pacific Ocean: Hawaiian Is.	273,77 ± 192,69	4,06 - 590,63	95,55 ± 31,19	47,19 - 143,13	3661,01 ± 2410,57	290,00 - 7656,25	4,99 ± 2,03	0,47 - 8,72	[38]
Pacific Ocean: Mexico	60,04	6,79 - 133,00	62,91	1,32 - 166,00	14,35	BDL - 1765,00			[42]
Pacific Ocean: Mexico									[43]

Table 2 (Continued)

Tissue - Location	Cadmium		Lead		Mercury		Arsenic		References
	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	
Pacific Ocean: Mexico	76,52	6,79 - 128,00	90,95	41,81 - 109,00	350,00	BDL - 671,00	0,24	BDL - 5,31	[44]
Pacific Ocean: Australia**			124,06 ± 9,38	52,19 - 289,69					[48]
Pacific Ocean: Japan	156,88 ± 98,75	13,34 - 353,13	94,69 ± 22,28	54,69 - 147,19	1440,63 ± 812,50	102,19 - 3968,75	5,81 ± 2,63	2,19 - 16,91	[50]
Pacific Ocean: Japan	34,75		182,19		423,44		5,91		[51]
Pacific Ocean: Japan	139,00 ± 86,00	36,40 - 340,00	87,20 ± 30,60	42,60 - 166,00			4,74 ± 2,06	2,28 - 11,10	[11]
Pacific Ocean: Japan	155,94	57,50 - 406,25	104,06	69,38 - 176,88					[52]
Pacific Ocean: China	9,17 ± 2,89	(A)	211,60 ± 30,57	(A)			10,50 ± 4,03	(A)	[56]
	133,00 ± 148,60	(J)	128,90 ± 63,92	(J)			16,27 ± 13,81	(J)	[56]
Pacific Ocean: Australia	283,22 ± 36,38	119,44 - 479,38	113,44 ± 6,75	64,78 - 147,63					[61]
Kidney									
Mediterranean Sea: Italy	34,17 ± 17,50	20,00 - 59,59	109,97 ± 43,80	60,71 - 160,35					[19]
Atlantic Ocean: Costa Rica	8,34 ± 1,75		77,40 ± 1,90		300,00 ± 25,00		5,75 ± 0,28		[24]
Pacific Ocean: Hawaiian Is.	15,14 ± 11,41	4,58 - 43,75	92,89 ± 31,33	52,09 - 158,76	180,36 ± 228,22	36,67 - 745,89	4,00 ± 1,32	2,00 - 5,79	[38]
Pacific Ocean: Mexico	5,67	1,59 - 20,36	128,00	1,59 - 330,00	44,09	BDL - 516,00	0,31	BDL - 8,12	[42]
Pacific Ocean: Mexico	5,83	1,98 - 11,60	189,00	102,00 - 281,00	93,16	BDL - 547,00	1,51	BDL - 7,73	[44]

Pacific Ocean: Australia**			88,76 ± 2,92	64,17 - 132,51					[48]
Pacific Ocean: Japan	8,96 ± 3,58	3,96 - 16,42	123,34 ± 30,79	72,92 - 186,26	95,01 ± 60,84	47,50 - 246,69	5,04 ± 1,33	3,00 - 9,58	[50]
Pacific Ocean: Japan	6,33		141,68		57,92		5,50		[51]
Pacific Ocean: Japan	8,27 ± 4,06	2,41 - 18,90	169,00 ± 61,00	85,50 - 352,00			5,60 ± 1,38	2,78 - 8,36	[11]
Pacific Ocean: China	15,20 ± 7,22	(J)	143,10 ± 12,42	(J)			11,59 ± 4,79	(J)	[56]
Pacific Ocean: Australia	10,71 ± 1,00	5,83 - 18,96	119,80 ± 9,29	74,59 - 200,81					[61]
Muscle									
Pacific Ocean: Mexico	0,03	BDL - 13,76	38,26	10,44 - 134,00	20,99	BDL - 225,00	0,003	BDL - 7,75	[42]
Pacific Ocean: Japan	1,67 ± 1,95	0,38 - 12,62	41,86 ± 26,24	15,71 - 159,05	25,14 ± 21,91	4,62 - 140,96	0,52 ± 0,29	0,24 - 1,71	[50]
Pacific Ocean: Japan	1,24		46,05		54,24		1,29		[51]
Pacific Ocean: Japan	0,88 ± 0,42	0,54 - 2,06	47,70 ± 18,60	24,40 - 69,20			0,46 ± 0,10	0,32 - 0,61	[11]
Pacific Ocean: China	1,56 ± 0,11	(A)	238,70 ± 101,60	(A)			5,04 ± 3,14	(A)	[56]
	3,74 ± 1,64	(J)	147,70 ± 12,62	(J)			1,28 ± 0,52	(J)	[56]
Pacific Ocean: Australia	1,62 ± 0,33	0,71 - 5,05	58,86 ± 13,48	28,33 - 185,43					[61]
Blood									
Pacific Ocean: Australia	5,20 ± 0,50	2,01 - 8,26	40,39 ± 34,07	17,95 - 62,53					[61]
Fat									
Atlantic Ocean: Costa Rica	0,45 ± 0,09		62,10 ± 8,40		45,20 ± 6,10		0,83 ± 0,13		[24]

Table 2 (Continued)

Tissue - Location	Cadmium		Lead		Mercury		Arsenic		References
	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	
Pacific Ocean: Japan	0,37		60,64		22,34		0,24		[51]
Pacific Ocean: Mexico	0,01	BDL - 9,48	49,82	19,51 - 163,00	2,63	BDL - 154,00	0,003	BDL - 0,79	[42]
Pacific Ocean: China	0,99 ± 0,38	(J)	105,00 ± 17,48	(J)			0,19 ± 0,06	(J)	[56]
Stomach content									
Pacific Ocean: Mexico		0,02 - 5,70		8,82 - 60,96		31,81 - 317,00		4,02 - 13,08	[44]
Pacific Ocean: Japan	9,41 ± 5,88	1,51 - 16,90	6,42 ± 2,37	3,79 - 9,53			7,63 ± 6,03	2,45 - 18,90	[11]
Pacific Ocean: China	10,42 ± 2,46	(J)	254,60 ± 121,40	(J)			28,82 ± 32,58	(J)	[56]
Egg									
Pacific Ocean: Hawaiian Is.	Shell: 34,89		Shell: 15,25		Shell: 34,40		Shell: 0,76		[38]
Pacific Ocean: Japan*	Egg: 0,78		Egg: 20,30		Egg: 10,90		Egg: 0,38		[51]
Pacific Ocean: Japan	Yolk: 1,68		Yolk: 126,02		Yolk: 65,15		Yolk: 1,52		[51]
	Albumen: 5,93		Albumen: 47,78		Albumen: 39,63		Albumen: BDL		[51]
	Shell: 11,57		Shell: 1,37		Shell: 4,83		Shell: 3,25		[51]
Pacific Ocean: Hong Kong	Yolk: 0,91 ± 0,11	0,45 - 2,06	Yollk: 120,15 ± 9,61	66,75 - 181,56	Yolk: 120,15 ± 7,74	77,43 - 176,22	Yolk: 0,72 ± 0,11	0,27 - 1,42	[57]

Pacific and Indian Ocean: Malaysia*	Albumen: 2,22 ± 0,37 Shell: 3,17 ± 0,81 Egg:	1,11 - 6,67 0,59 - 10,25 0,06 - 1,07	Albumen: 11,11 ± 2,22 Shell: 2,93 ± 0,56 Egg:	1,48 - 27,78 0,76 - 6,34 1,33 - 39,48	Albumen: 140,75 ± 27,78 Shell: 5,61 ± 0,95	17,04 - 348,18 0,37 - 13,91		Albumen: 1,85 ± 0,37 Shell: 0,66 ± 0,17	0,74 - 5,19 0,07 - 1,88	[57] [57] [60]
Liver									
Pacific Ocean: Hawaiian Is.		BDL	3,10 ± 2,85	0,43 - 10,59				BDL - 1,56	[38]
Pacific Ocean: Mexico	0,01	BDL - 7,40							[42]
Pacific Ocean: Mexico	0,00	BDL - 30,88							[44]
Pacific Ocean: Australia**			3,69 ± 0,50	0,22 - 8,38					[48]
Pacific Ocean: Japan		0,19 - 0,97							[50]
Pacific Ocean: Japan	0,22								[51]
Pacific Ocean: Japan			5,10 ± 2,30	2,00 - 9,90	2,20 ± 0,60			1,50 - 3,50	[11]
Pacific Ocean: Japan			10,31	2,72 - 23,44					[52]
Pacific Ocean: China	0,71 ± 0,03	(A)	12,43 ± 0,78	(A)	0,85 ± 0,21			(A)	[56]
	0,27 ± 0,25	(J)	25,65 ± 28,60	(J)				BDL - 1,11 (J)	[56]
Pacific Ocean: Australia			12,34 ± 3,38	1,63 - 32,50					[61]
Kidney									
Pacific Ocean: Hawaiian Is.		BDL - 4,17	1,93 ± 1,78	0,66 - 6,58				BDL - 1,67	[38]
Pacific Ocean: Mexico	1,15	BDL - 26,43							[42]
Pacific Ocean: Mexico	3,19	1,19 - 25,13							[44]
Pacific Ocean: Australia**			2,46 ± 0,38	0,38 - 7,71					[48]
Pacific Ocean: Japan	2,58 ± 1,54	0,50 - 5,54							[50]
Pacific Ocean: Japan	2,13								[51]
Pacific Ocean: Japan			5,30 ± 2,40	2,10 - 11,00	2,20 ± 0,70			1,40 - 3,80	[11]

Table 2 (Continued)

Tissue - Location	Nickel		Selenium		Chromium		*References*
	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	
Pacific Ocean: China	0,20 ± 0,13	(J)	5,89 ± 1,26	(J)	1,07 ± 0,40	(J)	[56]
Pacific Ocean: Australia			6,96 ± 2,04	1,21 - 21,29			[61]
Muscle							
Pacific Ocean: Mexico	0,03	BDL - 4,00					[42]
Pacific Ocean: Japan	BDL						[50]
Pacific Ocean: Japan		BDL - 0,29					[51]
Pacific Ocean: Japan			3,20 ± 1,00	1,00 - 4,80	1,40 ± 0,20	1,20 - 1,80	[11]
Pacific Ocean: China	1,07 ± 0,97	(A)	17,02 ± 20,17	(A)	2,18 ± 2,55	(A)	[56]
	0,22 ± 0,21	(J)	4,49 ± 1,09	(J)	2,71 ± 2,85	(J)	[56]
Pacific Ocean: Australia			5,57 ± 1,48	0,90 - 17,14			[61]
Blood							
Pacific Ocean: Australia			12,50 ± 3,21	0,35 - 46,34			[61]
Fat							
Pacific Ocean: Mexico	0,02	BDL - 13,42					[42]
Pacific Ocean: Japan		BDL - 0,07					[51]
Pacific Ocean: China	0,15 ± 0,05	(J)	0,72 ± 0,11	(J)	0,91 ± 0,24	(J)	[56]
Stomach content							
Pacific Ocean: Mexico		0,90 - 9,67					[44]
Pacific Ocean: Japan			1,00 ± 0,50	0,37 - 1,80	1,30 ± 0,80	0,53 - 2,80	[11]
						BDL - 1,19	
Pacific Ocean: China	0,39 ± 0,43	(J)	8,07 ± 1,78	(J)		(J)	[56]
Egg							
Pacific Ocean: Hawaiian Is.	Shell: BDL				Shell: 0,98		[38]
Pacific Ocean: Japan*	Egg: BDL						[51]
Pacific Ocean: Japan	Yolk: BDL						[51]

	Albumen: BDL						[51]
	Shell: BDL						[51]
Pacific Ocean: Hong Kong	Yolk: 0,51 ± 0,08	0,19 - 1,01	Yolk: 9,35 ± 1,60	3,47 - 20,29	Yolk: 2,51 ± 0,32	0,99 - 4,54	[57]
	Albumen: 0,74 ± 0,37	0,02 - 2,59	Albumen: 10,00 ± 2,22	2,96 - 27,04	Albumen: 1,85 ± 0,74	0,37 - 5,19	[57]
	Shell: 29,28 ± 3,66	6,34 - 51,24	Shell: 6,10 ± 2,03	1,10 - 26,84	Shell: 1,15 ± 0,24	BDL - 2,93	[57]
Pacific and Indian Ocean: Malaysia*			Egg:	0,05 - 0,84			[60]

BDL = bellow detection limit; * = µg g^{-1} wet weight; ** = Mean ± SEM; J = juvenile; A = adult; Egg = whole egg.

In the case of mercury and cadmium, these levels followed the patterns of metal accumulation that were described for other marine vertebrates [65, 66]. As we observed in loggerhead sea turtles, mercury levels tended to be highest in liver tissue and cadmium levels tended to be highest in kidney tissue.

Several studies have emphasized that food is the main source of exposure to heavy metals as cadmium, and therefore carnivorous animals (as loggerhead sea turtle) often show metal levels comparatively higher than herbivorous (as green sea turtle) [17, 67]. However, in this review we observe similar ranges in cadmium levels in loggerhead and green sea turtles from Mediterranean Sea, Atlantic Ocean and Pacific Ocean (see Table 1 and 2). Both species showed the highest cadmium levels in specimens from Pacific Ocean, where a major cadmium enrichment can be present.

Concerning essential metals, a large variety of these have been measured in tissues of *Chelonia mydas*, although the most frequently analyzed are zinc, copper and manganese (in that order). The tissues mainly used on this matter are liver, kidney and muscle (see Table 2). The highest copper levels were found in liver [11, 19, 24, 38, 42, 44, 50, 51, 52, 56, 61], while bone and carapace contained considerable levels of zinc (176.50 and 319.50 μg g^{-1} wet weight, respectively) [51]. The highest manganese levels were found in liver [24, 38, 50, 51, 56] and stomach content [11, 56]. If we observe the levels of these elements in whole eggs, we can see that zinc and iron exhibited the highest levels, while mercury, cadmium, lead, arsenic, copper, manganese, nickel and selenium showed the lowest ones [51, 60]. Metals in eggs were mainly accumulated in the yolk. Levels of lead, mercury, arsenic, copper, zinc, iron, manganese, nickel, selenium and chromium were higher in yolk than in albumen [51, 57], whereas eggshell contained the highest levels of copper, iron and nickel [38, 51, 57].

LEATHERBACK SEA TURTLE (*DERMOCHELYS CORIACEA*)

The available data about levels of metals and other inorganic elements (Cd, Pb, Hg, As, Cu, Zn, Fe, Mn, Ni, Se and Cr) in samples of leatherback sea turtle are shown in Table 3. Liver, kidney, muscle, blood, fat and eggs are the samples mainly analyzed. The elements most frequently analyzed in samples of this species are cadmium, copper and zinc, followed by lead, mercury and selenium. Referring to the eleven elements included in this chapter, there is no information about iron, manganese and chromium, which were not measured by any of the consulted studies.

Table 3. Heavy metal and essential element levels (µg g^{-1} dry weight) in tissues and other samples of *Dermochelys coriacea* from different locations

Tissue - Location	Cadmium		Lead		Mercury		Arsenic		References
	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	
Liver									
Atlantic Ocean: United Kingdom	0,22 ± 0,02		0,12 ± 0,02		0,39 ± 0,04		0,58 ± 0,11		[26]
Atlantic Ocean: France	21,38 ± 11,44	1,88 - 45,94							[10]
Kidney									
Atlantic Ocean: France	126,26 ± 117,09	35,29 - 258,35							[10]
Muscle									
Atlantic Ocean: United Kingdom	0,06 ± 0,01		0,31 ± 0,03		0,12 ± 0,06		0,21 ± 0,07		[26]
Atlantic Ocean: France	1,67 ± 0,95	0,76 - 4,76							[10]
Blood									
Atlantic Ocean: French Guiana	0,41 ± 0,15		0,92 ± 0,26		0,05 ± 0,02				[35]
Atlantic Ocean: US	0,36 ± 0,10	0,26 - 0,61	0,66	0,36 - 1,02	0,05 ± 0,00	0,05 - 0,10			[37]
Fat									
Atlantic Ocean: United Kingdom	BDL		0,04 ± 0,03		0,11 ± 0,02		1,28 ± 0,18		[26]

Table 3 (Continued)

Tissue - Location	Cadmium		Lead		Mercury		Arsenic		References
	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	
Egg									
Atlantic Ocean: French Guiana	Egg content:	0,1 ± 0,005	Egg content:	0,21 ± 0,005	Egg content:	0,05 ± 0,02			[35]
Pacific Ocean: Mexico	Shell:	0,90 ± 0,61	Shell:	11,60 ± 26,00					[39]
Liver									
Atlantic Ocean: United Kingdom	0,15 ± 0,04		2,62 ± 0,15		2,13 ± 0,16		1,41 ± 0,02		[26]
Atlantic Ocean: France	26,91 ± 13,75	3,28 - 61,56	91,25 ± 12,81	68,44 - 114,06					[10]
Kidney									
Atlantic Ocean: France	11,17 ± 1,38	9,83 - 12,58	107,09 ± 32,09	77,09 - 140,84					[10]
Muscle									
Atlantic Ocean: United Kingdom	0,26 ± 0,05		1,89 ± 0,10		1,62 ± 0,21		3,61 ± 0,48		[26]
Atlantic Ocean: France	4,52 ± 2,33	1,90 - 12,19	123,34 ± 28,10	87,14 - 177,62					[10]
Blood									
Atlantic Ocean: French Guiana	6,83 ± 1,43		56,61 ± 1,43				50,90 ± 0,26		[35]
Atlantic Ocean: US	5,05 ± 0,71	3,52 - 6,07					38,51 ± 14,43	20,15 - 68,49	[37]

Fat									
Atlantic Ocean: United Kingdom	0,06 ± 0,02		0,08 ± 0,01		0,07 ± 0,02		BDL		[26]
Egg									
Atlantic Ocean: French Guiana	Egg content:	3,28 ± 0,52	Egg content:	73,75 ± 11,61			Egg content:	7,50 ± 1,98	[35]
Pacific Ocean: Mexico	Shell:	8,90 ± 1,26	Shell:	11,90 ± 10,00	Shell:	7,90 ± 5,11			[39]

BDL = bellow detection limit.

The highest cadmium levels were found in kidney [10], whereas blood exhibited the highest lead concentrations [35, 37], although no data are available on lead concentrations in kidney. The majority of the mercury burden was found in liver [26].

In spite of the limited available data, cadmium and mercury followed the expected patterns of metal accumulation [17]: cadmium tends to accumulate mainly in kidney and mercury in liver. In the case of lead, a higher accumulation in calcified tissues would be expected, but no data on bones or carapaces of leatherback sea turtle are available.

The diet of leatherback sea turtles consists almost exclusively on jellyfish [67]. Caurant et al. [10] suggested that the ingestion of great quantities of jellyfish could imply a high exposure to cadmium for the leatherback sea turtles than for other fish-eating or herbivorous vertebrates [10, 17], although this prey has been very poorly studied. However, we observe that even the herbivorous green turtles can accumulate similar or higher cadmium levels than leatherback sea turtles in different areas of the world (range of cadmium levels in kidney of green turtles from Asia-Pacific area and Indian Ocean: 7.08 – 421.91 μg g^{-1} dry weight, see Table 2). These changes in cadmium accumulation can be related with differences among the diet of sea turtles depending on different stages of development and different distribution areas. For these reasons, it is important to study sea turtles throughout their growth and in the different regions of the world where each species is distributed.

Concerning essential elements, the highest copper and selenium concentrations were found in liver and blood, respectively, while considerable zinc concentrations were found in muscle, kidney and liver [10]. Calcified tissues, as bone and carapace, could contain higher zinc concentrations [17], although currently no data on these samples have been found. On the other hand, fat tissue showed low zinc levels [26] in contrast with the higher ones detected in other species of sea turtles [18, 22, 24, 42, 51, 56].

Only two works have studied metal levels in leatherback sea turtle eggs: Vazquez et al. [39] analyzed eggshells from the Pacific coast of Mexico and Guirlet et al. [35] analyzed egg contents from the Atlantic coast of French Guiana. It is difficult to compare both studies due to they were carried out in different areas and dates. However, these results seem to reflect that lead and copper are mainly concentrated in eggshells, whereas zinc shows the highest levels in egg contents. In the case of zinc, this great presence in yolk and albumen may be in relation to necessary requirement for embryonic development [17]. Referring to lead, levels in eggshell [39] were two orders of magnitude higher than those found

in other tissues and samples (see Table 3). This fact can reflect that eggshell is an important way for lead excretion in this species.

HAWKSBILL SEA TURTLE (*ERETMOCHELYS IMBRICATA*)

The available data about levels of inorganic elements (Cd, Pb, Hg, As, Cu, Zn, Fe, Mn, Ni, Se and Cr) in different tissues (liver, kidney, muscle, fat) and stomach content of hawksbill sea turtles are shown in Table 4. Nowadays, there are no data on levels of these elements in blood and egg of this species.

The tissues mainly analyzed are liver, kidney and muscle, whereas cadmium, lead, arsenic, copper and zinc are the metals mainly analyzed. Only one study measured iron and nickel levels [42] and another one measured chromium levels [11]. In spite of hawksbill sea turtles have a circumglobal distribution throughout waters of the Atlantic, Indian and Pacific Ocean [1], all the consulted studies were carried out in the Pacific coasts (Mexico, Japan and Australia).

The highest cadmium levels were detected in kidney (maximum level: 310.00 $\mu g\ g^{-1}$ dry weight) [11], whereas lead showed similar levels in all the studied samples (range: BDL – 1.12 $\mu g\ g^{-1}$ dry weight, see Table 4). The majority of the mercury burden was found in kidney and liver [11], and arsenic showed especially high levels in muscle [49, 59].

Cadmium levels which were found in the stomach contents of hawksbill sea turtles from Pacific coasts of Japan were greatly lower than those measured in kidney of these specimens (22.47 – 885.71 times lower, see Table 4). The interpretation of these results is not simple, due to the diet shifts that happen in sea turtles throughout their life cycle and among individuals [11, 50]. For example, although the concentration of cadmium in the diet of juvenile specimens could be higher than in adults, cadmium levels in the renal tissue of an adult specimen reflect intakes from the current and previous diet. This is due to the very long biological half-life that heavy metals present in the organisms. With respect to the trophic transfer of elements in this species, cadmium and mercury showed the highest trophic transfer coefficient (TTC) [11]. This fact suggests that bioaccumulation or biomagnification phenomenoms could be happening for these metals in hawksbill sea turtles. Besides, renal cadmium concentrations in some specimens were close to or above values that can cause renal damages [11].

Table 4. Heavy metal and essential element levels (μg g^{-1} dry weight) in tissues and other samples of *Eretmochelys imbricata* from different locations

Tissue - Location	Cadmium		Lead		Mercury		Arsenic		Refs.
	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	
Liver									
Pacific Ocean: Mexico	0,49		BDL						[42]
Pacific Ocean: Australia**		7,50 - 19,38				0,13 - 0,16		0,56 - 5,78	[48]
Pacific Ocean: Japan							15,30 ± 8,77	4,94 - 32,80	[49]
Pacific Ocean: Japan	7,05 ± 6,37	1,80 - 33,60	0,17 ± 0,13	0,02 - 0,53	0,87 ± 1,87	0,05 - 8,70			[11]
Pacific Ocean: Japan	3,84	1,97 - 5,69	0,09	0,03 - 0,19					[52]
Pacific Ocean: Japan							13,75 ± 7,81	2,06 - 23,44	[55]
Pacific Ocean: Japan							25,00 ± 32,00		[59]
Kidney									
Pacific Ocean: Mexico	4,2		BDL						[42]
Pacific Ocean: Australia**		15,00 - 52,92				0,13 - 0,17		0,54 - 3,88	[48]
Pacific Ocean: Japan							28,30 ± 9,82	8,62 - 36,60	[49]
Pacific Ocean: Japan	93,70 ± 76,30	19,10 - 310,00	0,27 ± 0,24	0,09 - 1,12	1,30 ± 1,20	0,08 - 5,00			[11]
Pacific Ocean: Japan							45,00 ± 38,00		[59]
Muscle									
Pacific Ocean: Mexico	1,02		0,38						[42]
Pacific Ocean: Japan							153,00 ± 65,10	23,10 - 205,00	[49]

Pacific Ocean: Japan	0,07 ± 0,04	0,02 - 0,15	0,04 ± 0,05	0,003 - 0,15	0,04 ± 0,03	BDL - 0,09			[11]
Pacific Ocean: Japan							210,00 ± 140,00		[59]
Fat									
Pacific Ocean: Mexico	0,43		BDL						[42]
Stomach content									
Pacific Ocean: Japan	0,59 ± 0,21	0,35 - 0,85	0,21 ± 0,09	0,06 - 0,31	0,04 ± 0,03	BDL - 0,09			[11]
Pacific Ocean: Japan							22,00		[59]
Liver									
Pacific Ocean: Mexico	2,47		25,89		71,88		74,00		[42]
Pacific Ocean: Australia**				55,31 - 94,69					[48]
Pacific Ocean: Japan	54,90 ± 116,00	8,28 - 570,00	109,00 ± 54,00	52,30 - 306,00			8,29 ± 4,26	3,76 - 17,90	[11]
Pacific Ocean: Japan	26,94	14,66 - 59,06	79,69	69,38 - 92,19					[52]
Kidney									
Pacific Ocean: Mexico	3,89		82,45		362,00		7,62		[42]
Pacific Ocean: Australia**				55,00 - 87,09					[48]
Pacific Ocean: Japan	7,04 ± 2,79	4,89 - 17,90	120,00 ± 32,00	81,20 - 200,00			13,20 ± 2,75	6,09 - 17,70	[11]
Muscle									
Pacific Ocean: Mexico	3,68		102,00		258,00		1,78		[42]
Pacific Ocean: Japan	0,96 ± 0,32	0,61 - 1,46	48,60 ± 26,10	25,80 - 111,00			0,70 ± 0,52	0,42 - 2,08	[11]

Table 4 (Continued)

Tissue - Location	Cadmium		Lead		Mercury		Arsenic		Refs.
	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	
Fat									
Pacific Ocean: Mexico	0,72		42,39		11,14		2,53		[42]
Stomach content									
Pacific Ocean: Japan	5,02 ± 1,60	3,22 - 7,10	24,20 ± 18,20	6,84 - 50,80			3,55 ± 2,17	0,94 - 6,30	[11]
Liver									
Pacific Ocean: Mexico	2,48								[42]
Pacific Ocean: Australia**					8,38 - 11,41				[48]
Pacific Ocean: Japan			49,00 ± 37,00		12,00 - 150,00	0,85 ± 0,68	0,21 - 2,10		[11]
Pacific Ocean: Japan			59,38		9,06 - 100,00				[52]
Kidney									
Pacific Ocean: Mexico	1,61								[42]
Pacific Ocean: Australia**					9,25 - 10,38				[48]
Pacific Ocean: Japan			28,00 ± 19,00		9,00 - 76,00	1,60 ± 0,80	0,30 - 2,90		[11]
Muscle									
Pacific Ocean: Mexico	BDL								[42]
Pacific Ocean: Japan			11,00 ± 10,00		2,60 - 33,00	1,10 ± 1,10	0,12 - 3,00		[11]
Fat									
Pacific Ocean: Mexico	BDL								[42]
Stomach content									
Pacific Ocean: Japan			14,00 ± 10,00		3,60 - 29,00	0,61 ± 0,19	0,40 - 0,89		[11]

BDL = bellow detection limit; ** = Mean ± SEM.

The essential elements copper and selenium showed the highest levels in liver [11, 42, 52], whereas zinc was mainly accumulate in kidney and muscle [11, 42].

The high copper levels that were found in liver of hawksbill sea turtles (maximum level: 570.00 μg g^{-1} dry weight) [11] exceeded in some cases the copper levels that are homeostatically controlled in other marine animals (10 – 100 μg g^{-1} dry weight in liver of marine mammals) [68]. These high levels could exert adverse effects in the biological systems of sea turtles, such as the formation of reactive oxygen species or the irreversibly and non-specifically association with thiol groups in proteins [69].

Besides the study of carcasses, the development of studies about metal levels in eggs and blood of this species in the future will be of great interest. These studies will contribute to the standardization of non-destructive methods for long-term monitoring of metals in hawksbill sea turtles.

KEMP'S RIDLEY SEA TURTLE (*LEPIDOCHELYS KEMPII*)

The available data about levels of serveral inorganic elements (Cd, Pb, Hg, Cu, Zn, Se and Cr) in different samples (liver, kidney, muscle and blood; no available data on fat, stomach content or eggs) of Kemp's ridley sea turtles are shown in Table 5. No data on arsenic, iron, manganese and nickel levels have been found.

All the published studies were carried out in North Atlantic [10, 30, 34, 36] since the distribution of this species is restricted to this area [1].

Blood is the most used sample for metal determination in the studies that included samples of Kemp's ridley sea turtles in their analysis. Cadmium, lead, mercury, copper and zinc are the most studied elements (see Table 5).

Regarding non-essential heavy metals, cadmium and mercury were mainly accumulated in liver and kidney (kidney > liver for cadmium and liver > kidney for mercury), whereas lead showed similar levels in all the analyzed soft tissues.

In the case of cadmium, levels in kidney (a target organ for cadmium accumulation) were considerably higher in other species of sea turtles (see Tables 1, 2, 3, 4 and 6) than cadmium levels measured in kidney of Kemp's ridley sea turtles (see Table 5), even for sub-adult and adult specimens (Kemp's ridley sea turtles SCL range: 33.90-64.00 cm) [34]. This carnivore species preys primarily upon crustaceans such as blue crabs (*Callinectes sapidus*). Blue crabs from American Atlantic coasts exhibited cadmium levels ranging from 0.001 to 1.76 μg

Table 5. Heavy metal and essential element levels (μg g^{-1} dry weight) in tissues and other samples of *Lepidochelys kempii* from different locations

Tissue - Location	Cadmium		Lead		Mercury		Copper		References
	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	
Liver									
Atlantic Ocean: US	2,13 ± 1,53	0,60 - 5,74	0,15 ± 0,15	0,03 - 0,48	1,23 ± 1,96	0,02 - 6,11	14,70 ± 7,65	2,42 - 26,70	[34]
Atlantic Ocean: US (J)					0,22 ± 0,22	0,06 - 0,66			[36]
Kidney									
Atlantic Ocean: US	2,41 ± 4,07	0,13 - 11,90	0,16 ± 0,15	0,03 - 0,47	0,74 ± 0,43	0,21 - 1,31	7,78 ± 3,35	3,19 - 13,00	[34]
Muscle									
Atlantic Ocean: France	0,43 ± 0,43	0,05 - 1,24					4,67 ± 2,38	2,10 - 8,81	[10]
Atlantic Ocean: US	0,05 ± 0,04	0,01 - 0,16	0,14 ± 0,18	0,01 - 0,59	0,43 ± 0,34	0,01 - 0,97	2,35 ± 1,54	0,33 - 5,89	[34]
Blood									
Atlantic Ocean: US			0,05	0,00 - 0,15	0,1	0,003 - 0,36	2,65	1,12 - 6,63	[30]
Atlantic Ocean: US	0,1	0,002 - 0,26	0,15	0,004 - 0,87	0,1	0,003 - 0,92	7,40	0,92 - 23,26	[34]
Atlantic Ocean: US (J)					0,10 ± 0,05	BDL - 0,20			[36]
Atlantic Ocean: US* plasma (J)	BDL						0,69 ± 0,68	0,33 - 3,18	[36]

BDL = bellow detection limit; * = μg g^{-1} wet weight; J = juvenile.

Table 5 (Continued)

Tissue - Location	Zinc		Selenium		Chromium		References
	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	
Liver							
Atlantic Ocean: US	69,00 ± 18,60	45,20 - 108,00			0,02 ± 0,01	0,006 - 0,05	[34]
Kidney							
Atlantic Ocean: US	134,00 ± 22,40	98,90 - 172,00			0,02 ± 0,01	0,01 - 0,04	[34]
Muscle							
Atlantic Ocean: France	78,10 ± 15,71	63,33 - 97,62					[10]
Atlantic Ocean: US	31,40 ± 13,80	10,30 - 60,80			0,05 ± 0,09	0,0005 - 0,30	[34]
Blood							
Atlantic Ocean: US	38,25	16,73 - 96,39					[30]
Atlantic Ocean: US	87,67	1,79 - 268,77			0,10	0,0005 - 1,07	[34]
Atlantic Ocean: US* plasma (J)	2,29 ± 3,55	0,51 - 13,07	0,49 ± 0,06	0,40 - 0,61			[36]

* = µg g^{-1} wet weight; J = juvenile.

g^{-1} wet weight [34]. These levels are similar or lower than other ones measured in the stomach contents of other species of sea turtles (see Tables 2 and 4). The scarce and fragmented information available about cadmium levels in their preys are not enough to explain the important inter-specific differences that were observed in renal tissues of sea turtles. Deeper investigations are needed to understand if these differences are due to different cadmium levels in their preys or different pollution levels in the distribution areas. As well as metabolic and physiological differences among these species could be involved too [10].

Referring to lead, the burden of this metal in carapace samples of Kemp's ridley sea turtles from American Atlantic coasts (1.39 ± 2.61 μg g^{-1} dry weight) [34] was one order of magnitude higher than the lead burden measured in soft tissues of the same specimens (see Table 5). These results point out that calcified tissues such as carapace accumulate lead largely. It can be useful for long-term biomonitoring of this element, which is a quite reliable indicator for assess the presence of anthropogenic pollution [64].

Copper and zinc are the most studied essential elements and showed the higher levels in liver and kidney, respectively (see Table 5). Concerning copper, this pattern of major accumulation in liver is similar in all the species of sea turtles (see Tables 1, 2, 3, 4, 6), except for *Natator depressus* (no available data). Probably these high copper levels in liver are related to specific features that are present in sea turtles. These features could allow the accumulation of higher hepatic levels of copper in sea turtles than in other marine organisms [17].

Besides the results showed in Table 5, high zinc levels were detected in carapace samples of Kemp's ridely sea turtles from American Atlantic coasts (270 ± 170 μg g^{-1} dry weight) [34]. This fact indicates that, similar to lead, zinc presents high affinity for calcified tissues.

OLIVE RIDLEY SEA TURTLE (*LEPIDOCHELYS OLIVACEA*)

There is not abundant information about concentrations of heavy metals and essential elements in samples of olive ridley sea turtle. The available data (Cd, Pb, Hg, Cu, Zn, Fe, Mn, Ni and Cr) are present in Table 6.

The metals most frequently analyzed are cadmium, lead and mercury (in that order), and the tissues mainly analyzed are liver, kidney and muscle (see Table 6). As in other species of sea turtles, the highest cadmium and lead concentrations were found in kidney [41, 42, 48], and mercury tended to be higher in liver [43].

Several essential elements have been measured in samples of *Lepidochelys Olivacea*, although copper, zinc and nickel were the most studied (see Table 6). The highest copper levels were found in liver [41], while blood [45] and muscle [42] contained considerable levels of zinc. Manganese showed relatively high levels in kidney and fat tissue [42].

Concerning metal levels in eggs, some of them were mainly present in the albumen: levels of lead, copper and nickel were higher in albumen than in yolk [45, 46], whereas shell contained considerable levels of iron, nickel and lead [45, 46, 47].

FLATBACK SEA TURTLE (*NATATOR DEPRESSUS*)

The available information about levels of heavy metals and essential elements in this species is very scarce (see Table 7). Flatback sea turtle has been slightly studied in contrast with other species of sea turtles, since this species is present in a limited geographical area [1].

Several elements (Cd, Pb, Hg, As, Cu, Zn, Mn, Ni, Se and Cr) have been measured in samples of blood and egg [62]. Yolk and albumen contained considerable zinc levels, which could be related to requirements for embryonic development [17]. Cadmium, lead, mercury and nickel showed non-detectable levels (see Table 7).

CONCLUSION

Cadmium and copper showed especially high levels in renal and muscle tissues, respectively, and these levels could even cause adverse effects in some specimens of sea turtles. Zinc and lead tended to accumulate high concentrations in calcified tissues, as bone and carapace, although these tissues have not been enough studied in sea turtles. Further assessment of the presence of metals in these tissues is recommended, as well as the standardization of non-invasive procedures for sampling (use of carapace, non-viable eggs or blood), which will allow the development of long-term monitoring programmes for these protected species. Furthermore, a deeper knowledge of metal presence in some species of sea turtles is needed (especially *Dermochelys coriacea*, *Lepidochelys kempii* and *Natator depressus*), since the available information is really scarce.

Table 6. Heavy metal and essential element levels (µg g^{-1} dry weight) in tissues and other samples of *Lepidochelys olivacea* from different locations

Tissue - Location	Cadmium		Lead		Mercury		Copper		References
	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	
Liver									
Pacific Ocean: Mexico	13,12 ± 1,50		13,30 ± 2,10				33,50		[41]
Pacific Ocean: Mexico	17,89	4,98 - 148,00	BDL				36,73	16,99 - 100,00	[42]
Pacific Ocean: Mexico					0,21	0,06 - 0,80			[43]
Pacific Ocean: Australia**	20,00								[48]
Kidney									
Pacific Ocean: Mexico	15,84 ± 1,20		13,40 ± 1,90				17,16		[41]
Pacific Ocean: Mexico	60,03	0,81 - 274,00	0,03	BDL - 2,63			4,86	0,81 - 53,40	[42]
Pacific Ocean: Mexico					0,14	0,03 - 0,37			[43]
Pacific Ocean: Australia**	124,18								[48]
Muscle									
Pacific Ocean: Mexico	2,48 ± 0,40		8,90 ± 1,00				16,15		[41]
Pacific Ocean: Mexico	0,48	BDL - 8,85	BDL				1,28	0,70 - 4,37	[42]
Pacific Ocean: Mexico					0,05	0,02 - 0,14			[43]

Table 6 (Continued)

Tissue - Location	Cadmium		Lead		Mercury		Copper		References
	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	
Blood									
Pacific Ocean: Mexico			0,95 ± 0,18						[46]
Pacific Ocean: Mexico	0,45 ± 0,20						2,28 ± 0,40		[45]
Fat									
Pacific Ocean: Mexico	0,69	0,33 - 2,54	BDL				0,83	0,47 - 2,54	[42]
Pacific Ocean: Mexico					0,03	BDL - 0,16			[43]
									[43]
Egg									[43]
Pacific Ocean: Mexico			Yolk: 0,80 ± 0,10						[46]
			Albumen: 1,08 ± 0,20						[46]
			Shell: 1,05 ± 0,20						[46]
Pacific Ocean: Mexico	Yolk: 0,24 ± 0,10						Yolk: 2,20 ± 1,47		[45]
	Albumen: 0,22 ± 0,09						Albumen: 3,53 ± 2,87		[45]
	Shell: 0,47 ± 0,09						Shell: 7,48 ± 2,60		[45]
Indian Ocean: India	Shell:	2,44 - 4,88	Shell:	19,52 - 36,60			Shell:	14,64 - 19,52	[47]

Liver										
Pacific Ocean: Mexico	47,14	18,66 - 85,75	731,00	119,00 - 9201,00	0,10	BDL - 9,20	0,58	BDL - 3,88		[42]
Pacific Ocean: Australia**	46,25									[48]
Kidney										
Pacific Ocean: Mexico	6,68	0,43 - 114,00	193,00	40,37 - 667,00	5,31	3,93 - 7,52	0,02	BDL - 2,46		[42]
Pacific Ocean: Australia**	78,34									[48]
Muscle										
Pacific Ocean: Mexico	85,78	49,89 - 107,00	93,09	57,35 - 319,00	0,77	BDL - 4,34	0,01	BDL - 0,41		[42]
Blood										
Pacific Ocean: Mexico	58,40 ± 4,70						2,80 ± 1,30			[45]
Fat										
Pacific Ocean: Mexico	3,70	0,41 - 16,65	27,91	6,37 - 236,00	2,10	0,88 - 3,65	0,03	BDL - 0,51		[42]
Egg										
Pacific Ocean: Mexico	Yolk: 72,30 ± 10,90						Yolk: 3,30 ± 0,30			[45]
	Albumen: 33,60 ± 6,10						Albumen: 4,00 ± 3,90			[45]
	Shell: 12,40 ± 1,50						Shell: 48,50 ± 12,90			[45]
Indian Ocean: India	Shell:	14,64 - 36,60	Shell:	92,72 - 129,32	Shell:	4,88 - 14,64	Shell:	21,96 - 41,48	Shell: 7,32 - 26,84	[47]

BDL = bellow detection limit; ** = Mean ± SEM.

Table 7. Heavy metal and essential element levels (µg g^{-1} dry weight) in tissues and other samples of *Natator depressus* from different locations

Tissue - Location	Cadmium		Lead		Mercury		Arsenic		Copper		References
	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	Mean ± SD	Min - Max	
Blood Pacific Ocean: Australia	BDL		BDL		BDL				0,04 ± 0,0005		[62]
Egg Pacific Ocean: Australia	Egg content:	BDL	Egg content:	BDL	Egg content:	BDL	Egg content:	3,49 ± 0,10	Egg content:	2,14 ± 0,02	[62]
Blood Pacific Ocean: Australia	0,77 ± 0,005				BDL						[62]
Egg Pacific Ocean: Australia	Egg content:	54,16 ± 0,31	Egg content:	0,89 ± 0,005	Egg content:	BDL	Egg content: 1,56 ± 0,01		Egg content: 0,89 ± 0,02		[62]

BDL = bellow detection limit.

This study shows up that sea turtles are potential biological indicators for monitoring the inorganic marine pollution, since they are long-lived species that present several special ecological features. However, sea turtles will be more reliable indicators if the presence of metals is studied in specimens at different stages of development throughout their life cycle and they are studied in the different distribution areas.

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In: Turtles: Anatomy, Ecology and Conservation ISBN: 978-1-61470-554-3
Eds: M. J. Cosgrove and S. A. Roe

Chapter 2

ANTHROPOGENIC CAUSES OF MORTALITY OF SEA TURTLES IN THE CANARY ISLANDS: A MULTIDISCIPLINARY APPROACH TO THE CONSERVATION OF ENDANGERED SEA TURTLES

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ABSTRACT

Because all species of sea turtles are included on the Red List of the World Conservation Union, the efforts to conserve sea turtles, the advances in their medical management, and the studies on diseases and causes of mortality and/or stranding of sea turtles must to be increased. A multidisciplinary approach to this focus carried out by veterinarians, biologists, and scientists, is necessary in order to compile data that prove the main threats for these endangered reptiles, and to design adequate strategies of conservation.

This chapter lists the pathological findings and causes of mortality of 49 sea turtles (46 *Caretta caretta*, 2 *Chelonia mydas*, and 1 *Dermochelyscoriacea*) stranded on the coasts of the Canary Islands, Spain, between 2002 and 2009.Of these, 12 turtles (24.49%) had died of spontaneous diseases including different types of hepatitis, pneumonia, and septicemic processes. However, 37 turtles (75.51%) died from lesions associated with human activities: ingestion of hooks and monofilament lines (34.69%), entanglement in fishing nets (24.49%), and boat-strike injuries (16.33%).

In addition, polychlorinated biphenyls (PCBs 28, 31, 52, 101, 138, 153, 180, and 209) and DDT and its metabolites (*o,p'*-DDT, *o,p'*-DDE, and *o,p'*-DDD) were measured in liver and fat from 32 sea turtles. Tissues from these turtles contained higher levels of PCBs and *o,p'*-DDT than those reported in turtles from other geographical regions. Statistically, a positive correlation was detected between ∑PCBs concentration and cachexia, and between ∑DDT concentrations and cachexia. No histological lesions exclusively attributed to the acute effects of PCBs and DDTs were described. However, chronic effects of organochlorines can not be discarded.

INTRODUCTION

Two families and seven species of sea turtles are currently recognized (Pritchard 1997). The family Cheloniidae includes the green turtle (*Chelonia mydas*), loggerhead (*Caretta caretta*), hawksbill (*Eretmochelysimbricata*), Kemp's ridley (*Lepidochelyskempi*), olive ridley (*Lepidochelysolivacea*), and flatback turtle (*Natatordepressus*). The family Dermochelyidae includes only the leatherback (*Dermochelyscoriacea*). Only five species have been reported in the Canary Islands: loggerhead, green turtle, leatherback, hawksbill, and Kemp's ridley sea turtle (Mateo *et al*. 1997, Barbadillo*et al.* 1999). The most common species in the Canary Islands is the loggerhead sea turtle (Mateo *et al*. 1997). However, evidence of a decline in the population of loggerhead sea turtles in the Canary Islands has been reported (López-Jurado& González 1983, Blanco & González 1992).

All species of sea turtles are included on the Red List of the World Conservation Union (IUCN/SCC2010). The IUCN emphasized as a priority issue in its document entitled *A Global Strategy for the Conservation of Marine Turtles* (1995) the research on diseases of sea turtles.Thus in recent

years, increased efforts have been devoted to the conservation of sea turtles, including medical management and pathological studies on stranded animals.

Sea turtles can be affected by several diseases. Some of these health problems are naturally occurring processes and are observed in both wild and captive turtles. Other conditions, such as certain nutritional deficiencies, are essentially a result of prolonged captivity in wildlife rehabilitation centers (George 1997).

There are previous reports of disease surveys of wild sea turtles in Australia (Glazebrook & Campbell 1990b, Raidal*et al*. 1998), Hawaii (Work *et al*. 2003, 2004), France (Duguy*et al*. 1998), Florida (Smith & Coates 1938), and the Canary Islands (Orós *et al*. 2004, 2005). However, usually these types of surveys do not include toxicological studies on stranded sea turtles. Regarding the previous studies on sea turtles stranded in the Canary Islands, it is remarkable that in addition to pathological, parasitological and microbiological studies, chemical analyses were carried out to determine the level of heavy metals in the tissues of 78 loggerhead sea turtles (Torrent *et al*. 2004).

Recent studies have reported baseline levels of persistent contaminants (polychlorinated biphenyls and organochlorine pesticides) on turtle populations of the Canary Islands (Monagas *et al*. 2008, Orós *et al*. 2009). Other studies on persistent contaminants have been focused on sea turtles from Long Island (Lake *et al*. 1994), Virginia (Rybitski*et al*. 1995), Scotland (Mckenzie*et al*. 1999), the Hawaiian Islands (Miao *et al*. 2001), the Baja California peninsula (Gardner *et al*. 2003), North Carolina (Keller *et al*. 2004, 2006), and the Mediterranean Sea (Corsolini*et al*. 2000, Storelli & Marcotrigiano 2000, Perugini *et al*. 2006, Storelli *et al*. 2007).

Polychlorinated biphenyls (PCBs) and DDT and its metabolites (OC-DDTs) have a particular significance because of their undesirable effects on environmental quality and animal health (Ahlborg*et al*. 1994). PCBs were manufactured from the 1930s to the 1970s for several industrial applications, such as liquid coolants for electrical transformers or as softeners in the production of plastics and as components of hydraulic fluids and lubricating oils. PCBs are able to bioaccumulate through the food chain and their effects have been reported on the immune, endocrine, and reproductive systems of different animal species (Fox 2001). OC-DDTs were used extensively all over the world as a domestic and agriculture pesticides (Ecobichon 1995). In most countries, DDT was banned in the 1970s due to its long residual life and its accumulation in food chains (Bolt &Degen 2002). In order to compile data

that prove the main threats for sea turtles, and to design adequate strategies of conservation, it is necessary a multidisciplinary approach to the study of causes of mortality and/or stranding of sea turtles, carried out by veterinarians, biologists, and scientists. This chapter lists the pathological findings and causes of mortality of 49 sea turtles (46 *Caretta caretta*, 2 *Chelonia mydas*, and 1 *Dermochelyscoriacea*) stranded on the coasts of the Canary Islands, Spain, between 2002 and 2009. In addition, concentrations of polychlorinated biphenyls (PCBs 28, 31, 52, 101, 138, 153, 180, and 209) and DDT and its metabolites (*o,p'*-DDT, *o,p'*-DDE, and *o,p'*-DDD) in the liver and the adipose tissue of 32 sea turtles are presented.

TURTLES AND METHODOLOGY

Between January 2002 and December 2009, 49 sea turtles that stranded on the coasts of the Canary Islands [Gran Canaria (n=41; 83.67%), Tenerife (n=4; 8.16%), y El Hierro (n=2; 4.08%)] were submitted for necropsy to the Veterinary Faculty, University of Las Palmas de Gran Canaria (ULPGC). Some of them had been previously submitted to the Tafira Wildlife Rehabilitation Center (TWRC) for health evaluation, medical and surgical management, and possible rehabilitation.

Species identifications were made according to Frick (1996). Turtles belonging to three different species were examined: 46 loggerhead turtles (*Caretta caretta*) (93.87%), 2 green turtles (*Chelonia mydas*) (2.04%), and 1 leatherback turtle(*Dermochelyscoriacea*) (2.04%). Species, sex, age group, and biometrics data are shown in Table 1. Age group was determined on the basis of straight carapace length (SCL) (Bjorndal*et al.* 2001, Seminoff*et al.* 2004) and sexual maturity (estimated from the appearance of their gonads).

The toxicological study was limited to 32 sea turtles necropsied before 2005: 30 loggerhead turtles (93.70%), 1 green turtle (3.10%), and 1 leatherback turtle (3.10%).

Pathological Study

Necropsies were performed at the Veterinary Faculty (ULPGC). The gross postmortem examinations were carried out using the procedures previously described (Wolke& George 1981, Campbell 1996, Orós & Torrent 2001).

The condition of the turtles ranged from freshly dead to moderately autolyzed. Macroscopic lesions were recorded and tissue samples from all major organs were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm for light microscopy and stained with hematoxylin and eosin (HE). Special stains performed on selected cases included Gram-stain for bacteria, Ziehl Neelsen (ZN) for acid-fast organisms, periodic acid-Schiff (PAS) for protozoa and fungal hyphae, Grocott's methenamine silver nitrate (GMS) for fungi, and von Kossa stain for calcium (Bancroft & Stevens 1996).

Table 1. Species, sex, age group and biometric data (mean ± standard deviation) of the turtles analyzed

Species	Sex	Age group	Weight (kg)	SCL (cm)
Caretta caretta (n = 46)	Female (n = 39; 84.78 %) Males (n = 7;15.21 %)	Pelagic juvenile (n = 18; 39.13 %) Juvenile (n = 28; 60.86 %)	12.7 ± 6.6	43.2 ± 10.5
Chelonia mydas (n = 2)	Female	Juvenile	22 ± 1.1	51.5 ± 0.9
Dermochelys coriacea (n = 1)	Female	Adult	231.5	nm

SCL: straight carapace length.
n: number of turtles.
nm: not measured.

OrganochlorineCompounds Analysis

Polychlorinated biphenyls (IUPAC nos. 28, 31, 52, 101, 138, 153, 180, and 209) and OC-DDTs (*o,p'*-DDT, *o,p'*-DDE, and *o,p'*-DDD)were analyzed according to the method described by Tanabe *et al*. (1994). Tissues samples (liver and celomic fat) were collected during necropsy. After collection, the samples were wrapped in aluminium foil and stored at -20 ºC until analysis.

Prior to analysis, tissue samples were homogenized using a commercial blender. The validity of analytical methods was confirmed with Standard Reference Materials (CARP-2: ground whole carp, *Cyprinuscarpio*) obtained from the National Research Council of Canada. Precision and accuracy are reported in Table 2.

Aliquots (4 -7 g) of the homogenized samples were ground with anhydrous sodium sulphate in a mortar, and extracted using Soxhlet apparatus for 6 h with 300 ml of diethyl ether:hexane (3:1) solvent mixture. Extracts were concentrated in volume to 10 ml in Kuderna-Danish and the aliquots (2 ml) were transferred to a glass column packed with 20 g of Florisil and dried by passing through nitrogen gas. Organochlorines adsorbed on Florisil were eluted with 150 ml of 20 % hexane-washed water in acetonitrile and transferred to a separatory funnel containing 600 ml of hexane-washed water and 100 ml of hexane. After partitioning, the hexane layer was concentrated, cleaned up with sulphuric acid, and passed through a 12 g Florisil packed glass column for separation.

Table 2. Precision and accuracy of analytical methods obtained using a certified ground whole carp Cyprinuscarpio (CARP-2)[a]

PCBs	CARP-2	
	Certified	Found[b]
PCB 28	34 ± 4.0	31 ± 3.1
PCB 52	138 ± 43	119.7 ± 13.9
PCB 101	145 ± 48	150.1 ± 24.1
PCB 138	103 ± 30	99.5 ± 18.7
PCB 153	105 ± 22	116 ± 16.6
PCB 180	53.3 ± 13.0	59.2 ± 10.7
PCB 209	4.6 ± 2.0	4.8 ± 3.6
o,p'-DDE	2.9 ± 0.5	2.4 ± 0.3
o,p'-DDD	21.8 ± 0.7	25.1 ± 1.1

[a]The concentrations are given in ng/g wet weight.
[b]Number de replicates is 4.

Final determination of organochlorine compounds PCBs was carried out using a Varian 3600 gas chromatograph fitted with an electron capture detector (GC-ECD). All analyses used a fused-silica capillary column Supelco (length = 30 m, inside diameter 0.53 mm and film thickness 0.50 μm). The column oven was programmed from 60 to 160 ºC, held for 10 min, and then

increased to 260 ºC at a rate of 2 ºC/min and held for 20 min. Injector and detector temperatures were set at 260 and 280 ºC respectively. Nitrogen was used as carried gas with 63.3 ml/min. The patrons used as internal standards were the PCB-Mix 12, Iso-octane, and the *o,p'*-DDT and metabolites Mix 32500 (Lab. Dr. Ehrenstorfer) for PCBs and OCs respectively. Quantification interval used for organochlorine compounds was from 1 ng/g (instrumental detection limit) to 50000 ng/g (optimum linear limit). Concentrations of PCBs and OC-DDTs, means of four measurements, are presented as ng/g on a wet weight basis. The data were evaluated using the Statistics Software SPSS V. 13.0.

Mann-Whitney's U test was conducted to determine whether the difference in the levels of PCBs and OC-DDTs were related to the tissues. Spearman's rho correlation method was used to calculate the correlation between ∑PCB concentrations and ∑OC-DDT in both tissues and physical conditions such as cachexia and septicemia. According to the necropsy reports, values of 0, 1, and 2 were given to turtles with absence of cachexia, mild cachexia, and severe cachexia respectively. Values of 0, 1, and 2 were also given to turtles with absence of infections, infection in one organ, and severe septicemia.

PATHOLOGICAL STUDY

Table 3 lists the lesions observed in the surveyed sea turtles. Cachexia was diagnosed in 8 turtles (16.32%) based on muscle atrophy, sunken eyes and concave plastron.

Ulcerative skin lesions attributed to trauma were the most common gross lesions, occurring in 22.44% of turtles examined. The distribution of the skin lesions was multifocal, with multiple lesions (1 to 4 cm in diameter) especially on the dorsum of the neck, head, and flippers. Histologically skin lesions were characterized by the ulceration of the epidermis, necrosis of the epidermis and dermis with infiltrating degenerate granulocytes and bacteria, and an underlying layer of multinucleated giant cells. Bacterial colonies were detected in all cases of ulcerative skin lesions. In addition, in the 63.63% of the turtles suffering from ulcerative skin lesions, other lesions of bacterial origin were detected in other major organs. These traumatic injuries were the sources from which pathogenic bacteria entered the tissues and the bloodstream resulting in fatal septicemia. In a survey of the diseases of farmed sea turtles in northern

Australia (Glazebrook & Campbell 1990a), ulcerative dermatitis attributed to trauma was observed in 66/102 turtles (63.5%), being associated with opportunistic *Vibrio alginolyticus*, *Aeromonas hydrophila*, *Pseudomonas* sp., and *Chryseobacterium* sp. infection. *Vibrio alginolyticus* is regarded as a normal inhabitant of seawater (Roberts 1978), while *Aeromonas hydrophila* has long been recognized as an opportunistic pathogen of reptiles (Reichenbach-Klinke & Elkan 1965, Rosenthal &Mader 1996).

Multifocal granulomatous pneumonia was the most frequently observed respiratory lesion (10.20%), being associated with granulomatous pleuritisin several cases (6.12%) (Figure 1A). These lesions were characterized by the presence of well defined granulomas containing central areas of eosinophilic necrotic debris mixed with heterophils and macrophages. Multinucleated giant cells surrounding the necrotic debris were observed in some granulomas. All granulomas were negative for mycobacteria (ZN), and one case was positive for fungi (GMS, PAS)(Figure 1B). Exudative bronchopneumonia was characterized by the presence of numerous heterophils in the central lumen of bronchi and bacterial colonies. In the majority of the cases the pneumonic lesions were unilateral.

Table 3. Systemic lesions and diseases of sea turtles (n = 49) stranded in the Canary Islands

System	Lesions	n	%	System	Lesions	n	%
General status	Cachexia	8	16.3	Digestive			
	Anemia	7	14.3	Oral cavity	Ulcerative glossitis	1	2.04
Integument				Esophagus	Ulcerative		
Skin					esophagitis	10	20.4
	Traumatic ulcerative				Fibrinous		
	dermatitis	11	22.4		esophagitis	2	4.1
	Subcutaneous						
	edema	3	6.1		Traumatic esophageal		
					perforation	5	10.2
Respiratory				Stomach	Catarrhal gastritis	2	4.1
Trachea	Tracheitis	1	2.04		Parasitic gastritis	4	8.2
Lungs	Granulomatous				Traumatic		
	pneumonia	5	10.2		gastric		

System	Lesions	n	%	System	Lesions	n	%
					perforation	2	4.1
	Interstitial pneumonia	1	2.04	Intestine	Catarrhal enteritis	3	6.1
	Exudative pneumonia	2	4.08		Necrotizing enteritis	5	10.2
	Pulmonary edema	3	6.1		Fibrinous enteritis	1	2.04
	Granulomatous pleuritis	3	6.1		Fibrinoussero sitis	2	4.1
	Fibrinouspleuri tis	2	4.1		Intussusceptio n	5	10.2
Cardiovasc.				Liver	Fibrinousperi		
Heart					hepatitis	3	6.1
	Hydropericardi um	2	4.1		Granulomato us hepatitis	8	16.3
	Fibrinous pericarditis	2	4.1				
Blood					Hepatic	3	6.1
vessels	Thrombosis	2	4.1		lipidosis		
				Pancreas	Chronic		
Immune					interstitial		
Spleen					pancreatitis	1	2.04
	Necrotizing splenitis	3	6.1	Celomicwall	Fibrinouscelo mitis	4	8.2
	Granulomatous splenitis	2	4.1	Endocrine			
	Splenomegaly	2	4.1	Adrenal glands	Adenomegaly	1	2.04
Peyer's patches	Hyperplasia	5	10.2	Reproductiv e	Ovarian follicular cyst	1	2.04
Excretory				Muscle	Necrotizing myositis	4	8.2
Kidneys	Granulomatous nephritis	3	6.1		Cachetic myopathy	5	10.2
	Chronic interstitial nephritis	2	4.1	Bone	Fractures	4	8.2
	Tubulonephros is	3	6.1		Traumatic amputation	6	12.2
Bladder	Catarrhal cystitis	1	2.04	Carapace/ Plastron	Lack of ossification	5	10.2
	Granulomatous serositis	2	4.1		Traumatic erosions	6	12.2
Salt glands	Purulent adenitis	5	10.2		Fractures	5	10.2

Table 3. (continued)

System	Lesions	n	%	System	Lesions	n	%
					Deformation (kyphosis/ lordosis)	2	4.1
				Nervous	Spinal cord compression	1	2.04
					Meningel hemorrhages	2	4.1
				Eye	Ulcerative keratitis	3	6.1
					Purulent kerato-conjunctivitis	4	8.2

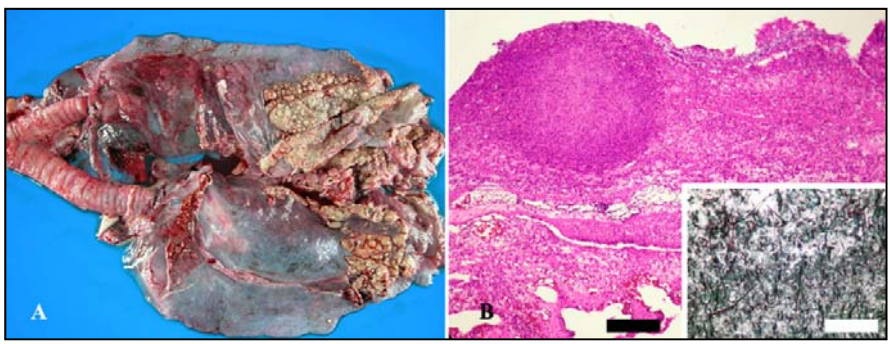

Figure 1. (A) Granulomatous pleuritis on the caudal area of both lungs of a loggerhead turtle. (B) Granulomatous inflammatory reaction in the pleura. HE. Bar = 200 µm. Inset: Micotic hyphae in the center of the granuloma. GMS. Bar = 60 µm.

In a previous study on sea turtles stranded in the Canary Islands, several bacteria including *A. hydrophila*, *Bacillus* sp., *Burkholderiacepacia*, *Citrobacter* sp., *Pasteurella* sp., *Proteus* sp., *Pseudomonas* sp., *Staphylococcus* sp., *Streptococcus* sp., and *V. alginolyticus* were associated with exudative bronchopneumonia and/or granulomatous pneumonia (Orós *et al*. 2005). Similar bacteria were isolated from bronchopneumonic lesions of turtles in Australia (Glazebrook & Campbell 1990a,b). *Vibrio alginolyticus*, *A. hydrophila*, and *Chryseobacterium* sp. have been repeatedly isolated from cases of ulcerative obstructive stomatitis and the rhinitis-pneumonia disease complex in hatchling and juvenile loggerhead and green turtles (Glazebrook *et*

al. 1993). Granulomatous focal pneumonia has been frequently associated with *Paecilomyces* sp. infection in green turtles (Jacobson *et al*. 1979). The high prevalence of pulmonary lesions observed in sea turtles may indicate a predisposition to suffer from these pathologic processes. George (1997) considered the aspiration of seawater as one of the primary modes by which bacteria entered the respiratory system and caused clinical disease. In other cases the pulmonary lesions were secondary to traumatic injuries in the carapace, as a consequence of the position of the lungs, those being dorsally attached to the carapace and the vertebral column (Wyneken 2001). The majority of the pneumonic lesions observed in our survey were unilateral, and it seemed likely that the unilateral pneumonia in most cases was due to aspiration or full thickness shell trauma. Buoyancy problems observed in the turtles may indicate a pulmonary disease (Jacobson 1997). However, buoyancy disorders of sea turtles are not always associated with pulmonary lesions; they may also result from excessive gas in the gastrointestinal tract, such as occurs during an obstruction (Campbell 1996, Jacobson 1997).

Lesions in the cardiovascular system were not frequent. Fibrinous exudative pericarditis (4.08%) was associated with bacteria and fibrinous lesions in other major organs. Thus, these lesions were expression of a septicemic status, rather than a specific cardiovascular disease. The most common cardiovascular pathology described in sea turtles is spirorchid fluke infection (Glazebrook *et al*. 1989, George 1997, Gordon *et al*. 1998). High prevalences of sea turtle spirorchidiasis have been reported in other surveys around the world (Glazebrook *et al*. 1989, Glazebrook & Campbell 1990b, Gordon *et al*. 1998). Adult flukes have been found in the heart and major blood vessels, causing lesions such as mural endocarditis, arteritis, thrombosis or aneurysms. Dissemination of the fluke eggs throughout the vascular system results in vasculitis and microgranulomas wherever they lodge (Wolke*et al*. 1982, Glazebrook *et al*. 1989, Gordon *et al*. 1998). However, none of the sea turtles included in our study had intravascular adult flukes or trematode eggs in tissues. All loggerhead and green turtles included in our survey were juvenile and subadult specimens, whereas other studies in which spirorchidiasis was reported included adult turtles. Different alimentary habits of the juvenile and subadult turtles on the coasts of the Canary Islands can also explain the absence of spirorchid fluke infection. Sea turtles acquire the flukes by ingesting unknown cercaria-rich intermediate hosts. The life cycle of spirorchids in sea turtles is unknown but is probably similar to that of fresh water turtles (Holliman & Fisher 1968).

Multifocal necrotizing splenitis was the most frequent lesion that affected the spleen (6.12%).All cases of multifocal necrotizing splenitis were associated with septicemic processes of bacterial origin. Thus, the lesions involving the spleen were an expression of a septicemic status, rather than a specific splenic disease.

Several lesions were observed in the excretory system. Lesions included, granulomatous nephritis, chronic interstitial nephritis, and tubulonephrosis, affecting 6 turtles (12.24%). Chronic interstitial nephritis was characterized by the presence of aggregates of lymphocytes in the renal interstitium. Granulomatous nephritis was characterized by small, yellow-white, granulomatous foci scattered randomly throughout the kidney. Microscopically, granulomas were characterized by central foci of necrosis surrounded by macrophages and multinucleated giant cells. In two cases the renal lesions were secondary to traumatic injuries in the caudal aspect of the carapace, related to the anatomical location of the kidneys, retrocelomically and dorsally attached to the caudal carapace (Wyneken 2001).In two cases, the renal lesions were attributed to a septicemic status with multiorganic involvement.In a previous study on sea turtles stranded in the Canary Islands, granulomatous nephritis and renal abscesses were associated with *A. hydrophila*, *Citrobacter* sp., *E. coli*, *Proteus* sp., *V. alginolyticus*, and *Staphylococcus* sp. infections (Orós *et al*. 2005).

Exudative salt gland adenitis was observed in 9 loggerhead turtles (18.36%) (Figure 2).Lesions ranged from the presence of abundant eosinophilic material associated with bacterial colonies in the lumen of the central ducts of the glandular lobules to the destruction of the glandular tissue and presence of abundant eosinophilic material composed by heterophils and cell debris, lined by multinucleated giant cells. Multinucleated giant cells were detected around the inflammatory foci in all cases. Because the kidney of the sea turtles cannot produce hypertonic urine, excess salt entering the body down the concentration gradient, ingested in the food or by drinking seawater is largely excreted by paired salt glands (Reina *et al*. 2002). Salt glands are composed of specialized, secretory cells that concentrate sodium and chloride from the blood to the lumen of secretory tubules through an energy-dependent process (Abel & Ellis 1966). Sea turtle salt glands secrete a solution composed almost entirely of sodium chloride at approximately 1500-1800 mosmol/l (Marshall & Cooper 1988, Nicolson & Lutz 1989, Reina & Cooper 2000) in response to increasing plasma sodium concentration, and their activity is regulated by microcirculatory changes in or near the glands (Reina 2000).

Very few descriptions of salt gland diseases in wild sea turtles could be found in the literature. Common lesions include pale spots or calculi, which indicate malfunction in solute dissolution, which may occur in severely dehydrated marine turtles. This manifests as hard rugose deposits associated with necrosis of surrounding tissues (Flint *et al*. 2009). Granulomas associated with spirorchiid eggs, of variable severity, are also common in stromal tissue that surrounds central canals of lobules and may sometimes extend into and disrupt the glands themselves (Flint *et al*. 2009). Glazebrook & Campbell (1990a) reported *Pseudomonas* sp. infections in the salt glands of two farmed green sea turtles due to the removal of foreign material from the main excretory duct leading from the posterior orbit. The procedure involved the use of forceps which introduced bacteria and led to infection.

Ulcerative and fibrinous esophagitis and traumatic esophageal perforation were the most frequently observed lesions in the esophagus, being associated in all cases with ingestion of fishing hooks. Hooks were identified as belonging to the longline tuna fishery. Lesions were characterized by the ulceration of the esophageal mucosa, coating in eosinophilic fibrillar material consistent with fibrin, with infiltrating degenerate granulocytes and bacteria in the lamina propria and/or submucosa, and an underlying layer of multinucleated giant cells. The estimated total take of sea turtles by the longline tuna fishery in the international waters in the North of the Canary Islands for 1983 to 1991 was 3000 turtles (López-Jurado& González 1983, Blanco & González 1992). Although many of the turtles are liberated, it is estimated that approximately 15-50% of the turtles die due to the severe lesions caused by the fishing hooks (Lizana&Barbadillo 1997). Many of the turtles with traumatic lesions in the esophagus and stomach had also systemic lesions due to septicemia (Figure 3). The lesions induced by the fishing hooks in the esophagus and stomach were the primary routes by which pathogenic bacteria entered the tissues and the bloodstream resulting in fatal septicemia.

Larval nematodes from Anisakidae family caused gastritis in 4 turtles (8.16%)(Figure 4A). The migration of larvae from the lumen of the stomach to the celomic cavity was characterized by the presence of granulomas in the lamina propria, submucosa (Figure 4B), lamina muscularis and serosa of the stomach and mucosal ulceration. The diagnosis of a parasitic gastritis in sea turtles caused by the larval migration of nematodes belonging to the family Anisakidae has been described for the first time by Carr & Main (1973) and Rodgers & Burke (1982). Turtles act as paratenic hosts for third stage larvae unable to complete their life cycle when confined to the tissues of an

ectothermic species (Glazebrook & Campbell 1990a). The migration of larvae from the lumen of the stomach to the celomic cavity resulted in ulceration of the gastric mucosa, and granulomatous inflammatory reactions. This pathologic condition was not considered a direct cause of mortality in any of the turtles examined in our survey.

Figure 2. Purulent salt gland adenitis in a loggerhead turtle.

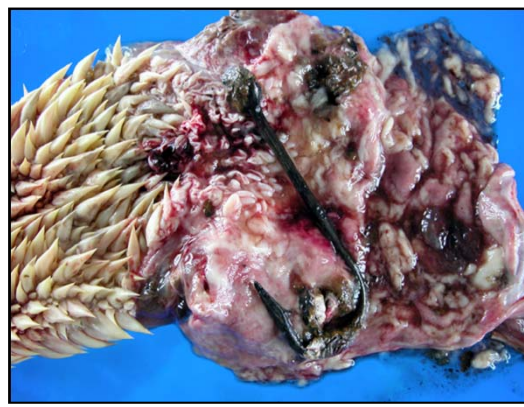

Figure 3. Lesion in the cardial area of the stomach of a loggerhead turtle caused by a fish hook.

Different histological types of enteritis included catarrhal enteritis, fibrinous enteritis, necrotizing enteritis, and fibrinous intestinal serositis, affecting 9 turtles (18.36%). In addition, all turtles with fibrinous intestinal

serositis also demonstrated fibrinouscelomitis, being also associated with multisystemicsepticemic lesions. All cases of necrotizing enteritis were associated with intestinal intussusception caused by ingestion of monofilament fishing lines(Figure 5). Ingestion of monofilament fishing lines is a serious threat to sea turtles. Monofilament line can stop normal gut function and results in death when a strand that extends along a portion of the intestine becomes lodged at the anterior end.

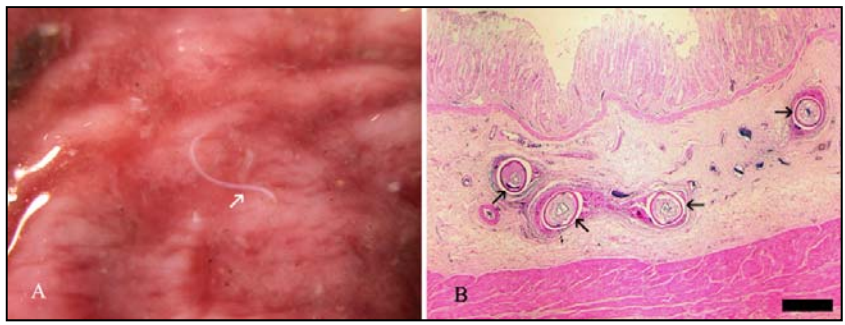

Figure 4. (A) Larval nematode (arrow) on the stomach of a loggerhead turtle. (B) Larval nematodes (arrows) in the gastric submucosa. Note the inflammatory reaction around the parasites. HE. Bar = 1200 µm.

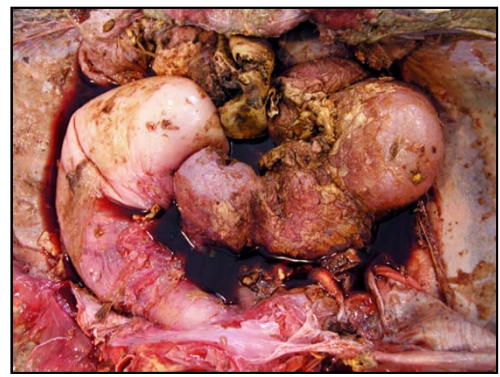

Figure 5. Intestinal intussusception and fibrinopurulentserositis associated to a monofilament fishing line in a loggerhead turtle.

Normal peristalsis of the intestine and movements of the digesta will result in the intestine becoming gathered along the length of the line (Bjorndal*et al.* 1994). Intestinal volvuli associated with different causes have

been frequently reported in sea turtles (Schumacher *et al*. 1996, Hasbún*et a*l. 1998, Helmicket al. 2000). However, this lesion was not observed in the present survey.

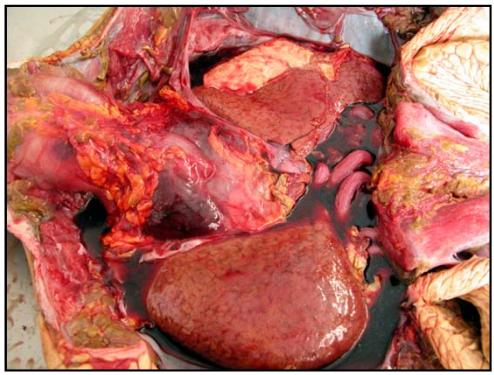

Figure 6. Fibrinopurulentperihepatitis (left hepatic lobe) in a loggerhead turtle.

Multifocal granulomatous hepatitis was the lesion most commonly observed in the liver (16.32%). Granulomatous hepatitis was characterized by small, yellowish-whitish, granulomas scattered randomly throughout the liver. Microscopically, these granulomas were characterized by necrotic centers surrounded by macrophages and multinucleated giant cells. These hepatic lesions were attributed to multisystemicsepticemic status (Figure 6).In only one case these lesions were associated with fungi. In a previous study on sea turtles stranded in the Canary Islands,necrotizing and/or granulomatous hepatitis were commonly observed (26/93 or 27.95%), being associated with *A. hydrophila*, *Citrobacter* sp., *E. coli*, *Proteus* sp., *Staphylococcus* sp., and *V. alginolyticus* infections (Orós *et al*. 2005).

Cachetic myopathy was characterized by generalized atrophy of skeletal muscles, affecting 5 turtles (10.20%).Lesions of focal necrotizing myositis (8.16%) were attributed to entanglement in fishing nets or boat strikes, and were characterized by necrosis of the myofibers with infiltrating degenerated heterophils and bacteria, and an underlying layer of multinucleated giant cells. These lesions were similar to those described by George (1997). Calabuig (1998) reported that 56% (45/72) of the turtles submitted to the TWRC in 1998 had similar lesions due to entanglement. These lesions have been previously described by others (Schroeder 1987, Teas 1994, George 1997).

Affections of the skeletal system included traumatic amputation of one or two flippers (12.24%), and multiple bone fractures (8.16%). These amputations were mainly related to entanglement in fishing nets.

Traumatic erosions and/or fractures of the carapace/plastron were frequently observed (22.44%)(Figure7). These lesions were mainly attributed to boat strikes.

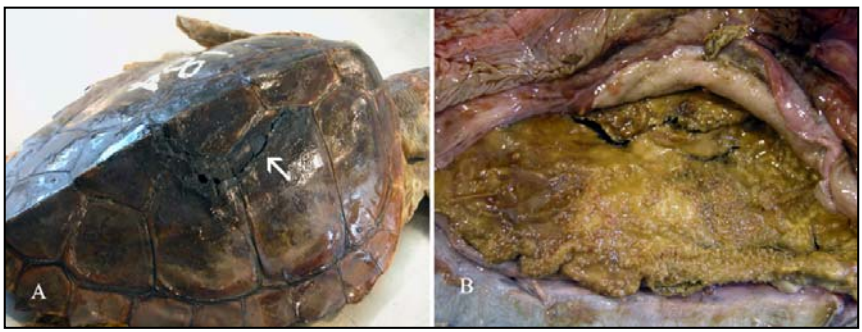

Figure 7. (A) Traumatism (arrow) in the carapace of a loggerhead turtle. (B) Purulent lesions in the right lung and thoracic cavity.

Diseases of the nervous system were uncommon and included compression of the spinal cord (associated with ciphosis) and meningeal hemorrhages associated with cranial trauma.

Eye lesions included heterophilic keratoconjunctivitis and ulcerative keratitis, affecting 6 turtles (12.24%). Heterophilic keratoconjunctivitis was characterized by the presence of large amounts of heterophils in the corneal stroma infiltrating the conjunctiva. Ulcerative keratitis was characterized by loss of epithelium of the cornea and the presence of large numbers of mixed inflammatory cells in the stroma. In all cases bacterial colonies were associated with the lesions. Histological examination of the ocular and periocular lesions did not reveal intranuclear inclusion bodies indicative of a herpesvirus infection (Jacobson *et al*. 1986).

The absence of fibropapillomatosis in the sea turtles stranded in the Canary Islands during this survey is remarkable because fibropapillomatosis of sea turtles is a disease of global distribution. However, fibropapillomatosis affects several populations in epidemic proportions throughout the world, and many observations have been reported in Florida, Hawaii, Puerto Rico, Barbados, Virgin Islands, Panama, Colombia, Venezuela, Belize, Australia

and Indonesia (Balazs 1991, Jacobson 1991, Adnyana*et al*. 1997, Work *et al*. 2004). The prevalence started to increase in the 1980's at multiple sites including Florida and Hawaii. The prevalence of fibropapillomatosis in sea turtle populations in Hawaii has been observed as high as 92% (Balazs 1991). In the Indian River Lagoon System of east central Florida, 57% of green turtles collected between 1985 and 1986 hadfibropapillomatosis (Jacobson *et al*. 1989).

TOXICOLOGICAL STUDY

PCBs

PCB concentrations in both tissues (liver and fat) are shown in Table 4. The turtle tissues analyzed contained high levels of PCBs compared to concentrations reported in turtles collected in other locations around the world (McKim& Johnson 1983, Lake et al. 1994, Rybitski et al. 1995, Mckenzie et al. 1999, Corsolini et al. 2000, Storelli&Marcotrigiano 2000, Gardner et al. 2003, Keller et al. 2004, Perugini et al. 2006, Storelli et al. 2007).

Loggerheads showed the highest hepatic ∑PCB (IUPAC nos. 28, 52, 101, 138, 153, 180) mean concentration (1980 ± 5321 ng/g wet wt.), followed by the only specimen of leatherback turtle analyzed (445 ng/g wet wt.). ∑PCB concentration in the liver of the only specimen of green turtle analyzed was lower (116 ng/g wet wt.) than those detected in loggerheads and leatherback turtles. Loggerheads also showed the highest ∑PCB mean concentration in the adipose tissue (448 ± 1700 ng/g wet wt.).

The high degree of variation of concentrations of PCBs detected in our study may be attributed to the different exposure of individual turtles to pollutants and to the physical condition of the animals. Sea turtles visit different areas with different degree of contamination during migration, resulting in differences in exposure for each animal (Ziswiler 1986).According to recent studies, loggerhead turtles stranded on the coasts of the Canary Islands come from two different migration routes: from the Western Atlantic arriving to the Canary Islands by the Gulf Stream (Pérez-Jiménez 1997), and from Cape Verde (Monzón-Argüello 2009). In addition, turtles included in this study varied widely in physical condition, ranging from turtles that had been killed incidentally through human interaction, to turtles that showed severe emaciation and septicemia after prolonged illness.

Table 4.PCBs concentrations (range, mean ± standard deviation) (ng/g wet wt) in liver and fat of the three species of sea turtles analyzed

	Caretta caretta n = 30		*Chelonia mydas* n = 1		*Dermochelyscoriacea* n = 1	
	Liver	Fat	Liver	Fat	Liver	Fat
∑PCB	BDL – 34900	BDL – 9828				
	1980 ± 5320	448 ± 1700	116	144	445	77
PCB	BDL – 587	BDL – 38				
28,31	42 ± 131	3.8 ± 9	BDL	5.7	71.5	9.7
PCB 52	BDL – 1012	BDL – 56				
	98 ± 262	5.5 ± 12.5	BDL	7.2	8.7	11.4
PCB	BDL – 671	BDL – 84.5				
101	32 ± 130	6 ± 19	BDL	BDL	BDL	BDL
PCB	BDL – 13300	BDL – 2039				
138	456 ± 2426	83± 370.5	BDL	BDL	BDL	BDL
PCB	BDL – 15440	BDL – 2604				
153	915 ± 3178	133 ± 477	BDL	58	251	56
PCB	BDL – 3887	BDL – 5006				
180	440 ± 1016	217 ± 907	116	73	114	BDL
PCB	BDL – 14877	BDL – 218				
209	1194 ± 3117	15 ± 45.5	BDL	BDL	526	501

BDL: below detection limit.

∑PCB = sum of PCBs nos. 28, 52, 101, 138, 153, and 180.

Loggerheads showed the highest hepatic PCB 209 mean concentration (1194 ± 3117 ng/g wet wt.). Six loggerhead turtles had individual PCB concentrations higher than 2000 ng/g, and seven turtles showed individual PCB concentrations higher than 900 ng/g. All these turtles showed severe septicemia and/or severe cachexia. Statistically, a positive correlation was detected between ∑PCBs concentration and cachexia. Although sea turtles are long-lived high trophic organisms that would be expected to contain high concentrations of PCBs (Lake et al. 1994), the poor physical condition of these animals could be the reason of these high PCB levels. It has been described in cetaceans that many diseases affect metabolic centers and thus the capacity to metabolize or excrete pollutants may be affected, questioning whether PCB concentrations in the tissues of these specimens are representative of normal conditions (Aguilar et al. 1999).

As revealed by statistical analysis, turtles showed significant higher PCBs 28,31, PCB 153, PCB 209 and ∑PCBs concentrations in the liver than in the adipose tissue. However, no significant differences between concentrations in

both tissues were detected for PCB 138 and PCB 180. According to other authors, the pattern of tissue distribution of PCBs reported in turtles and other marine species is the following: adipose tissue > liver > kidney > muscle (Tanabe et al. 1983, Martineau et al. 1987, Colborn& Clement 1992, Rybitski et al. 1995, Mckenzie et al. 1999). This is attributed to the highest total lipid content and triglycerides for the adipose tissues and the strong correlations between these and their burden of lipophilic pollutants (Cockroft et al. 1989). However, we detected in the liver ∑PCBs concentrations higher than in the adipose tissue. Thirteen turtles showed PCB levels in the liver higher than in the fat. All these turtles showed severe cachexia and/or septicemia. According to different studies, animals with abundant fat deposits can accumulate and tolerate higher concentrations of toxic chemicals because lipophilic pollutants are stored in the adipose tissue and are less available to target organs and receptors. In contrast, emaciated animals which have mobilized their lipid stores may be more susceptible to toxic effects as a result of remobilization of the pollutants, resulting in higher concentrations of PCBs in the remaining tissues like the liver (Bernhoft&Skaare 1994). Thus, the cachexia observed in our turtles may indicate a previous remobilization of the fat and the pollutants, and could explain the high concentration of PCBs in the liver. In addition, the septicemic condition reported in these turtles could be caused by a possible immunosupression as one of the most distinguished effects of organochlorine compounds.

The congener most frequently detected in the liver was PCB 180 (46.8%), followed by PCB 153 (31.2%), PCBs 28, 31 (25%), PCB 52 (25%), PCB 209 (15.6%), PCB 138 (15.6%), and PCB 101 (12.5%). The congeners most frequently detected in the adipose tissue were PCB 180 (50%) and PCB 153 (50%), followed by PCBs 28,31 (34.4%), PCB 52 (34.4%), PCB 209 (31.2%), PCB 138 (28.1%), and PCB 101 (18.7%).

PCB profiles in the loggerhead tissues were dominated by the higher chlorinated congeners. Hexachlorobiphenyls (PCBs 138 and 153) were predominant and accounted for 43.6% of the total PCBs, followed by decachlorobiphenyls (PCB 209) (33%) and heptachlorobiphenyls (PCB 180) (18%). Concerning individual congeners, the most abundant were PCB 209 (33%) followed by PCB 153 (29%), PCB 180 (18%), and PCB 138 (15%). The isomer pattern of PCBs in loggerhead turtles was different among liver and fat. In the liver the most abundant were PCB 209 (37.6%), followed by PCB 153 (29%), PCB 138 (14%) and PCB 180 (14%).In the fat the most abundant were PCB 180 (47%), followed by PCB 153 (28.6%), PCB 138

(18%) and PCB 209 (3%). Hexaclorobiphenyls and heptaclorobiphenyls are predominant in turtles and marine mammals (Muir et al. 1988, Rybitski et al. 1995, Colborn&Smolen 1996, Alam& Brim 2000, Corsolini et al. 2000, Storelli&Marcotrigiano 2000, Miao et al. 2001, Perugini et al. 2006, Storelli et al. 2007). However, Gardner et al. (2003) reported in Chelonia mydas a profile dominated by lower-chlorinated congeners. Differences in PCB patterns in turtles may be attributed to differences in the congener compositions of environmental media among regions, dietary differences or differences in the abilities of the various species and populations to metabolize PCBs (Gardner et al. 2003).

Regarding PCB profiles in the only specimen of green turtle analyzed, the most abundant was PCB 180 (73%) followed by PCB 153 (22%) and PCB52 (3%). Although PCB 180 was the most abundant in both tissues, this congener was the only PCB detected in the liver of this turtle.

Regarding PCB profiles in the only specimen of leatherback turtle analyzed, the most abundant was PCB 209 (66%) followed by PCB 153 (20%) and PCB 180 (7%). The isomer pattern of PCBs in this turtle was slightly different among liver and fat. In the liver the most abundant was PCB 209 (54%) followed by PCB 153 (26%) and PCB 180 (12%).In the fat the most abundant was PCB 209 (87%), followed by PCB 153 (10%), and PCB 52 (2%).

Hepatic ∑PCBs concentrations were positively correlated with cachexia ($r = 0.250$, $P = 0.04$). No significant correlation was observed between ∑PCBs concentrations and septicemia.

OC-DDTs

OC-DDTs concentrations measured in fat and liver and frequencies of detection of OC-DDTs in both tissues of loggerhead turtles are reported in Table 5. Due to the limited number of studies on tissue distribution of OC-DDTs in sea turtles, it is difficult to compare the results obtained in this study. The majority of surveys have been dedicated to *p,p'*-DDT compounds, and very little information has been published on *o,p'*-DDT compounds. Only Perugini*et al*. (2006) reported *o,p'*-DDT concentrations in the liver (BDL-21.7 ng/g) and fat (BDL-9.5 ng/g) of loggerhead turtles from the Adriatic Sea. In our study, *o,p'*-DDT concentrations in both tissues were higher than those

detected by Perugini*et al*. (2006). It may imply a relatively larger usage of technical DDT in our study area in recent years.

Table 5.*o,p'*-DDT concentrations (range, mean ± standard deviation) (ng/g wet wt) and frequencies of detection (percentage) in liver and fat of the three species of sea turtles analyzed

	Caretta caretta n = 30		*Chelonia mydas* n = 1		*Dermochelyscoriacea* n = 1	
	Liver	Fat	Liver	Fat	Liver	Fat
∑DDT	BDL – 810.8 78.8 ± 163.8 (43.47%)	BDL – 449 33.8 ± 84.3 (26.08%)	250.9	BDL	BDL	BDL
o,p'-DDT	BDL – 243.8 25.2 ± 60.7 (17.39%)	BDL – 327.2 26.7 ± 70.3 (21.73%)	250.9	BDL	BDL	BDL
o,p'-DDD	BDL – 798.8 49.8 ± 156.6 (30.43%)	BDL – 121.8 6.1 ± 24.6 (13.04%)	BDL	BDL	BDL	BDL
o,p'-DDE	BDL – 26.2 3.18 ± 6.75 (13.04%)	BDL	BDL	BDL	BDL	BDL

BDL: below detection limit (1 ng/g).
∑DDT = *o,p'*-DDT + *o,p'*-DDE + *o,p'*-DDD.

Although DDT was banned in the 1970s in North America and Europe, it has not been well documented when its use was definitely ended in the Canary Islands (Zumbado*et al*. 2005). It is well known that in underdeveloped African countries, OC-DDTs are still running, and it is necessary to have into account that the Canary Islands are located very close to Africa (Zumbado*et al*. 2005). In addition, studies of detection of organochlorine pesticides in pine needles (Villa *et al.* 2003) and serum samples from people from the Canary Islands (Zumbado*et al*. 2005) allow those authors hypothesize that DDT has been used recently in the Canary Islands, particularly in the islands devoted to intensive agriculture (Zumbado*et al*. 2005).

The high degree of variation of concentrations of OC-DDTs detected in our study may be attributed to the different exposure of individual turtles to pollutants and to the physical condition of the animals. Turtles included in this study varied widely in physical condition, ranging from turtles that had been

killed incidentally through human interaction, to turtles that showed severe emaciation after prolonged illness.

As revealed by statistical analysis, OC-DDTs were differentially distributed in both tissues with the liver having the highest concentration. Regarding the pattern of tissue distribution of OC-DDTs in two grouped categories [emaciated turtles (n =8) and turtles with normal weight conditions (n= 24)], emaciated turtles showed higher average ∑DDT concentrations in the liver (98.56 ± 55.31ng/g) than in the fat (15.31 ± 45.56 ng/g), whereas turtles with normal body conditions showed higher average ∑DDT concentrations in the fat (39.76 ± 69.61ng/g) than in the liver (32.36 ± 57.13ng/g). The differences in the pattern of OC-DDTs among liver and fat are attributed to the hepatic metabolization.

The pattern of tissue distribution of OC-DDTs reported in sea turtles is the following: adipose tissue > liver > muscle (Rybitski*et al*. 1995, Mckenzie *et al*. 1999, Perugini*et al*. 2006). However, we detected in the liver ∑DDT concentrations higher than in the adipose tissue. Eleven turtles showed ∑DDT levels in the liver higher than in the fat. All these turtles showed severe cachexia. The cachexia observed in our turtles may indicate a previous remobilization of the fat and the pollutants, and could explain the high ∑DDT concentration in the liver.

Of the different organochlorine pesticides, *o,p'*-DDD was predominant and accounted for 53.15% of the total OC-DDTs, followed by *o,p'*-DDT (44.18%) and *o,p'*-DDE (3.76%). The pattern of OC-DDTs was different among liver and fat. In the liver the most abundant was *o,p'*-DDD(66.13%), followed by *o,p'*-DDT (30.09%), and *o,p'*-DDE (3.76%). In the fat the most abundant was *o,p'*-DDT (78.01%), followed by *o,p'*-DDD (22.08%). No detectable concentrations for *o,p'*-DDE were found in the fat samples.*o,p'*-DDD is a component of chlordane, a widely used pesticide. However, according to the bibliography, *p,p'*-DDE is, in general, the pesticide present in the greatest concentrations in sea turtles due to its highly persistent nature (Lake *et al*. 1994, Rybitsky *et al*. 1995, Mckenzie *et al*. 1999, Storelli & Marcotrigiano 2000, Keller *et al*. 2004, Perugini *et al*. 2006,Storelli *et al*. 2007). In our study, the predominance of *o,p'*-DDD may imply a species-specific metabolic capability and/or a specific exposure in reductive (anoxic) conditions. It is also remarkable that in the survey published by Gardner *et al*. (2003) *p,p'*-DDD contributed most to ∑DDT. The very low concentration for *o,p'*-DDE has been reported previously in human beings from the Canary Islands (Zumbado *et al*. 2005).

CONCLUSION

According to the stranding reports, clinical reports, gross and microscopic lesions, and toxicological results, we attempted to establish the causes of mortality of the turtles examined in this survey (Table 6). Only 12 turtles (24.49%) died from spontaneous diseases including different types of pneumonia, hepatitis, and septicemic processes. However, 37 turtles (75.51%) died from lesions associated with human activities such as ingestion of hooks and monofilament lines (34.69%), entanglement in derelict fishing nets (24.49%), and boat strike injuries (16.33%).

Table 6. Estimated causes of mortality in the stranded sea turtles in this study

Cause		n	%
Spontaneous diseases		12	24.49
Anthropogenic causes		37	75.51
	Hooks and monofilament lines	17	34.69
	Entanglement	12	24.49
	Traumatism	8	16.33

Net entanglement is the most important cause of sea turtle stranding in the Canary Islands and frequently the turtles land with a part of the net (Calabuig 1998). Entanglement in fishing nets resulted in severe ulcerative dermatitis, amputation of flippers, and septicemic processes. A comparison of our survey and the data provided by the sea turtle stranding registry of the TWRC indicates that turtles with lesions induced by entanglement usually have a better prognosis for rehabilitation that those with traumatic lesions in the carapace or plastron. Affection of vital organs such as lungs and kidneys, because the anatomical location, dorsally attached to the carapace, explain the generally poor prognosis for turtles with severe traumatic injuries in the carapace.

To assess the impact of organochhorine compounds in the turtles of our study is difficult because the most frequent causes of death were attributed to fishing activity. In addition, few toxicological studies have been published regarding organochlorines in reptiles and no safe concentrations have been established. It is remarkable that almost all turtles with severe septicemia showed very high levels of PCBs. Immunosupression as result of PCBs

pollution has been previously described (Keller *et al*. 2004, 2006). However, the presence in our study of other factors different to PCBs concentrations, as incidental fishing, nutritional status, and exposition to different micro-organisms makes difficult to establish a clear association between PCB concentrations and causes of death. According to the histopathological study no lesions exclusively attributed to acute effects of PCBs were described. All the histological lesions were attributed to bacterial and/or fungal infections mainly associated with gross lesions caused by fishing activity. However, chronic effects of PCBs are much difficult to determine.

Histologically, we detected hepatic lesions in four loggerhead turtles with high ∑DDT concentrations. Lesions included diffuse vacuolar hepatic degeneration and hepatic lipidosis. These lesions were not exclusively attributed to acute effects of OC-DDTs because clinical history of these turtles also included severe septicaemia. However, chronic effects of OC-DDTs can not be discarded.

The detection of high levels of PCBs and OC-DDTs in these turtles makes necessary to continue to monitor the levels of these chemicals in turtles from the Canary Islands to contribute to the protection of these currently endangered species. In addition, studies of detection of other persistent organochlorines in loggerhead turtles stranded in the Canary Islands are necessary.

A multidisciplinary approach to this focus carried out by veterinarians, biologists, and scientists, is necessary in order to compile data that prove the main threats for these endangered reptiles, and to design adequate strategies of conservation.

ACKNOWLEDGMENT

The authors would like to thank members of the Tafira Wildlife Rehabilitation Center (Cabildo de Gran Canaria), specially P. Calabuig, for providing us the turtles. This study was partially supported by the Consejería de Medio Ambiente del Gobierno de Canarias. We are grateful to Elsevier for the permission to use the data of the toxicological studies previously published by us in the journals *Marine Pollution Bulletin* and *Chemosphere*.

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In: Turtles: Anatomy, Ecology and Conservation ISBN: 978-1-61470-554-3
Eds: M. J. Cosgrove and S. A. Roe

Chapter 3

THE PAINTED TURTLE AS A MODEL OF NATURAL ANOXIA TOLERANCE: ROLE OF THE NEUROTRANSMITTER GABA

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ABSTRACT

Painted turtles range throughout the USA and Canada. The most northern member of this species is the western painted turtle *Chrysemys picta bellii;* which is also the most anoxia-tolerant vertebrate identified, surviving for up to 6 months buried in the mud of ice covered pond at 3˚C. *C. picta* provides a working model of an anoxia-tolerant vertebrate, and of particular interest - a brain that functions without oxygen. We have recently been exploring the role of GABA, the primary vertebrate inhibitory neurotransmitter, in anoxia tolerance. Increases in endogenous GABAergic signalling appear to mediate neuronal electrical depression in

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C. picta and in a number of other anoxia-tolerant species. Unlike most other vertebrate species that undergo severe neural injury within minutes of an anoxic episode, *C. picta* neurons reduce action potential frequency and suppress metabolism rapidly with the onset of anoxia, and avoid injury. Anoxia-sensitive mammals lack a hypoxia/anoxia-mediated increase in GABA concentrations and suffer electrical hyper-excitability and excitotoxic cell death following the onset of an anoxic/ischemic episode. Experimental interventions aimed at increasing inhibitory GABAergic signalling prevent over-excitation and rescue mammalian neurons from anoxic/ischemic cell death. Drawing upon evidence from a number of studies on a variety of naturally anoxia-tolerant species we will explore in detail the role of GABA in neuroprotection. The anoxic response could be interpreted as a natural anaesthetic mechanism and is achieved by a reduction in excitatory neurotransmitter receptor activity (glutamate) and an increase in inhibitory receptor activity (GABA). An understanding of the complex regulation of GABAergic signaling in facultative anaerobes may provide insight into interventions that could prove efficacious against low oxygen situations in mammals, such as ischemic insult due to stroke.

Keywords: facultative anaerobe, shunting inhibition electrical depression, anoxia, spike arrest, snail, killifish

INTRODUCTION

Painted turtles (*Chrysemys picta*) range throughout the USA and into southern Canada from west to east and north of Lake Superior. The north-south distribution of painted turtles reveals an interesting relationship between latitudinal position and anoxia tolerance. The more northern species being much more anoxia-tolerant than southern species and this correlates with extended ice cover in northern waters. Indeed, the increased anoxia tolerance in the northern species is almost certainly the result of natural selection as they spread northward following the Wisconsinan glaciation about 18,000 to 20,000 years ago. The western painted turtle (*Chrysemys picta bellii*) occupies the most northern range and is the most anoxia-tolerant, surviving for up to 6 months submerged in nitrogen gassed water at 3˚C (Ultsch and Jackson 1982). We and others have been studying the physiological and biochemical mechanisms by which this remarkable tolerance is achieved. Unlike most other vertebrate species that undergo severe neural injury within minutes of an

anoxic episode, *C. picta* neurons reduce action potential frequency and suppress metabolism rapidly with the onset of anoxia and avoid injury. It is clear that the reversible suppression of metabolism by greater than 90% of normoxic resting levels is key; however, how this is achieved is less clear (Bickler and Buck 2007). Nevertheless, considerable progress has been made and in this review we will highlight recent advances and focus on a unique mechanism we have uncovered in the brain. We will also focus on the unique opportunity provided by an anoxia-tolerant brain to explore natural mechanisms by which mammal brain can be protected from the debilitating consequences of ischemic stroke.

Stroke is characterized by a sudden impairment of blood flow to a particular brain region, preventing delivery of oxygen and thus rendering the region functionally anoxic. This lack of oxygen availability presents severe challenges to the exquisitely oxygen-sensitive mammalian brain, which suffers ATP loss, membrane potential depolarization and irreversible cell damage within minutes of anoxia (Donnelly et al., 1992). During an anoxic episode, oxidative phosphorylation ceases and cells must rely entirely upon glycolysis to generate ATP, essentially sacrificing 90% of their ATP-producing capacity (Buck and Pamenter, 2006). Therefore, defense of ATP supplies is likely the most important aspect of surviving anoxic insults without detriment. In normoxic rat brain the primary consumers of ATP are excitatory electrical events, including action potential (AP) generation and propagation, and post-synaptic potentials (PSPs). Together these processes account for ~81% of neuronal energy expenditure (Attwell and Laughlin, 2001). Thus electrical depression of both APs and PSPs is critical to preventing ATP depletion and surviving transient low-oxygen insults in mammalian brain.

Some vertebrates, including freshwater turtles and some fish and amphibian species, display a remarkable tolerance to anoxic insults (Bickler and Buck, 2007). Anoxia-tolerance is achieved via multiple protective mechanisms including increased glycogen stores, enhanced buffering capacity, ion channel arrest, and perhaps most importantly: electrical depression (Bickler and Buck, 2007; Hochachka, 1986; Jackson, 2002; Perez-Pinzon et al., 1992a). One mechanism in particular that appears to be employed ubiquitously in facultative anaerobes is a large and transient elevation in extracellular GABA concentration $[GABA]_e$ during anoxic perfusion (see below). GABA is the predominant inhibitory neurotransmitter in the adult mammalian CNS and in anoxia-tolerant species thus far characterized, and mediates wide-spread electrical depression in most neurons (Lutz and Milton,

2004; Martyniuk et al., 2005; Turner and Whittle, 1983). GABA activates two specific receptors: $GABA_B$ receptors which act via an associated G protein, and $GABA_A$ receptors which contain a membrane-spanning chloride (Cl^-) channel that is also conducive to bicarbonate (HCO_3^-) (Kaila et al., 1993). Activation of either of these receptors is electrically inhibitory in adult mammalian neurons, and GABAergic interneurons make widespread connections throughout the nervous system and are therefore well positioned to regulate electrical activity. Furthermore, GABAergic interneurons may be relatively tolerant to hypoxia, potentially enabling them to regulate hypoxic-protective mechanisms without loss of function or cell death (Nitsch et al., 1989).

In this review we discuss GABA-based strategies of electrical depression utilized in the anoxia tolerance of several facultative anaerobes, and compare these strategies with the mechanisms of GABA-mediated electrical depression that have begun to emerge in mammalian models of stroke tolerance. Furthermore, we discuss the role of GABA inhibition in facultative anaerobes and immature mammalian neurons in which GABA is an excitatory neurotransmitter. Major findings from these studies are summarized in Tables 1 and 2 and are discussed below.

GABA AND ANOXIA-TOLERANCE: ENDOGENOUS DEFENSE MECHANISMS

Facultative anaerobes commonly experience prolonged hypoxia or anoxia in their natural environments. GABA-mediated inhibitory signaling is highly conserved in evolution and differential regulation of GABA receptors, GABA synthesis and uptake mechanisms, as well as cellular Cl^- gradients may underlie differences in oxygen sensitivity between facultative anaerobes and oxygen-sensitive adult mammals. Indeed mammalian neurons possess the same basic components of GABA-mediated signaling as facultative anaerobe neurons. Thus, mechanisms of neuroprotection that occur naturally in facultative anaerobes may be induced with experimental intervention in mammalian neurons, conferring neuroprotection (Figure 1). Anoxia-tolerant vertebrates exhibit common patterns of neurotransmitter release during anoxia. In general, excitatory amino acid (EAA) concentrations, in particular of the primary EAA glutamate, remain constant or decrease slightly with anoxia,

while inhibitory amino acid (IAA) concentrations, in particular of the primary IAA GABA are greatly elevated during anoxia. These changes have been consistently observed in all identified facultative anaerobes in which GABA is inhibitory, including fish, reptile and amphibian species (see Table 1 and discussion below) and also persist during the developmental stage of at least one facultative anaerobe.

Table 1. GABA: Critical inhibitory neurotransmitter in endogenous mechanisms of anoxia tolerance and ischemic preconditioned neuroprotection

Anoxia Tolerant Species				
Facultative Anaerobe	**Change in GABA**	**GABA-mediated responses to anoxia**	**Neuroprotection**	**References**
Freshwater turtles (*C. Picta bellii, T. Scripta Elegans*)	↑ $[GABA]_e$ ↑ $GABA_A$ B_{Max}	↓ Electrical activity ↓ Glutamate release	Survives > 6 months of anoxia at low temperatures	(Lutz and Leone-Kabler, 1995; Nilsson et al., 1990; Thompson et al., 2007; Ultsch and Jackson, 1982)
Freshwater fish (*C. carassius, C. auratus*)	↑ $[GABA]_e$	↓ Electrical activity ↓ Glutamate release	Survives months of hypoxia at low temperatures	(Hylland and Nilsson, 1999; Johansson et al., 1997; Nilsson, 1990; Suzue et al., 1987)
Anoxic shore crab (*C. maenas)*	↑ $[GABA]_e$	↓ Electrical activity (putative)	Survives 12 hours in N_2-bubbled water at warm temperatures	(Nilsson and Winberg, 1993)
Killifish embryo (*A. limnaeus*)	↑ $[GABA]_e$	↓ Electrical activity (putative)	Survives months in anoxic mud in a diapaused state	(Podrabsky et al., 2007)
Anoxia Intolerant Species				
Mammalian Ischemia Model	**Response to Ischemic Insult**	**GABA-targeted intervention**	**Effect due to ischemic insult**	**References**
Rat cortex (*R. norvegicus*)	↓ $GABA_A$ B_{Max} $[GABA]_e$ unchanged ↑ Glutamate release ↑ Cell death	↑ $[GABA]_e$ $GABA_A$ agonism	↓ Glutamate release	(Mielke and Wang, 2005; Ouyang et al., 2007)
		Ischemic Preconditioning*	↑ $[GABA]_e$ ↓ Glutamate release ↓ Cell death	(Johns et al., 2000)

Table 1 (Continued)

Anoxia Intolerant Species				
Mammalian Ischemia Model	**Response to Ischemic Insult**	**GABA-targeted intervention**	**Effect due to ischemic insult**	**References**
Rat hippocampus	$[GABA]_e$ unchanged ↑ Glutamate release ↑ Cell death	Ischemic Preconditioning	↑ $[GABA]_e$ ↓ Glutamate release ↓ Cell death	(Dave et al., 2005)
Gerbil hippocampus (*M. unguliculatus*)	$[GABA]_e$ unchanged ↓ $GABA_A$ mRNA ↓ $GABA_A$ B_{Max} ↑ Glutamate release ↑ AMPAR B_{Max} ↑ NMDAR B_{Max} ↑ Cell death	Ischemic Preconditioning*	↑ $GABA_A$ B_{Max} ↓ AMPAR B_{Max} ↓ NMDAR B_{Max} ↓ Cell death	(Li et al., 1993; Sommer et al., 2002; Sommer et al., 2003)
		Blockade of GABA uptake mechanisms	↑ $[GABA]_e$ ↓ Glutamate release ↓ Cell death	(Inglefield et al., 1995)
		↑ $[GABA]_e$ $GABA_A$ agonism	↑ $[GABA]_e$ ↓ Glutamate release ↓ Cell death	(Hall et al., 1997; Schwartz-Bloom et al., 2000; Sternau et al., 1989)

Comparison of endogenous GABA-mediated mechanisms of anoxic tolerance in facultative anaerobe brain with up-regulated GABA-mediated mechanisms of neuroprotection in ischemic mammalian brain. *GABAA receptor antagonists blocked neuroprotective effects attributable to ischemic preconditioning in these studies. Abbreviations: [GABA]e – extracellular GABA concentrations, BMax – receptor binding affinity.

Evidence for GABA-mediated mechanisms in facultative anaerobes will be discussed in the proceeding sections.

Anoxic GABA Changes in Fish: Roles in Developing and Adult Brain

Anoxia-tolerant fish have received considerable attention in the study of GABA-mediated mechanisms of anoxia tolerance. In their natural environment, Crucian carp (*Carassius carassius* L.) and their slightly less anoxia-tolerant cousins the common goldfish (*Carassius auratus*) survive in an active

state for weeks of anoxia at low temperatures while over-wintering under frozen surfaces of lakes and ponds in northern Europe (Nilsson, 2001).

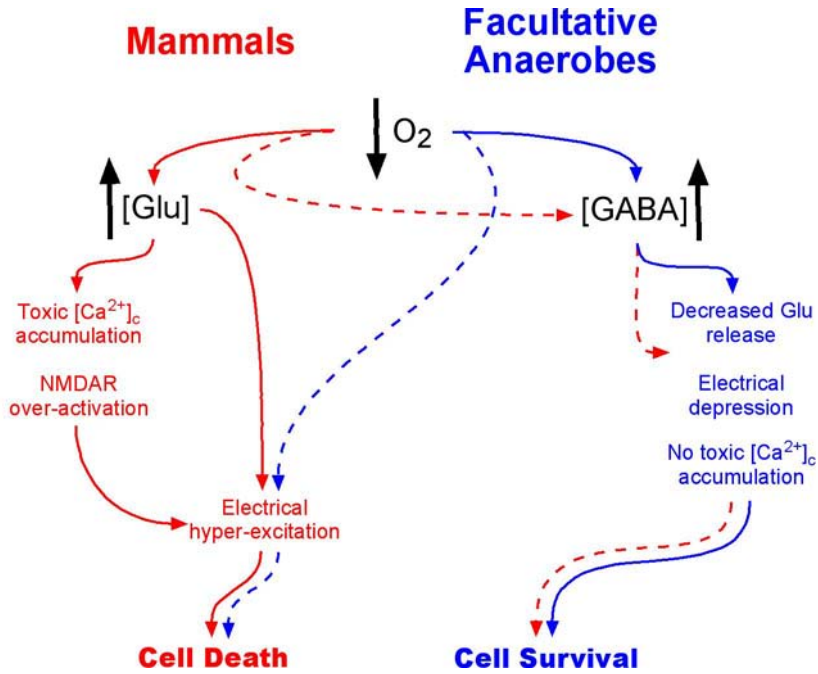

Figure 1. Schematic of inhibitory GABAergic mechanisms of neuroprotection in anoxia tolerant facultative anaerobe and preconditioned mammalian neurons. Ischemic preconditioning (IPC)- or GABA-induced neuroprotective mechanisms resemble endogenous mechanisms in facultative anaerobes: Facultative anaerobes (blue pathway) utilize endogenous GABA-mediated neuroprotective mechanisms (solid blue lines) when exposed to anoxia. Ischemic mammalian neurons (red pathway) undergo excitotoxic cell death (solid red lines) but can be rescued by experimental interventions including IPC, GABA perfusion or $GABA_A$ receptor agonism (dashed red lines). Blockade of $GABA_A$ receptors during anoxic or ischemic insult abolish neuroprotective mechanisms in facultative anaerobes (dashed blue lines) and in IPC- or GABA- pretreated mammalian neurons.

Anoxia tolerant fish rely entirely on glycolytic energy production and their brains undergo metabolic arrest in response to anoxia without significant depletion of ATP supplies (Johansson and Nilsson, 1995; Johansson et al., 1995). There is evidence supporting decreased electrical activity in the anoxic brain of these species from both visual and auditory inputs and this decreased

excitability likely contributes to ATP conservation and may be mediated by enhanced GABAergic inhibitory signaling (Johansson et al., 1997; Suzue et al., 1987). Indeed, during anoxia $[GABA]_e$ in crucian carp brain doubles within 6 hours and increases a further 5-fold within two weeks of anoxic exposure (Hylland and Nilsson, 1999; Nilsson, 1990). These large increases in $[GABA]_e$ likely reduce electrical excitability and thus ATP demand to allow the crucian carp to maintain energy balance.

Interestingly, in both goldfish and crucian carp, expression of metabotropic glutamate (mGlu) receptors 2 and 3 are up-regulated in comparison to oxygen-sensitive fish species (i.e. trout) (Poli et al., 2003). These authors also report that in goldfish exposed to periods of anoxia sufficient to induce cell damage, the incidence of cell death was inversely proportional to the expression of mGlu2/3 receptors, and blockade of these receptors aggravated glutamate release and increased cell death due to anoxia. mGluRs have been linked to enhanced GABA release in preconditioned mammalian brain exposed to ischemic insult (Saransaari and Oja, 2004; Saransaari and Oja, 2005), and endogenously high expression of these receptors in the brain of facultative anaerobes may partially underlie rapid and prolonged elevations in GABA with anoxic exposure. Thus correlative evidence suggests a critical role for GABA in anoxia tolerance in fish, but the specific mechanisms of protection remain unexplored.

Facultative anaerobes may also utilize GABA-based mechanisms of neuroprotection during maturation and development. An example comes from the gulf killifish (*Fundulus grandis*), which has recently been identified as a facultative anaerobe (Virani and Rees, 2000). These fish are found in ephemeral ponds in the Maracaibo Basin of Venezuela and produce drought-resistant diapausing embryos during the dry season (Podrabsky et al., 2001). Diapaused embryos that are buried in mud experience severe hypoxia or even anoxia for months, and in response metabolic rate decreases 80-90% and protein synthesis is also depressed (Podrabsky and Hand, 1999; Podrabsky and Hand, 2000). Diapausing embryos also exhibit large increases in GABA that are maintained throughout the anoxic episode. This increase is accompanied by a decrease in glutamate, likely due to conversion to GABA (Podrabsky et al., 2007). These results indicate GABA may be an inhibitory neurotransmitter at all stages of development in facultative anaerobe brain. Thus even in early developmental stages, facultative anaerobes utilize endogenous GABA-based mechanisms of electrical inhibition to provide neuroprotection against low oxygen insults.

Anoxic GABA Changes in Freshwater Turtles

While the data supporting an increase in $[GABA]_e$ in anoxia-tolerant species is compelling, knowledge of the protective mechanisms regulated by GABA in these species is minimal and based primarily on mammalian studies of the inhibitory effects of GABA on nervous electrical activity. However, recent studies have begun to elucidate the mechanisms of GABA-mediated protection in a pair of facultative anaerobes: the painted and red-eared freshwater turtles (*Chrysemys picta belli* and *Trachemys scripta elegans*).

Freshwater turtles are the most anoxia tolerant vertebrate species identified. In nature, turtles over-winter in the anoxic mud beneath frozen ponds. In the laboratory, they can survive anoxic episodes lasting days at 25ºC and up to six months at 3ºC (Musacchia, 1959; Ultsch and Jackson, 1982). As with the facultative anaerobes discussed previously, anoxia causes rapid and prolonged elevations of $[GABA]_e$ in turtle brain (Hitzig et al., 1985; Nilsson et al., 1990; Nilsson and Lutz, 1991). Furthermore, $GABA_A$ receptor binding affinity (B_{Max}) is increased during anoxic exposure, likely due to increased receptor density on the plasma membrane (Lutz and Leone-Kabler, 1995; Sakurai et al., 1993).

Recently, studies have begun to examine the underlying mechanisms of GABA-mediated protection in the turtle brain. GABA appears to be involved in regulation of electrical depression in the anoxic cortex and also of glutamate release in early anoxia. Turtle brain exhibits a significant electrical depression with anoxic exposure, as characterized by reductions in: transmembrane potentials, Na^+ spike thresholds, AP frequency, field potentials, and EEG activity (Fernandes et al., 1997; Perez-Pinzon et al., 1992a; Perez-Pinzon et al., 1992b). Recent results from our lab indicate that GABA, acting through the $GABA_A$ receptor mediates electrical depression in the turtle cortex. Normoxic to anoxic transitions resulted in significant electrical depression including: increased whole-cell conductance, decreased AP frequency, increased AP firing threshold, increased inhibitory post-synaptic potential (IPSP) activity, and a mild membrane potential depolarization. GABA perfusion (30-750 µM) induced similar changes and both the GABA-mediated and the anoxia-mediated changes were blocked by the $GABA_A$ inhibitor gabazine. Furthermore, cells treated simultaneously with anoxia and gabazine underwent repeated seizure-like events and suffered severe membrane potential depolarizations and cell death characteristic of ischemic rat neurons (Pamenter et al., 2011).

These data suggest that elevated $[GABA]_e$ in the anoxic turtle brain activates $GABA_A$ receptors, which mediate an inhibitory Cl^- conductance (G_{Cl}). Their activation leads to increased whole-cell conductance and since the reversal potential for Cl^- is near neuronal resting membrane potential, increases in G_{Cl} oppose excitatory events and tend to clamp the membrane potential near the chloride ion's reversal potential (~ -80 mV in adult turtle brain). Thus $GABA_A$ receptor activation during anoxia opposes APs, as indicated by the decrease in AP frequency and increase in IPSP amplitude, by a process known as "shunting inhibition". Furthermore, increased cell death in anoxic neurons treated with gabazine highlights the critical role of GABA in anoxia tolerance in this facultative anaerobe and offers the first conclusive evidence that the elevation of GABA in an anoxia-tolerant species mediates specific protective mechanisms (Figure 1).

In addition to initiation of electrical depression, elevations in GABA may regulate glutamate release in the anoxic turtle brain. Glutamate release from turtle brain tissue decreases 40% in the first 1.5 hours of anoxia, and glutamate re-uptake mechanisms remain active (Milton et al., 2002a). During longer duration anoxic exposures (up to 5 hours), glutamate levels decrease further to 30% of control levels (Thompson et al., 2007). The early reduction is maintained by the coordinated action of ATP-sensitive K^+ channels and adenosine receptors; however, the later reduction is mediated by the coordinated action of $GABA_A$ receptors and adenosine (Milton et al., 2002b; Thompson et al., 2007). GABA-mediated changes in glutamate release have also been demonstrated in mammalian brain exposed to ischemic insult (see discussion below). Therefore, mechanisms regulating naturally occurring GABA-induced reductions in glutamatergic activity in the turtle may have homologous pathways in mammalian systems that can be switched on with appropriate interventions, and may explain some of the neuroprotection afforded by $GABA_A$ agonists in mammalian experiments (see below).

GABAergic Systems in Anoxia-sensitive Non-mammalian Vertebrates

Despite these examples, anoxia-tolerance is not a common trait in non-mammalian vertebrates. Interestingly, non-mammalian vertebrate species that are intolerant to anoxia exhibit changes in amino acids that closely resemble changes in anoxia-intolerant mammals, such that release of excitatory

neurotransmitters is greater than release of inhibitory neurotransmitters. For example, in rainbow trout exposed to anoxic insult amino acid concentrations do not change until after the loss of ion homeostasis due to cell death as determined by K^+ efflux. Furthermore, glutamate levels increased prior to GABA release (Hylland et al., 1995). Another example comes from the frog *Rana pipiens*, which can survive hours of anoxia, but which does not exhibit elevations of amino acids until several hours into the anoxic period when all amino acids become elevated following total depletion of ATP (Lutz and Reiners, 1997). Thus while anoxic death of the frog is delayed, cell death does occur and in a fashion similar to mammalian neurons with regard to amino acid release patterns.

GABA AND ISCHEMIC PRECONDITIONING: MODELS OF INDUCIBLE ANOXIA TOLERANCE

There is overwhelming correlative evidence suggesting a critical role for GABA in neuronal low oxygen survival in facultative anaerobes. GABA-mediated electrical inhibition is common in both ectothermic and mammalian vertebrate species and likely relies on similar mechanisms of GABA synthesis, uptake, and release (Martyniuk et al., 2007). Thus understanding of the protective mechanisms employed by facultative anaerobes may offer GABA-based strategies to combat ischemic damage in mammals. Recently, interest in GABA's role in ischemia-tolerance has increased and the results support the hypothesis that the mechanisms underlying anoxia tolerance in some species can be targeted and up-regulated to confer neuroprotection in mammals. (Table 1, Figure 1).

Ischemic preconditioning (IPC) is a phenomenon that occurs in anoxia-sensitive animals whereby mild anoxic insults induce mechanisms that protect cells against subsequent lethal anoxic insults (Murry et al., 1986). This phenomenon was originally described in canine heart but has proven to be transmittable to numerous models of neuroprotection (see below). Furthermore, IPC in the brain appears to be mediated by enhanced GABAergic inhibitory signaling. For example, rat cortical slices exposed to oxygen and glucose deprivation (OGD) exhibit glutamate levels that are elevated 5-fold while GABA levels are unchanged. However, in cells preconditioned with brief bouts of OGD, and subsequently exposed to continuous, otherwise lethal

OGD, GABA levels were considerably elevated and glutamate release and cell death were decreased (Johns et al., 2000).

A role for enhanced GABAergic activity in IPC-mediated neuroprotection is also supported by another interesting study that examined the binding affinity of glutamatergic and GABAergic receptors in ischemic hippocampal CA1 neurons. Five minutes of ischemia induced severe cell death; however, pretreatment with a 2.5-minute period of ischemia significantly reduced cell death induced by the subsequent 5-minute ischemic insult. In cells that survived ischemia the binding affinity (B_{Max}) of excitatory α-amino-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors decreased and that of the inhibitory $GABA_A$ receptor increased. In addition $GABA_A$ receptor B_{Max} increased progressively from 30 minutes to 48 hours after recirculation before decreasing (Sommer et al., 2002; Sommer et al., 2003). Furthermore, in rats preconditioned against OGD, elevations were observed in $[GABA]_e$ and in the activity of glutamate decarboxylase, the predominant source of GABA synthesis in the brain (Dave et al., 2005). Therefore, IPC renders mammalian neurons more anoxia-tolerant by mimicking endogenous changes observed in the anoxic turtle cortex (elevated $[GABA]_e$ due to increased synthesis and increased $GABA_A$ receptor B_{Max}).

Important from a clinical perspective, IPC can be induced pharmacologically by activation of GABA receptors. Pre-treatment with a variety of GABA-related pharmaceuticals is efficacious in preventing ischemic cell death in selectively vulnerable gerbil hippocampal neurons. GABA perfusion, specific activation of GABA receptors or prevention of GABA catabolism via inhibition of the GABA transaminase provide strong to moderate neuroprotection against bilateral cerebral ischemia (Grabb et al., 2002; Sternau et al., 1989). Furthermore, prevention of GABA reuptake reduces cell death in gerbil hippocampus (Inglefield et al., 1995).

There is particularly strong evidence for a central role for $GABA_A$ receptors in this mechanism. For example, activation of $GABA_A$ receptors with partial or complete agonists, such as diazepam, both prior to transient ischemia and immediately following the ischemic insult is neuroprotective against cell death in gerbil (Hall et al., 1997; Hall et al., 1998; Schwartz-Bloom et al., 1998; Schwartz-Bloom et al., 2000). Similarly, in rat hippocampal neurons exposed to 7-minutes of OGD, ATP concentrations decreased 70% and cytochrome c (an apoptotic factor released from the mitochondria prior to programmed cell death) concentrations in the cytosol

were increased 400%. However, in cells treated with diazepam during reoxygenation, ATP levels were completely restored and cytochrome c release was prevented (Galeffi et al., 2000). In addition to studies demonstrating protection mediated by $GABA_A$ agonism, there is evidence that $GABA_A$ *antagonism* prevents the neuroprotective effects of IPC. Also $GABA_A$ receptor activation with GABA or specific agonists were all abolished by $GABA_A$ blockade (Grabb et al., 2002). Taken together these data indicate direct GABAergic manipulation is a good candidate for interventions against ischemic damage in mammalian neurons.

PRECONDITIONING IN ANOXIA-TOLERANT SPECIES?

Sensitivity to oxygen appears to follow a sliding scale that is closely connected to temperature effects. At one end are facultative anaerobes that survive for months without oxygen at low temperatures and days of anoxia at warmer temperatures. At the opposite end of the scale are relatively anoxia-intolerant mammals that are intolerant to anoxia and to low temperatures, but which can survive anoxic insults provided natural mechanisms of neuroprotection are up-regulated in advance or immediately following ischemic insult. Interestingly, the epaulette shark may represent a mid-point on this scale in that it is hypoxia-tolerant at high temperatures but it's tolerance may rely on naturally-occurring preconditioning protocols resembling experimental preconditioning interventions in mammals (Nilsson and Renshaw, 2004).

The hypoxic epaulette shark (*Hemiscyllium ocellatum*), is found in coral-rich offshore regions. During nocturnal low tide, these regions can become cut-off from the greater ocean and due to the high metabolic activity of the coral reef; the water can become extremely hypoxic overnight (Routley et al., 2002). Thus the epaulette shark experiences repeated bouts of severe hypoxia at regular intervals and does not exhibit apparent neuronal damage as a result of this intermittent hypoxia (Renshaw et al., 2002). Indeed intermittent hypoxia may serve to up-regulate defensive mechanisms against ischemic damage and render the epaulette shark more tolerant to subsequent bouts of anoxia. Epaulette shark brain pre-treated with short bouts of hypoxia exhibits increased $[GABA]_e$ and $GABA_A$ B_{Max}, decreased glutamate release and increased tolerance to anoxic insult (Nilsson and Renshaw, 2004; Renshaw et al., 2002). Thus even in hypoxia tolerant species preconditioning elevates

neuroprotective pathways dependent on enhanced inhibitory GABAergic neurotransmission. This mechanism highlights the similarities between endogenous and induced anoxic protective mechanisms.

INHIBITORY OR EXCITATORY? CHLORIDE GRADIENTS AND $GABA_A$ RECEPTORS

Like most ions, chloride (Cl^-) is distributed unequally across the neuronal plasma membrane. In most mature neurons, extracellular Cl^- concentrations ($[Cl^-]_e$) are ~10-fold greater than intracellular concentrations and this gradient is maintained by the balance of extrusion of Cl^- though K^+-Cl^--cotransporters (KCC2), Cl^--bicarbonate exchangers, ATP-driven Cl^- pumps and voltage-sensitive Cl^- channels; with the accumulation of Cl^- through NKCC1 ($Na^+/K^+/2Cl^-$) (for review see (Delpire, 2000; Kaila, 1994). Primarily, it is the differences in expression of the opposing KCC2 and NKCC1 transporters that determine neuronal Cl^- gradients (E_{Cl}). Higher expression of KCC2 results in an E_{Cl} that is more negative than resting membrane potential. Thus increases in G_{Cl} results in inward Cl^- flux and membrane hyperpolarization. Therefore, $GABA_A$ receptor activation is inhibitory. Conversely, if NKCC1 expression is higher, E_{Cl} is reduced or even reversed and increases in G_{Cl} are depolarizing. Thus, $GABA_A$ receptor activation becomes excitatory.

In the CNS of adult mammals and most facultative anaerobes, the combination of expression of these transporters is such that the Cl^- reversal potential is generally negative with regard to resting membrane potential, and therefore activation of $GABA_A$ receptors hyperpolarizes the cells resting membrane potential and depresses cellular excitation (Table 1, Figure 2). Conversely, in some organisms at different stages in development, G_{Cl} is excitatory and GABA is a major excitatory neurotransmitter. In such systems, inhibition of GABAergic mechanisms is critical to preventing over-excitation during anoxic insults (Figure 2). Examples of this phenomenon are found in both the anoxia-tolerant and anoxia-intolerant species (Table 2).

Table 2. GABA is a critical excitatory neurotransmitter in pond snails and may regulate excitotoxicity in immature mammalian neurons exposed to ischemic insults

Species	Response to GABA during anoxia	Effect of GABAergic inhibition during anoxia	Neuroprotection	References
Anoxic pond snail (*L. stagnalis*)	Membrane potential depolarization ↑ Electrical activity	↓ $GABA_A$ activity ↓ NKCC1 activity ↓ Electrical activity	Survives days of anoxia at warm temperature	(Cheung et al., 2006; Rubakhin et al., 1996)
Immature rat (*R. norvegicus*) neohippocampus and neocortex	↑ $GABA_A$ activity ↑ Glutamate release ↑ Electrical excitation ↑ Cell Death ↑ $[Ca^{2+}]_c$	No information available	No information available	(Clayton et al., 1998a; Nunez et al., 2003; Zhao et al., 2005)

Comparison of endogenous inhibition of GABA-mediated electrical excitability in pond snail and of effects of GABA regulation on ischemic immature mammalian neurons. Interventions targetting depression of GABA mediated excitability have not been explored in mammalian brain but may prove to be effective against ischemic damage due to stroke. Abbreviations: $[GABA]_e$ – extracellular GABA concentrations, $[Ca^{2+}]_c$ - cytosolic calcium concentration.

REDUCTION IN GABA-MEDIATED EXCITABILITY IN THE ANOXIA TOLERANT POND SNAIL

In the pond snail *Lymnaea stagnalis* GABA is an excitatory neurotransmitter. The reversal potential of Cl^- (and thus $GABA_A$ receptors) in these cells is ~ -20 mV and GABA perfusion causes membrane potential depolarization mediated by a $GABA_A$ receptor-dependent inward Cl^- current (Rubakhin et al., 1996). These snails are reported to survive up to 40-hours in N_2-bubbled water and when made hypoxic undergo a ~38% depression in action potential firing as well as a membrane potential hyper-polarization that is reversed by reoxygenation and mimicked by $GABA_A$ receptor blockade (Cheung et al., 2006). Interestingly, NKCC1 blockade also mimicked the

hypoxic decrease in AP frequency, suggesting that in anoxia NKCC1 activity is reduced, elevating intracellular Cl^- ($[Cl^-]_i$) and thus decreasing the magnitude of GABA-mediated excitatory neurotransmission. Thus, just as anoxia-tolerant vertebrates that exhibit inhibitory responses to GABA possess endogenous mechanisms to upregulate inhibitory GABAergic systems, facultative anaerobes that exhibit excitatory GABAergic signaling possess endogenous mechanisms to *downregulate* excitatory GABA-mediated signaling. Furthermore, these mechanisms may be adaptable to immature mammalian brain to upregulate neuroprotection against excitatory GABAergic signaling during ischemia (see next section).

Cl^- Gradients and Excitotoxicity in Ischemic Developing Mammalian Neurons

While GABA is an inhibitory neurotransmitter in most mammalian neurons, there are a number of examples in which GABA becomes excitatory in the developing mammal and in regions of the adult mammalian CNS (for a review see (Ben-Ari, 2002). GABA is excitatory in some regions of the adult CNS where Cl^- gradients are rearranged. Depolarization due to GABA has been reported in neurons and interneurons from the hippocampus and dentate gyrus regions of the adult murine brain and can also be induced by repetitive stimulation or by prolonged exposure to GABA (Lambert and Grover, 1995; Michelson and Wong, 1991; Staley and Mody, 1992).

In the immature mammalian brain GABA is initially excitatory, primarily due to the low expression of KCC2, which results in high $[Cl^-]_i$ and a reversal potential for $GABA_A$ receptors that is depolarizing with regard to resting membrane potential (Figure 3). Thus, as with the pond snail, GABAergic signaling is excitatory and therefore undesirable during anoxia. This hypothesis is supported by data from embryonic murine cultures exposed to excitotoxicity, OGD or induced depolarization. Perfusion of extracellular GABA or direct agonism of GABAergic receptors increased neuronal cell death due to each of these treatments in immature neurons (Erdo et al., 1991; Lukasiuk and Pitkanen, 2000; Muir et al., 1996).

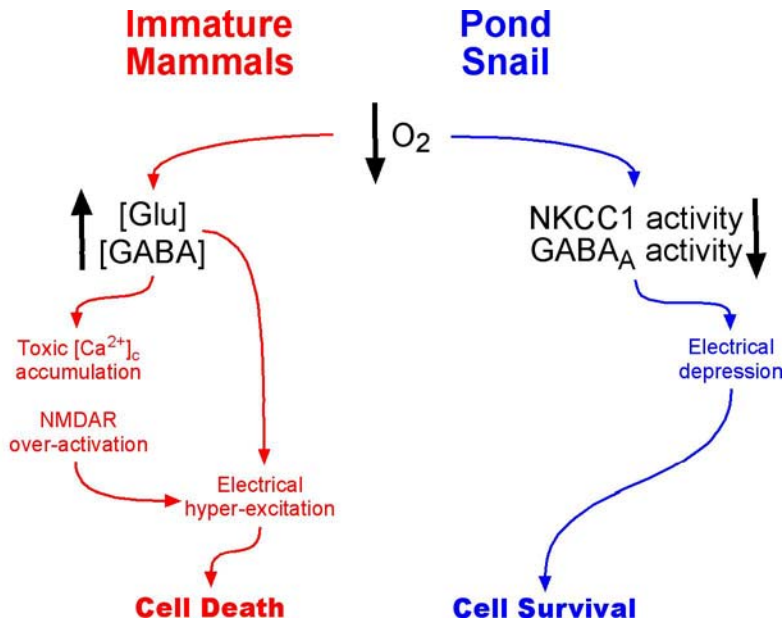

Figure 2. Schematic of excitatory GABAergic mechanisms in the anoxia- tolerant pond snail: potential neuroprotective roles in immature mammalian neurons? GABA is excitatory in neuronal circuits with small or reversed Cl^- gradients, such as in the pond snail and in immature mammalian neurons. In the anoxia tolerant snail, endogenous inhibition of GABAergic systems is neuroprotective (blue pathway). Conversely, ischemic immature mammalian neurons undergo GABA- and glutamate (Glu) mediated hyper-excitation and cell death (red pathway). The effect of GABA inhibition on survival in ischemic immature mammalian neurons has not been investigated.

In addition to inducing a hyper-excited state, inappropriate excitatory GABAergic signaling in immature rat cortex exposed to OGD leads to toxic accumulations of cytosolic Ca^{2+} ($[Ca^{2+}]_i$) that is prevented by NMDA receptors antagonists (Fukuda et al., 1998). Furthermore, glutamate induced $[Ca^{2+}]_i$ accumulation is increased in immature versus adult hippocampal neurons. The enhanced susceptibility to glutamate may be due to incomplete development of what will become inhibitory GABAergic inputs in dendritic regions (Azimi-Zonooz et al., 2006). Rather than underdeveloped inhibitor inputs an alternative explanation is that GABAergic neurons that will eventually become inhibitory are excitatory at this early stage of neuronal development.

As neurons mature, KCC2 expression increases and $[Cl^-]_i$ decreases, rendering GABA-mediated currents inhibitory (Figure 3) (Clayton et al., 1998a; Clayton et al., 1998b). Interestingly, expression of glutamic acid decarboxylase-67 (GAD67), an important enzyme in the production of GABA, is upregulated with brain development. Furthermore, ischemia results in a decrease in GAD67

Expression in immature brain but not in adult brain (Xu et al., 2006). These authors conclude that decreases in GAD67 expression represents a reduction in inhibitory signaling and thus reduced neuroprotection. However, it is more likely that the decrease in GAD67 expression in ischemic immature brain is a protective mechanism as GABA is excitatory at this stage of development. This interpretation is supported by the fact that perfusion of GABA (10-2000 mM) onto developing rat cortical neurons resulted in neuroprotection in mature neurons older than 20 days in culture, but enhanced cell death in less developed, immature neurons (Zhao et al., 2005). Therefore, just as inhibition of excitatory GABAergic signaling is neuroprotective in pond snail ganglia, GABAergic inhibition may also prove to be crucial to the protection of developing brain against hypoxic or ischemic insult, although they have received comparatively little study (Figure 2).

Cl^- Gradients and Hyper-excitability in Adult Mammalian Neurons Post-ischemia

While experiments with pond snails cannot be directly extrapolated to adult mammalian models, they offer insight into the rescue of critically damaged neurons that have already passed the window of conditioned protection or recovery based on GABA receptor manipulations. Mammalian cells that undergo ischemic insult experience periods of over-excitability during reoxygenation that may contribute to eventual cell death over the ensuing weeks following recovery, and appear linked to derangements in cellular GABA homeostasis and altered Cl^- gradients. Non-lethal administration of OGD or anoxic episodes induce transient elevations of $[Cl^-]_i$ and are not cytotoxic in rat neurons (Inglefield and Schwartz-Bloom, 1998; Inglefield and Schwartz-Bloom, 1999; Jiang et al., 1992).

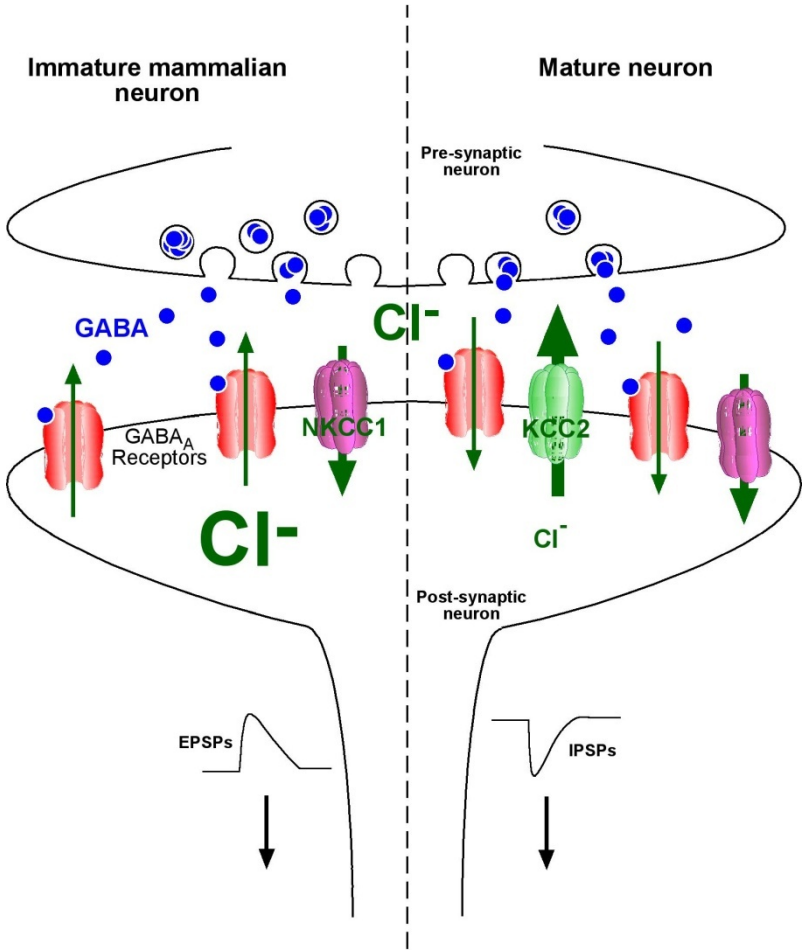

Figure 3. Schematic of the effect of chloride gradient reversal on the determination of GABAergic signaling in immature and mature mammalian neurons. In immature mammalian neurons (left side), $Na^+/K^+/Cl^-$ (NKCC1) is expressed but the K^+/Cl^- co-transporter (KCC2) is not and intracellular Cl^- (green) is high. Pre-synaptic GABA release (blue) leads to activation of post-synaptic $GABA_A$ receptors and passive extrusion of Cl^- ions out of the cell. This movement is depolarizing and results in the propagation of excitatory post-synaptic potentials (EPSPs) and increased neuronal excitation. In mature mammalian and facultative anaerobe neurons (right side), KCC2 expression is increased and intracellular Cl^- is low. Here, $GABA_A$ receptor activation results in the passive influx of Cl^- ions into the cell. This movement is hyperpolarizing and results in the propagation of inhibitory post-synaptic potentials (IPSPs) and decreased electrical excitability.

Conversely, longer durations of OGD or anoxic insult leads to loss of synaptic activity and a second, larger elevation in intracellular $[Cl^-]_i$ and cell death following reoxygenation (Galeffi et al., 2004; LoPachin et al., 2001; Zhu and Krnjevic, 1999). Pronounced elevations in $[Cl^-]_i$ can lead to reversal of $GABA_A$ receptors such that GABA becomes excitatory (Thompson and Gahwiler, 1989). Therefore, reversal of Cl^- gradients may contribute to hyper-excitability and cell death following ischemic insult.

These findings create a paradox in that excitatory GABA signaling enhances cell death attributable to ischemic insult, while cells treated with GABA agonists during or immediately post-ischemia experience neuroprotection. As with the pond snail, the answer may lie in the expression of the two opposing Cl^- transport mechanisms: KCC2 and NKCC1. In the reperfusion period following OGD, NKCC1 protein levels were unchanged but protein levels of the Cl^- extruder KCC2 were reduced by 30% at one hour and 70% by two hours post-insult. Interestingly, both the rise in intracellular Cl^- and the reduction in KCC2 protein levels were reversed by $GABA_A$ receptor agonism immediately following ischemic insult and cell death was avoided (Galeffi et al., 2004). These authors suggested that reduced KCC2 expression may be part of preprogrammed cell death and that $GABA_A$ receptor agonism may not directly affect KCC2 expression, but rather protects the cell and prevents cell death and the associated downstream mechanisms, including protein expression. Taken together, these data indicate that activation of $GABA_A$ receptors immediately after ischemic insults maintains KCC2 expression, which in turn is critical to maintaining neuronal Cl^- gradients. Failure to maintain this gradient likely not only removes the inhibitory mechanism mediated by GABAergic signaling, but may contribute to post-ischemic excitability by directly exciting neurons.

The loss of inhibitory signaling in post-ischemic neurons is likely further enhanced by decreased abundance of $GABA_A$ receptors and decreased expression of $GABA_A$ receptor subunit mRNA that is associated with extensive cell death in the recovering hippocampal area CA1 (Li et al., 1993). Mechanisms that limit GABA-mediated signaling in neurons that have deformed Cl^- gradients may help to reduce post-ischemic hyperexcitation and allow the cells to partially recover post anoxia. Thus a mechanism such as occurs in the pond snail, which reduces brain activity by limiting the uptake of Cl^- via NKCC1, may provide transient neuroprotection to neurons otherwise programmed to undergo apoptosis following ischemic insult.

CONCLUSION

The anoxia-tolerant western painted turtle provides an excellent model in which to explore natural neuroprotective mechanisms that may lead to improved stroke therapies; furthermore, our research indicates that GABA plays an import role in anoxia-tolerance and that GABA-based interventions against ischemic damage should be pursued. Previously, most treatments aimed at reducing ischemic cell death following stroke were targeted at excitatory NMDARs, which have been linked to neuronal death due to ischemia (Diemer et al., 1990). However, despite substantial evidence of the efficacy of NMDAR antagonists at preventing ischemic damage, no NMDAR antagonists have passed clinical trials due to a variety of psychotomimetic and toxic side effects, although recently mementine, a non-competetive partial antagonist of the receptor has shown promise (Lipton, 2004). Conversely, many of the compounds used to target GABA receptors in experimental models are already used clinically to treat other disorders including seizures (Caramia et al., 2000; Vincent et al., 2005), and may in fact regulate glutamate release following ischemic insults, thus circumventing the need to regulate NMDARs directly (Fukuda et al., 1998; Ouyang et al., 2007; Thompson et al., 2007).

Facultative anaerobes with high inwardly directed Cl^- gradients and hyperpolarizing responses to GABA exhibit a common response to anoxia involving moderate to extreme elevations of $[GABA]_e$, heightened $GABA_A$ B_{Max} and suppression of electrical activity including both AP and PSP frequencies. Mature mammalian neurons are sensitive to anoxia but experimental interventions that increase $GABA_A$ receptor activity prevent hyper-excitability and provide neuroprotection against ischemic insults. Thus, mimicry of the endogenous GABA-based defense mechanisms of anoxia-tolerant vertebrates results in increased anoxia-tolerance in adult mammalian neurons. A similar common mechanism may exist in the immature neurons of developing mammals in which GABA is an excitatory neurotransmitter. The pond snail possesses endogenous defense mechanisms that down-regulate the effect of excitatory GABA signaling, and mimicking these mechanisms may prove to be effective against ischemic insult in immature mammals.

It should be noted that in addition to elevated GABA, true facultative anaerobes employ a wide range of strategies to defend energy stores during anoxic insult (Bickler and Buck, 2007). Furthermore, these species are invariably ectothermic and are able to survive at low temperatures and

therefore take advantage of considerable Q_{10} effects. However, GABA elevations are a hallmark of anoxia-tolerance in facultative anaerobes and likely underlie inducible neuroprotective mechanisms in hypoxic mammalian models. Thus, parallels between endogenous and induced GABAergic mechanisms highlight the potential of targeting GABA-based pathways in preventing cell death following ischemic insult due to stroke.

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In: Turtles: Anatomy, Ecology and Conservation ISBN: 978-1-61470-554-3
Eds: M. J. Cosgrove and S. A. Roe

Chapter 4

AN OVERVIEW OF THE THREATENED PHYLOGENETIC DIVERSITY OF LIVING TESTUDINES BASED ON A REVIEW OF THE COMPLEX EVOLUTIONARY HISTORY OF TURTLES

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ABSTRACT

The history of turtle lineage began around 225 Mya. Since then, this group of reptiles developed diverse ecological strategies and colonized a wide range of environments, from marine to fully terrestrial habitats. In spite of their great ecological diversity, the bauplan of turtles is peculiar and little variable, with a body encased in a rigid shell consisting of a dorsal carapace and a ventral plastron. This fact has made the morphological comparison of turtles with other vertebrates very complicate. Thus, the understanding of the origins and phylogenetic relationships of turtles within the Amniota and the evolution of turtle biology are stimulating challenges for researchers. Several attempts in

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order to resolve these mysteries have been carried out from a multidisciplinary approach, but lacks consensus. The knowledge of the evolutionary history of turtle species and their inclusive groups is relevant for their conservation management, especially taking into account that many species are among moderate and high risk of extinction. In this chapter, we update and review the state of the art of the systematics of turtles from the point of view of several often conflicting disciplines, embryology, morphology, paleontology and molecular systematics. In addition, we sound out the amount of threatened phylogenetic diversity in the turtle tree of life based on fossil evidence, recent phylogenetic hypotheses and data from the red list of the International Union for the Conservation of Nature.

INTRODUCTION

The clade Testudines (= Chelonia *sensu* Gauthier et al., 1988) include about 289 species alive today split up into two primary groups: Pleurodira and Cryptodira (Joyce et al., 2004). Nowadays, cryptodires are more diverse than pleurodires (Figure 1), the latter being more restricted geographically, living only in the southern hemisphere (Gaffney et al., 2006). Cryptodires are also adapted to a wider range of habitats, from open marine to arid environments, whereas pleurodires are restricted to freshwater (Gaffney et al., 2006).

Turtles are mysterious animals for the biologists, especially as far as their obscure evolutionary history is concerned. The origins and phylogenetic relationships of turtles with the remaining amniotes have been explored from different approaches such as paleontology, comparative anatomy, developmental biology, and molecular phylogenetics. However, there is no consensus among different source of data making the position of turtles in the amniote tree of live still rather confusing (see below and Werneburg & Sánchez-Villagra, 2009 and references therein). Uncertain phylogenetic relationships remain unresolved among fossil and extant turtles and even among several living turtle families and higher taxa (Shaffer et al., 1997; Gaffney et al., 2006; Barley et al., 2010). In addition, the evolution of adaptation of turtles to terrestrial and aquatic environments is poorly known because of uncommon preservation of appendicular skeletal elements in extinct turtles and their incomplete fossil record (Scheyer & Sander, 2007). As an example, there has been an intense debate about the terrestrial (e.g. Joyce & Gauthier, 2004;

Scheyer & Sander, 2007) versus semiaquatic (e.g. Gaffney, 1990) origins of turtles.

The knowledge of the evolutionary history of organisms is crucial to characterizing the biological diversity. Phylogeny is a record of how biodiversity has come about and the understanding of the origins of a group of organisms can assist in their conservation by contrasting current versus historical patterns (Harvey et al., 1996; Purvis et al., 2005). Moreover, phylogenies can inform about the risk of lineage extinction and assess the amount of evolutionary history lost if extinction occurs (Purvis et al., 2000). Thus, the extinction of species-poor or monotypic highly divergent lineages containing endangered species would entail the loss of a significant part of the evolutionary history of the inclusive clade. It is also possible to extrapolate from the past and present to the future, in order to predict the state of biodiversity under different scenarios (Rosenzweig, 2001). In addition, well resolved phylogenies are quite important in wildlife management because provide legal basis for conservation programs (O'Brien, 1994).

According to IUCN (2010), near a half of turtle species are threatened. Among them, 59 species are classified as vulnerable, 40 species as endangered and 30 species are critically endangered, representing the 20%, 14% and 10% of the total number of turtle species respectively. Thus, a better understanding of the evolutionary history of turtles is urgently needed in order to estimate the amount of turtle diversity that is at risk of extinction. In this chapter, the evolutionary significance of turtles is discussed by means of a review of the most recent advances in the knowledge of turtle origins, systematics, and the evolution of ecological adaptation. In addition, available data on endangered species is analyzed from a evolutionary point of view in order to obtain a general view of the amount of evolutionary history of turtles that may be lost if no efficient conservation measures are taken.

THE MYSTERIOUS PHYLOGENETIC RELATIONSHIPS OF TURTLES WITHIN AMNIOTA

The origins of turtles and their body plan are one of the great riddles of reptile evolution. Turtles are characterized by a horny beak rather than teeth and a shell composed of an upper carapace and a lower plastron, joined together by a bony bridge. Their shell is like that of no other animal and it is a

composite structure derived from ribs, parts of the shoulder girdle and specialized dermal bones (Reisz & Head, 2008). The peculiar body plan of turtles and their highly derived anatomical features make their phylogenetic position within the amniotes obscure. In fact, there has been an intense debate if turtles evolved from a lineage within Parareptilia —a well supported clade of stem amniotes including pareiasaurs and procolophonoids among others— or belong to Eureptilia which include diapsid reptiles (Tsuji & Müller, 2009).

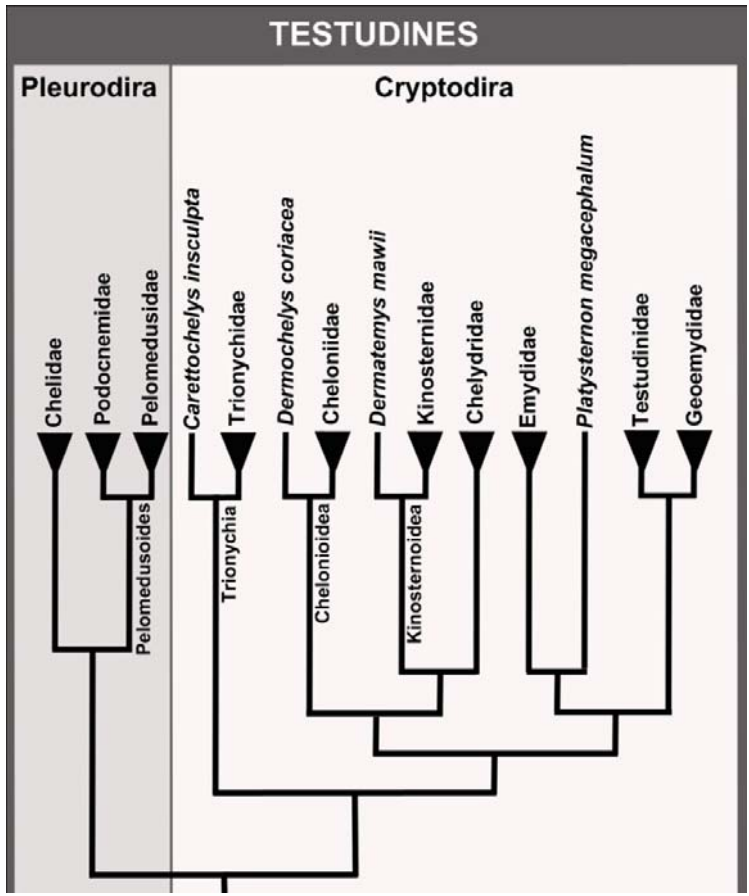

Figure 1. Phylogenetic tree of the main lineages of living turtles based on Joyce et al. (2004) and Barley et al. (2010).

Until today, up to ten phylogenetic hypotheses have been proposed, but none of them has a general acceptance (Figure 2). For instance, it has been

suggested that turtles are sister of Thecodontia (including Mammalia and Archosauria) (Gardiner, 1993), Sauria (crown diapsids) (Caspers et al., 1996), Sphenodontia (Fushitani et al., 1996), Archosauria (Kumazawa & Nishida, 1999), Crocodylia (Hedges & Poling, 1999), Lepidosauria (Zardoya & Meyer, 2000), and Aves (Pollock et al., 2000). However, most of these phylogenetic hypotheses are constructed ignoring extinct taxa. Extensive data including both extinct and extant amniotes suggest that turtles are part of a clade of basal terrestrial "anapsid" reptiles (Gauthier, 1994), are related to procolophonoids (Laurin & Reisz, 1995), or pareiasaurs (Lee, 1997) or they are sister taxa of sauropterygians within Eureptilia (de Braga & Rieppel, 1997). Disagreements among all these hypotheses probably are due to both morphological and molecular data are sensitive to taxon sampling, homology issues, rate heterogeneity and missing data (Lee et al., 2008).

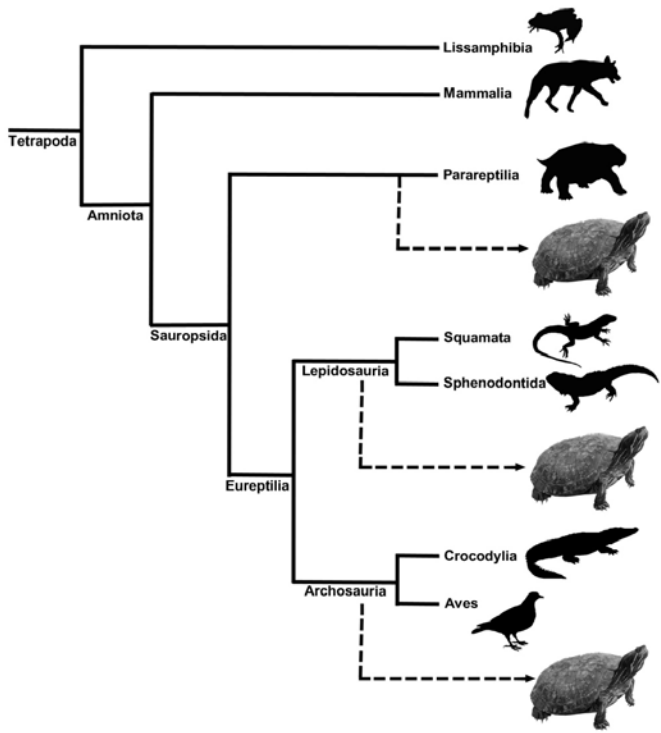

Figure 2. Three (out of ten) main hypotheses for the phylogenetic relationships of turtles with remaining amniotes.

Recent studies have not shed much light to the phylogenetic relationships of turtles within Amniota. New molecular analyses strongly support a turtle + archosaur relationship within Eureptilia (e.g. Hugall et al., 2007; Shedlock et al., 2007). In fact, since the beginnings of 2000s there has been decreasing support to the hypothesis of a turtle-parareptile relationship which is no longer considered valid among the majority of evolutionary biologists (see references in Tsuji & Müller, 2009). However, new embryological data (Werneburg & Sánchez-Villagra, 2009), combined molecular and morphological data (Lee et al., 2008) and fossil evidence (Lyson et al., 2010) place again turtles outside Diapsida. Specifically, in this latter study, turtles nest within parareptiles, forming a well supported clade with the genus *Eunotosaurus*, as the sister group to Diapsida (Figure 3). According to Lyson et al. (2010), basal-most stem turtles *Odontochelys semitestacea* and *Proganochelys quenstedti* have all six unequivocal synapomorphies diagnostic of Parareptilia which were listed by Tsuji & Müller (2009): 1) absence of a lacrimal-nasal contact, 2) absence of a caniniform region, 3) shortened postorbital region, 4) single median embayment of the posterior margin of the skull roof, 5) absence of a supraglenoid foramen and 6) absence of a subtemporal process of the jugal. Thus, the debate about the phylogenetic affinities of turtles with other amniotes seems still open and further analyses are needed to confirm the turtle-parareptile hypothesis as suggest these last studies.

OLDEST KNOWN TURTLES AND THE EVOLUTION OF SHELL

In spite of major controversies on their phylogenetic relationships, the evolutionary history of turtles began in the early Late Triassic, around 220 Mya. Since its discovery in 1884, *Proganochelys quenstedti* from the Late Triassic of Germany was considered the oldest turtle known (Gaffney, 1990). Nevertheless, only two years ago, *Proganochelys* was dethroned as the basal-most Testudinata due to new evidence from China. *Odontochelys semitestacea* is a relatively small turtle, about 40 cm long, approximately five million of years older than *Proganochelys* that was discovered near Guanling, Guizhou Province (southwestern China) (Li et al., 2008). According to these authors, *Odontochelys* is evolutively more primitive than *Proganochelys* in having teeth on premaxilla, maxilla and dentary; absence of fully formed carapace;

absence of acromial process on scapula and presence of long tail, among other characters. *Odontochelys* also shares primitive features with *Proganochelys* (see Li et al., 2008 for details).

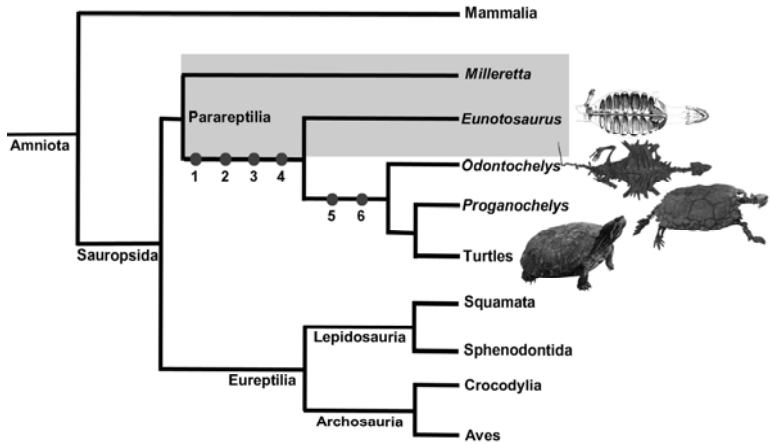

Figure 3. According to new phylogenetic analyses carried out by Lyson et al. (2010) turtles are closely related to the parareptile *Eunotosaurus* based on the following shared characters: 1, broad T-shaped ribs; 2, ten elongate trunk vertebrae; 3, cranial tubercles; 4, wide trunk. Two additional features are shared by *Odontochelys*, *Proganochelys* and extant turtles: 5, presence of plastron and 6, presence of neurals.

Turtle shell represents an evolutionary novelty and some authors propose that turtle evolution might be radical or saltatory instead of a gradual change (Gilbert et al., 2001; Rieppel, 2001). The comparison of rib cage of the parareptile *Eunotosaurus* and the shell of *Odontochelys* and *Proganochelys* provides valuable information about the evolution of turtle shell (Lyson et al., 2010). The dorsal part of turtle shell, or carapace, is derived from the ribs. The scapula is found under the carapace, contrary to the pattern of remaining amniotes in which the scapula is outside the rib cage (Figure 4 and Nagashima et al., 2009). In the Late Permian, *Eunotosaurus* had broadened ribs with apparent metaplastic ossification of the dermis and ribs joined by a second ossification. In the next stage, 44 Myr after, *Odontochelys* developed neural plates and a fully ossified plastron, containing portions of the shoulder girdle and gastralia. The carapace of *Odontochelys* resembles embryonic stages of extant turtles in that there is only some broadening and consequent flattening of the dorsal ribs, which do not expand into costal plates (Li et al., 2008;

Nagashima et al., 2009). Anterior ribs grew posteriorly in *Odontochelys* and the scapula remained anterior to the ribs (Figure 4). The dorsal ribs of *Odontochelys* seem arrested axially and they do not bend ventrally, suggesting that a caparacial-like ridge might be acquired in the flank of their embryos but not persisted and encircled the carapace margin in later development, as in modern turtles (Nagashima et al., 2009). The completion of the shell may have occurred rapidly, approximately in five million of years, which is the timespan that separate *Odontochelys* and *Proganochelys* (Lyson et al., 2010).

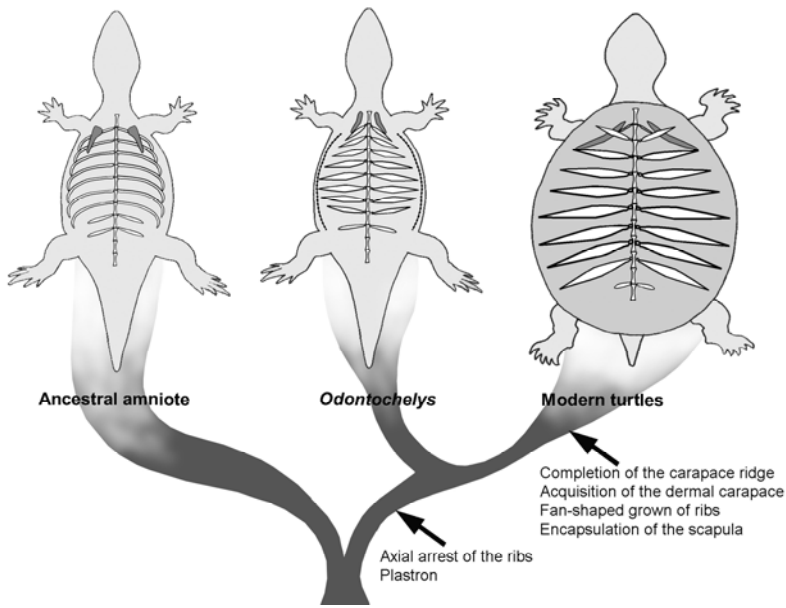

Figure 4. Evolution of turtle body plan. In the general pattern of amniotes (excluding turtles), scapulas (in dark grey) are outside the rib cage. In *Odontochelys*, scapulas are anterior to the ribs whereas in modern turtles they are encapsulated under the carapace. Dorsal ribs are arrested axially in *Odontochelys* and in modern turtles ribs are placed in a fan-like arrangement. The broken black line in *Odontochelys* indicates a carapacial-like ridge that it might be present in their embryos. Modified from Nagashima et al. (2009).

The genus *Proganochelys* is well known from several skeletons. These ancient turtles are characterized by a massive shell and spiked armor on the neck and tail (Reisz & Head, 2008). The shell is fully developed with carapace and plastron enclosing shoulder and pelvic girdles (Gaffney, 1990). The

carapace consists of fused ribs, neural bones with fused thoracic vertebrae and marginal bones. The plastron is formed from interclavicles, clavicle and five paired bones fused together. Other Late Triassic stem turtles are *Proterochersis robusta* from several localities of Germany (Lapparent de Broin, 2001) and *Palaeochersis talampayensis* from northwestern Argentina (Rougier et al., 1995).

TERRESTRIAL VERSUS AQUATIC ORIGINS OF TURTLES

Extant turtles inhabit a wide diversity of terrestrial and fresh- and sea-water environments such as land, ponds, lakes, streams, large rivers, estuaries and ocean. An aquatic origin of turtles has been suggested when they are considered to be closely related to extinct marine sauropterygian reptiles (de Braga & Rieppel, 1997). However, other authors indicate that oldest turtles were clearly terrestrial (Joyce & Gauthier, 2004; Scheyer & Sander, 2007).

The lifestyle of extinct turtles may be inferred from different source of evidence. The gross morphology of shell, shoulder girdle and limbs reveals adaptations to terrestrial or aquatic environments (Depecker et al., 2006). Terrestrial turtles have domed shell and long scapular prong and short coracoid associated with a mode of locomotion in which walking is predominant (Figure 5a). By contrast, in both highly aquatic freshwater and marine turtles the scapular prong is short and the coracoid is long and they are associated with flat shells and swimming locomotion (Figure 5b). However, Joyce & Gauthier (2004) pointed out that some exceptions exist to this general rule. For instance, the aquatic Asian box turtle (*Cuora amboinensis*) has highly domed shell and the terrestrial African pancake tortoise (*Malacochersus tornieri*) has a greatly flattened shell (Ernst & Barbour, 1989). Other commonly used indicator is the depositional environment. Although it is easy to discard marine habitat for turtles discovered in terrestrial sediments, turtles discovered in fluvial or marine sediments may be either aquatic or terrestrial because rivers can bury remains of terrestrial animals or transport them to marine environments (Joyce & Gauthier, 2004). In these cases, detailed taphonomic analysis is needed in order to infer the degree of transport undergone by turtle remains before burying. For instance, isolated plates with evident abrasion marks indicate long distance transport (Figure 6a), whereas articulated shells and lack of abrasion, scavenging and tramping marks suggest short biostratinomic history and little transport (Figure 6b, c) (Brand et al.,

2000; Bertini et al., 2006). In this latter example, the habitat of the fossil turtle can be inferred from the depositional setting (e.g. Marmi et al., 2009).

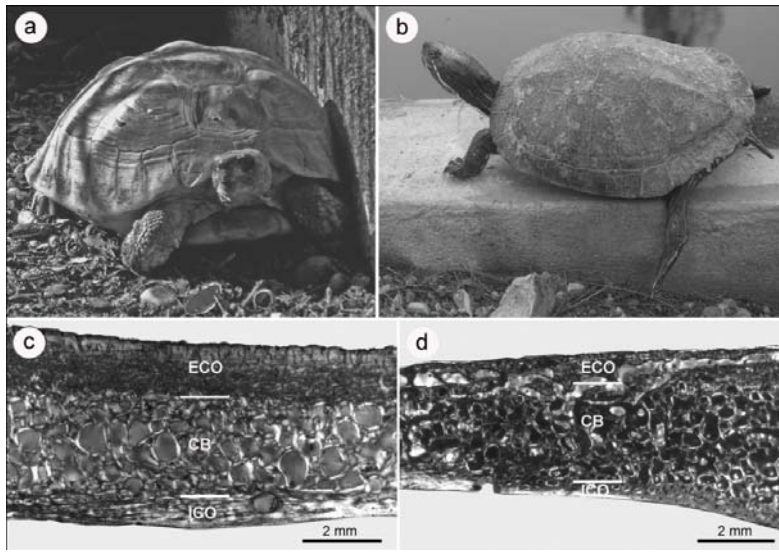

Figure 5. Adaptations to terrestrial and aquatic lifestyles: a, terrestrial *Testudo hermanni* with a moderate domed shaped carapace; b, semiaquatic *Trachemys scripta* with a flat shell; c, thin section of left xiphiplastron of *Testudo hermanni* showing wide and low vascularized external (ECO) and internal (ICO) cortices; d, thin section of a right xiphiplastron of *Trachemys scripta* with thin and highly vascularized external and internal cortices. The cancellous bone (CB) consists of short trabeculae and small to medium sized vascular spaces in both species. The pictures (a) and (b) have been taken at the Centre de Recuperació d'Amfibis i Rèptils de Catalunya (CRARC).

In living turtles, forelimbs usually reflect their lifestyle (Ernst & Barbour, 1989). Morphometric analysis of forelimbs revealed a close relationship between relative hand length and habitat preference (Joyce & Gauthier, 2004). Terrestrial turtles are short-handed to facilitate digital rollover during walking and highly aquatic turtles such as chelonioids are long-handed for swimming (Figure 7). However, after death, skull and limb bones separate from the body early and these elements are generally not preserved in fossil turtles (Brand et al., 2000; 2003). In these cases, histology provides an alternative way for testing the ecology of extinct turtles since the bone structure of the shell of living aquatic and terrestrial turtles reveal histological differences (Scheyer & Sander, 2007). Shell bones of terrestrial turtles exhibit a diploe structure with

well developed external and internal cortices, weak vascularization of the compact bone layers and dense interior cancellous bone with overall short traveculae (Figure 5c). On the contrary, aquatic turtles increase overall vascularization of the bone tissue reducing cortical bone layers and creating medium to large sized vascular spaces in the cancellous bone delimited by long and slender traveculae (Figure 5d). This pattern reported in aquatic turtles may be interpreted as an adaptation to aquatic environments in order to increase buoyancy. However, several exceptions of the general pattern exist, with terrestrial taxa showing moderate levels of vascularization (e.g. *Kinixys homeana* and *Chelonoidis carbonaria*) and aquatic taxa showing terrestrial-like bone microstructures (e.g. *Mauremys mutica* and *Pangshura tentoria*).

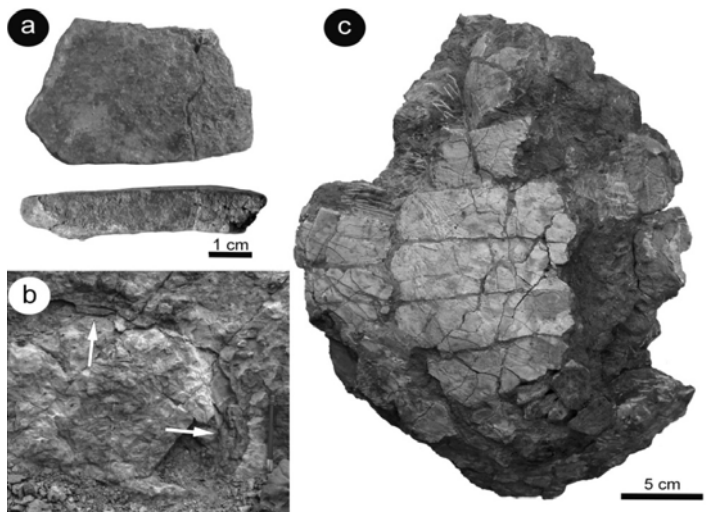

Figure 6. Different examples of preservation of turtle remains from the Late Cretaceous of Pyrenees: a, isolated plate with evident signs of abrasion in the external (top) and articulation (bottom) surfaces suggesting long distance transport (Barranc de Torrebilles site, Isona, southeastern Pyrenees); b, cast of a *Solemys* sp. carapace with remains of peripheral plates (arrowed) from Mina Esquirol site (Vallcebre, southeastern Pyrenees); c, partial shell of a bothremydid with carapace and plastron articulated from Barranc de Torrebilles site. Short transport or autochthony have been inferred for (b) and (c) and the depositional setting suggests that these extinct turtles inhabited lagoonal and fluvial environments, respectively (Marmi et al., 2009 and Marmi et al. under study). The pencil in (b) measures 14.6 cm.

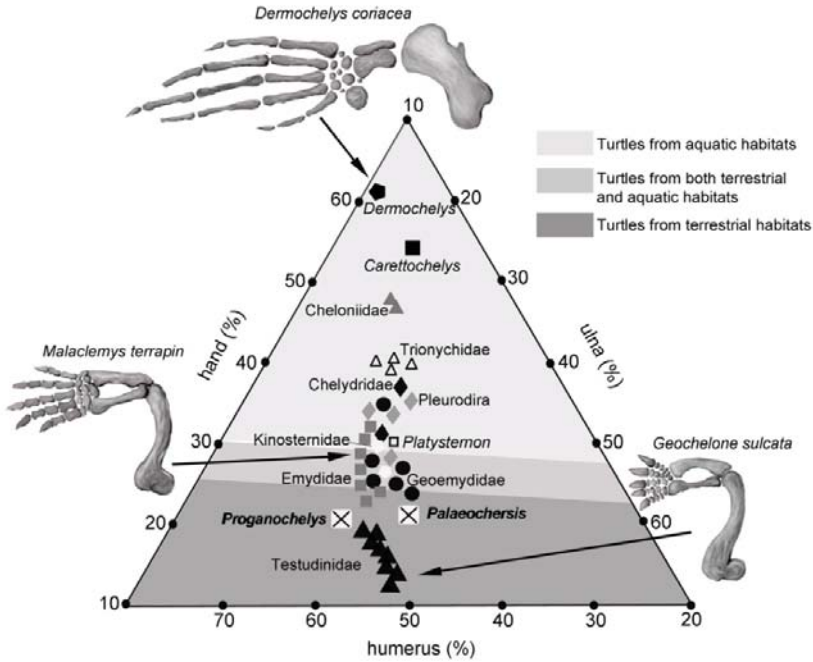

Figure 7. Distribution of taxa and habitat preferences on a ternary plot using forelimb measurements. There is large difference in hand sizes between specialized taxa such as marine *Dermochelys coriacea* and terrestrial *Geochelone sulcata*. Late Triassic turtles *Proganochelys* and *Palaeochersis* are included within the group of terrestrial taxa. Modified from Joyce & Gauthier (2004).

Gaffney (1990) suggested that *Proganochelys* inhabited freshwater as a bottom walker, being not exclusively aquatic or terrestrial, based on interpretations of the depositional environment and features of limb morphology. The analysis of forelimb proportion and depositional environment also indicate aquatic habits for *Odontochelys* and suggest a possible aquatic origin of turtles (Li et al., 2008). According to a recent phylogenetic analysis, stem turtles are closely related to the parareptile *Eunotosaurus* which lacks obvious aquatic adaptations and is only known from terrestrial sediments (Gow 1997; Lyson et al., 2010). In addition, forelimb proportions, shape of shell and histological data provide strong evidence for a terrestrial lifestyle in *Proganochelys* and *Palaeochersis* (Joyce & Gauthier, 2004; Scheyer & Sander, 2007). Thus, the putative marine ecology of *Odontochelys* might be interpreted as independently derived and

not ancestral to the subsequent radiation of turtles, supporting an origin of stem turtles in terrestrial environments (Lyson et al., 2010).

THE SCARCE JURASSIC FOSSIL RECORD AND THE ORIGINS OF CROWN TURTLES

The Jurassic is an outstanding period for understanding the evolution of turtles but their fossil record is fragmentary and sparse from the Late Triassic to the Late Jurassic, with only few known species spread around the world (Sterli, 2008 and references therein). However, as a whole, turtle remains have been collected from almost all continents —southern Africa, southern South America, North America, Europe, India and Central Asia (Sterli, 2008)— suggesting that chelonians were already distributed throughout the world. The Jurassic is also a key period to decipher the timing of the origin of crown groups of turtles (i.e. the clades delimited by living representatives). In this sense, two main hypotheses have been proposed (Figure 8).

The first hypothesis (Figure 8a) suggests that all turtles with the exception of *Proganochelys*, *Palaeochersis* and *Australochelys africanus*, from the Early Jurassic of South Africa, belong to one of the main groups of living turtles, Pleurodira or Cryptodira (Gaffney et al., 2007 and references therein). Based on this hypothesis, the crown turtles originated in the Late Triassic and *Proterochersis* would be interpreted as a stem pleurodire. On the contrary, other authors such as Joyce (2007) and Sterli (2008) interpret Late Triassic to Middle Jurassic turtles as stem groups suggesting that the origins of crown turtles were more recent, during the Middle to Late Jurassic (Figure 8b). According to Sterli & de la Fuente (2010) and references therein, Gaffney's hypothesis was supported by misleading interpretation of some key characters considered as synapomorphies of Pleurodira and Cryptodira. For instance, Gaffney (1975) includes the presence of a processus trochlearis oticum, a vertical flange on the transverse process of the pterygoid, and the presence of an epipterygoid among the synapomorphies that define Cryptodira. However, according to Joyce (2007) and Sterli & Joyce (2007) the former character may be a synapomophy of a more inclusive clade than Cryptodira and the two latter characters should be considered symplesiomorphies of turtles.

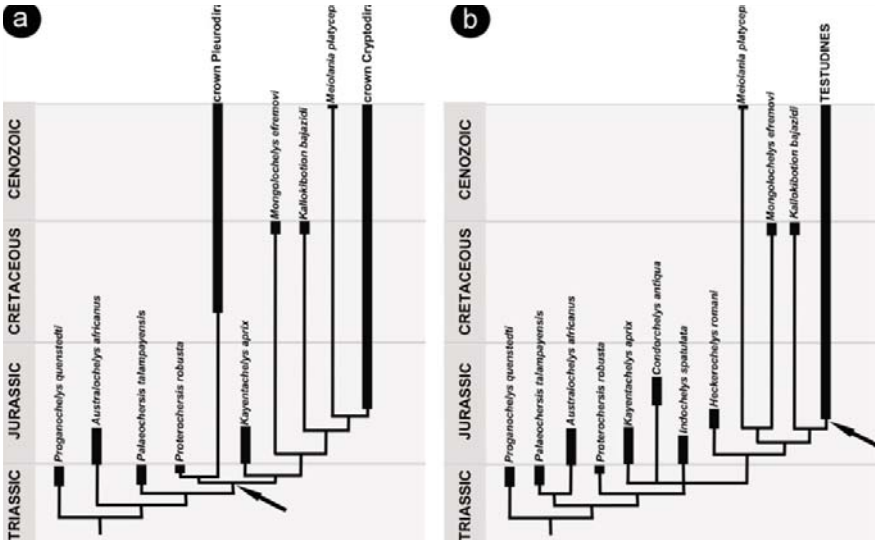

Figure 8. Two phylogenetic hypotheses about the origins of crown turtle taxa: a, lineages leading living turtles originated in the Late Triassic according to Gaffney et al. (2007); b, the origin of living turtle lineages occurred between the Middle and Late Jurassic, according to Joyce (2007) and Sterli (2008). Arrows indicate the crown group. Modified from Sterli & de la Fuente (2010).

ORIGINS AND DIVERSIFICATION OF PLEURODIRA

Extant pleurodires or "side necked turtles" are informally characterized by bending the neck in a horizontal plane (Gaffney et al., 2006). Nowadays, the lineage contains two major crown clades: Chelidae and Pelomedusoides that includes Pelomedusidae and Podocnemidae (Joyce et al., 2004). However, the extensive fossil record has revealed that pleurodires were significantly more complex and diverse in the past (Gaffney et al., 2006). Following Joyce et al. (2004) taxonomic proposal for turtles, only three Late Jurassic fossil species may be situated unambiguously along the phylogenetic stem of pleurodires: *Platychelys oberndoferi* from Europe, *Caribemys oxfordiensis* from Cuba, and *Notoemys laticentralis* from Argentina (de la Fuente & Iturralde-Vinent, 2001; Joyce et al., 2004). The large geographic distances among these three stem taxa indicate that the evolutionary history of the pleurodire lineage started in the Late Jurassic or even before.

The oldest representatives of living clades are reported since the Cretaceous. The clade Chelidae includes around 52 species of freshwater pleurodire turtles living in South America and Australasia that diversified in South Gondwana (Broin, 1988). Classically, the fossil record of this group was considered extremely poor and restricted to Tertiary (Joyce et al., 2004 and references therein). However, findings carried out in South America at the beginnings of 2000s have firmly established the presence of this crown clade in the Late Cretaceous. Thus, the oldest representatives of Chelidae are of Santonian (*Lomalatachelys*), Turonian-Campanian (*Bonapartemys* and *Prochelidella*) and Campanian-Maastrichtian (*Palaeophrynops* and *Yaminuechelys*) ages (De la Fuente et al., 2001; Lapparent de Broin & de la Fuente, 2001). However, Gaffney et al. (2006) recognizes this family since the Early Cretaceous.

Nowadays, Pelomedusidae are less diverse than Chelidae and contains eighteen living species (Joyce et al., 2004). The fossil record attributed to this group is scarce and rather confusing and specimens exhibit few diagnostic features (Joyce et al., 2004; Gaffney et al., 2006). *Teneremys lapparenti*, from the Aptian of Niger, is the only well recognized stem taxa of Pelomedusidae (Lapparent de Broin, 2000a). The crown clade is known at least since the Miocene (Lapparent de Broin, 2000b). According to Gaffney et al. (2006) the Podocnemidinura is sister of the extinct family Bothremydidae. Within the former, Podocnemidae are closely related to *Brasilemys* from the Albian of Brazil and *Hamadachelys* from the Cenomanian of Morocco. The crown taxa of Podocnemidae are recognized since the Late Cretaceous (Joyce et al., 2004). At present, Podocnemidae include eight living species.

ORIGINS AND DIVERSIFICATION OF CRYPTODIRA

Cryptodira is a morphologically diverse clade of turtles that differs from Pleurodira in the manner that retracts their head into the shell, bending their neck in a vertical plane to withdraw the head backwards. Thus, the majority of Cryptodira can put the head straight back into the shell, with some exceptions such as sea turtles or *Platysternon megacephalum*. Nowadays, the Cryptodira is the most extensive and rich group of Testudines that informally includes freshwater turtles, bigheaded turtles, Central American river turtles, pig noise turtles, snapping turtles, mud and musk turtles, box and wood turtles, tortoises, soft shell turtles, and sea turtles. The oldest taxa primitively inhabited

freshwater habitats, but later invaded marine environments at least once and at least four independent lineages diversified in fully terrestrial habitats (Joyce & Gauthier, 2004). Nowadays, the lineage contains eleven clades that can be grouped into four or five major crown clades: Chelonioidea, Trionychoidea, Chelydridae, Kinosternoidea and Cryptoderinea (Joyce et al., 2004; Barley et al., 2010). Although, the fossil record is extensive and fairly complete, the debate about the phylogenetic affinities of extinct Cryptodira with the major crown clades remains still open and further analyses are needed (Gaffney et al., 1987; Gaffney & Meylan, 1988; Joyce et al., 2004; Danilov & Parham, 2006; Anquetin et al., 2009; Joyce et al., 2009).

According to recent hypotheses, the phylogenetic stem of Cryptodira contains a series of common fossil taxa, such as Baenidae, Meiolanidae, Pleurosternidae, Plesiochelydiae, Sinemydidae, and Macrobaenidae (Gaffney, 1996; Gaffney et al., 1998; Parham & Hutchison, 2003; Joyce et al., 2004). However, other analyses suggest that most of these groups diverged before the diversification of Testudines (Sterli, 2010). Following Gaffney et al. (1987), only *Kayentachelys aprix* from the Early Jurassic of North America, may be situated unambiguously along the phylogenetic stem of Cryptodira, taking into account the following characters: presence of the processus trochlearis oticum and processus pterygoideus externus projecting posteriorly with a flat, vertical plate. Nevertheless, other authors propose Sinochelydae, from the Early Cretaceous of China and Mongolia, as the oldest reported Cryptodira based on characteristics of the shell (Hirayama et al., 2000).

Nowadays, the clade Chelonioidea includes seven or eight species of sea turtles belonging to Dermochelyidae and Cheloniidae. All species of this group are characterized by hard-shelled bodies, except for *Dermochelys coriacea* that is covered with horny scutes. The extensive fossil record has revealed that Chelonioidea was more abundant and diverse in the past, including several extinct families, such as Toxochelyidae, Osteopygidae, Thalassemyidae and Protostegidae (e.g. Gaffney et al., 2006). This last family of sea turtles contains the probable oldest representative of the living clade, *Santanachelys gaffneyi* from the Early Cretaceous of Brazil (Hirayama, 1998). This species had a primitive flipper that still possessed movable digits and a specialized skull with large interorbital foramina (Hirayama, 1998). As no other turtles are known from the phylogenetic stem, *Santanachelys gaffneyi* may also represent the oldest Chelonioidea (Joyce et al., 2004). However, it is important to note that new phylogenetic analyses place *Santanacheys* out of the Testudine clade (Sterli, 2010). The oldest remains of dermochelyids are

reported from the Campanian of North America and Japan (Joyce et al., 2004 and references therein) and *Toxochelys latiremys*, from the Late Cretaceous of North America, may be among the oldest stem cheloniids (Hirayama, 1998).

Trionychia is the sister taxa of different basal forms from the Early Cretaceous such as Adocidae and Nanhsiungchelyidae (Danilov & Parham, 2006). According to these authors, *Yehguia tatsunensis*, from the Late Jurassic of China, is placed on the stem of Trionychia, near the clades Adocidae and Nanhsiungchelyidae. Among the list of potential trionychians, *Sandownia harrisi* from the Early Cretaceous (Aptian) of Europe may be the oldest (Meylan et al., 2000). The crown clade Trionychia comprises the families Trionychidae and Carettochelyidae, informally called softshell and pig nosed turtles. The fossil record of the Carettochelyidae was well represented in the Tertiary period but actually only persists a single species, *Carettochelys insculptata*. The oldest representative of Carettochelyidae is *Kizylkumemys schultzi* from the middle Cretaceous (Albian and Cenomanian) of Central Asia (Nessov, 1977; Meylan, 1988). Trionychidae contains 23 especies of living softh-shelled turtles but has a poor fossil record. Despite this, the oldest hypothesized representatives of the trionychid clade are *Trionyx riabinin* and *"Trionyx" kansaiensis*, both reported from the Late Cretaceous (Santonian-early Campanian) of Asia and Kazakhstan (Vitek & Danilov, 2010), and *Aspideretoides allani*, *A. foveatus*, *A. splendidus* and *Apalone latus* from the Late Cretaceous (middle Campanian) of North America (Gardner et al., 1995).

Phylogenetic relationships among Kinosternoidea and other clades of cryptodires are unclear. Joyce et al. (2004) places Kinosternoidea within Trionychoidea, following Gaffney (1975). On the contrary, new molecular data supports a deep sister group relationship between Kinosternoidea and Chelydridae (Barley et al., 2010). The sister group relationship between *Dermatemys mawii* and Kinosternidae was only proposed within the last two decades, but currently it is strongly supported by morphological and molecular data (e.g., Gaffney & Meylan, 1988; Hutchison, 1991; Shaffer et al., 1997; Sterli, 2010). The oldest representatives along the stem lineage of *Dermatemys mawii* are *Haplochelys clark* and *Agomphus pectoralis* both from the Late Cretaceous (Maastrichtian) of North America (Knauss et al., 2010). This phylogenetic placement breaks with the tradition started by Hutchison & Bramble (1981), which suggest that *Agomphus* spp. is a stem-kinosternoid and *Hoplochelys* spp. is a representative of the kinosternid stem (Hutchison, 1991; Meylan & Gaffney, 1989; Joyce, 2007). The genus *Baptemys*, from the Eocene of North America, has been allied to *Dermatemys mawii* within

Dermatemydidae (Knauss, 2006). On the other hand, the oldest putative members of stem kinosternidae may be some undescribed remains from the Campanian of North America (Hutchison et al., 1998).

Taxonomic composition of Chelydridae and its relationships with remaining clades within Cryptodira are also still under debate. This family of turtles has a long fossil history from North America, Asia and Europe, far outside its present range. According to Eaton et al. (1999a, 1999b), the oldest crown chelydrid is *Protochelydra zangerli* from the Eocene of North Dakota and the oldest stem chelydrid is *Emarginachelys cretacea* from the Late Cretaceous (Turonian) of Montana. Following to Knauss et al., (2010), *Baltemys staurogastros*, *Xenochelys formosa* and *X. lostcabinensis*, from the Early Eocene of Wyoming, also belong to the crown clade Chelydridae. However, previous works placed *Baltemys* and *Xenochelys* within crown Kinosternidae (Joyce et al., 2004).

The crown clade Cryptoderinea contains *Platysternon megacephalum* and the crown taxa Testudinoidea (Joyce et al., 2004). *Platysternon megacephalum* is an extant freshwater turtle from Asia that forms a monotypic lineage sister to the Testudinoidea (Joyce et al., 2004; Krenz et al., 2005; Sterli, 2010). The fossil record of the *Platysternon* lineage is very fragmentary, with few and partial specimens coming from the Paleocene and Oligocene of Kazakhstan (Joyce et al., 2004 and references therein). Testudinoidea contains three major clades, Testudinidae, Emydidae and Geoemydidae called informally land tortoises and generally pond turtles. In addition to the last principal crown groups, Testudinoidea includes different basal families from the Cretaceous of Asia such as Haichemydidae, Sinochelyidae and Lindholmemydidae because of the development of an ossified bridge connecting the plastron with the carapace (Sukhanov, 2000; Danilov & Sukhanov, 2001).

The crow Testudinidae is represented by 43 extant species characterized by exclusive terrestrial habitats. This clade has an excellent fossil record but the majority of the basal forms, such as *Hadrianus*, *Stylemys*, *Manouria*, *Cheirogaster*, *Ergilemys* and others, lack skulls making phylogenetic analyses difficult. The oldest representatives are *Hadrianus majuscula* from the Early Eocene (Wasatchian) of New Mexico and *Achilemys cassouleti* from the Early Eocene (Ypresian) of France.

The crow Geoemydidae contains 61 species of freshwater, swamp, lagoon and humid environment turtles living in all the continents except to Australia and Antarctica.

Table 1. Conservation status of living turtles according to the International Union for Conservation of Nature (IUCN) database (IUCN, 2010). Numbers in parenthesis indicate the number of species within each turtle family. Remaining numbers are the number of species per genus classified within each threat category: CE, critically endangered; E, endangered; V, vulnerable; LR/NT, lower risk/near threatened; LR/LC, lower risk/least concern; DD, data deficient; NL, not listed in the IUCN Red List. The taxonomy of genera follows Joyce et al. (2004) appendixes

Major taxa		Genus	IUCN threat category						
			CE	E	V	LR/NT	LR/LC	DD	NL
Pleurodira	Chelidae (52)	*Acanthochelys*			1	3			
		Batrachemys	1						5
		Chelodina	1	1	1	3	2		5
		Chelus							1
		Elseya		1	1		1	2	3
		Elusor		1					
		Emydura					1		4
		Hydromedusa			1				1
		Mesoclemmys			1				1
		*Phynops**		1		2			4
		Platemys							1
		Pseudemydura	1						
		Rheodytes			1				
	Pelomedusidae (17)	*Pelomedusa*							1
		Pelusios			1		3	1	11
	Podocnemidae (8)	*Erymnochelys*	1						
		Podocnemis		1	3	1			1
		Peltocephalus			1				

Table 1 (Continued)

Major taxa		Genus	IUCN threat category						
			CE	E	V	LR/NT	LR/LC	DD	NL
Cryptodira	Dermatemydidae (1)	*Dermatemys*	1						
	Kinosternidae (25)	*Claudius*				1			
		Kinosternon			4	2	6	3	3
		Staurotypus				2			
		Sternotherus			1				3
	Trionychidae (24)	*Amyda*			1				
		Apalone					2		1
		Aspideretes			3				
		Chitra	1	1					
		Cyclanorbis				2			
		Cycloderma				2			
		Dogania					1		
		Lissemys					1	1	
		Nilssonia		1					
		Palea		1					
		Pelochelys		1	1				
		Pelodiscus			1				
		Rafetus	1	1					
		Trionyx							1
	Carettochelyidae (1)	*Carettochelys*			1				
	Chelydridae (3)	*Chelydra*			1		1		
		Macroclemys			1				
	Dermochelyidae (1)	*Dermochelys*	1						
	Cheloniidae (6)	*Caretta*		1					

Major taxa		Genus	IUCN threat category						
			CE	E	V	LR/NT	LR/LC	DD	NL
		Chelonia		1					
		Eretmochelys	1						
		Lepidochelys	1		1				
		Natator						1	
	Platysternidae (1)	*Platysternon*		1					
	Testudinidae (43)	*Chersina*							1
		Dipsochelys							1
		*Geochelone***	3		6		1		1
		Gopherus			3		1		
		Homopus			1	1			3
		Indotestudo		2	1				
		Kinixys			1	1		1	2
		Malacochersus			1				
		Manouria		1	1				
		Psammobates		1					2
		Pyxis	2						
		Testudo	1		2	1	1		
	Geoemydidae (61)	*Batagur*	2						
		Cuora	8	2	1				
		Cyclemys				1			3
		Geoclemys			1				
		*Geoemyda****		3					
		Hardella			1				
		Heosemys	2	1	1				
		Hieremys		1					
		Kachuga	1	2					

Table 1 (Continued)

Major taxa	Genus	IUCN threat category						
		CE	E	V	LR/NT	LR/LC	DD	NL
	Leucocephalon	1						
	Malayemys			1				
	Mauremys	1	3		1			2
	Melanochelys			1	1			
	Morenia			2				
	Notochelys			1				
	Orlitia		1					
	Pangshura		1		1	2		
	Rhinoclemmys				5			4
	Sacalia		2					
	Siebenrockiella			1				
Emydidae (48)	*Actinemys*			1				
	Chrysemys							1
	Clemmys			1				
	Deirochelys							1
	Emyoidea				1			
	Emys				1		1	
	Glyptemys		1	1				
	Graptemys		2	1	5			4
	Malaclemys				1			
	Pseudemys		1		2			4
	Terrapene		1		2		1	
	Trachemys		2	5	3			5

*, Includes genus *Rhinemys*

**, Includes genera *Astrochelys, Centrochelys* and *Chelonoidis.*

***, Includes genus *Vijayachelys.*

This group of turtles, informally called Eurasian pond, river turtles and Neotropical wood turtles, comprises a significant portion of the diversity of extant turtles and the major number of freshwater species. Nowadays, the oldest representative of geoemydids probably is *Echmatemys* ssp. from the Eocene of North America and Asia (Hirayama, 1985). Other undefined stem geoemydids are known from the Paleocene to Oligocene of various North American, Asian and probably European sites. Moreover, some basal taxa such as *Grayemy-Hokouchelys*, *Clemmydopsis*, *Epiemys* and *Elkemys* have been attributed to Geoemydidae but phylogenetic relationships among these taxa and extant forms are unclear (Mlynarsky, 1976).

The crow Emydidae contains 48 species of extant turtles most of which living in the New World, with the exception of *Emys orbicularis* from Europe, north Africa and western Asia. This group of turtles, informally called pond turtles, developed diverse ecological strategies and colonized a wide range of environments, from aquatic to fully terrestrial habitats. Nowadays, the oldest fossil emydid is *Gyremys sectabilis* from the Late Cretaceous of Judith River Formation of Montana, North America (Ernst & Barbour, 1989). Traditionally, a number of fossils have been associated (Mlynarsky, 1976) with this group but none has been integrated into a cladistic analysis and consequently cannot be referred with any confidence to Emydidae (Joyce et al., 2004). Recently, phylogenetic analyses have been carried out to clarify the phylogeny of some primitive forms, such as *Hummelemys, Palaeochelys, Juvemys, Merovemys* and others (Hervet, 2004). However, results do not elucidate their relationships with the Geoemydidae and Emydidae stem.

AN OVERVIEW OF THE TURTLE THREATENED PHYLOGENETIC DIVERSITY

Posadas et al. (2001) highlighted the fact that conservation of biodiversity requires the knowledge of its history. Phylogenies provide the ways to measure biodiversity, to quantify the evolutionary history of a set of species and to assess conservation priorities (Mace et al., 2003). Despite the large number of the recently published papers, key questions about the origins and evolutionary history of turtles remain unanswered, as it has been explained in the above paragraphs. Phylogenetic analyses have not resolved satisfactorily if turtles are sister to parareptiles (e.g. Lyson et al., 2010) or diapsids (e.g. Shedlock et al.,

2007). The Jurassic turtle fossil record is fragmentary and sparse making the understanding of the origins of extant lineages difficult (see Sterli, 2008). The phylogenetic relationships of extant Testudines are also unclear, with controversial results about the placement of Pleurodires (see Sterli, 2010 for an example) and other major lineages such as Chelonioidea, Kinosternoidea and Chelydridae (Barley et al., 2010 and references therein) in the tree of life of turtles. Some factors can explain disagreement in phylogenetic analyses carried out with different source of data (e.g. fossil evidence, morphology, DNA sequences). Firstly, morphological variation of turtles is still poorly understood from an evolutionary point of view. This is enhanced by large gaps in their fossil record, especially in key periods such as Jurassic, Cretaceous and Paleocene. Moreover, fossil turtles usually lack parts of the skeleton with characters of great systematic value (e.g. skulls). On the other hand, molecular trees show short internal branches, often weakly supported, and long terminal branches suggesting a rapid radiation of the living groups of turtles since the Late Jurassic (Sterli, 2010, but see Barley et al., 2010). This complicates the determination of interrelationships and the position of the root of major lineages of extant turtles.

Despite this bleak picture, we have estimated the amount of threatened phylogenetic diversity of turtles based, in part, on the results reported by Sterli (2010) and data from IUCN (2010) red list (Table 1, Figure 9). However, due the large disagreement among current studies, relationships among largest clades of living turtles are represented as a polytomy, especially within Cryptodira. Most of living major lineages of turtles are recognized in the fossil record since the Middle-Late Cretaceous (Figure 9). Only three of fourteen living lineages can be considered slightly endangered, two within Pleurodira (Chelidae and Pelomedusidae) and one within Cryptodira (Kinosternidae), containing 25% or less threatened species (Figure 9). At the opposite end, Podocnemidae, Carettochelyidae, Dermochelyidae, Cheloniidae, Dermatemydidae and Platysternidae are at high risk of extinction with 75% or more species threatened. The status is critical for Dermochelyidae, Dermatemydidae and Platysternidae because they are represented by a single species classified as endangered or critically endangered (Table 1).

This overview of the conservation status of the turtle tree of life clearly shows that most of the phylogenetic diversity generated during 90 million of years or more is in a real risk of extinction and the situation is extreme for an important number of lineages. Turtles agree with the prediction of Purvis et al. (2000) in that species on long branches (i.e. without close relatives) are particularly likely to be at risk.

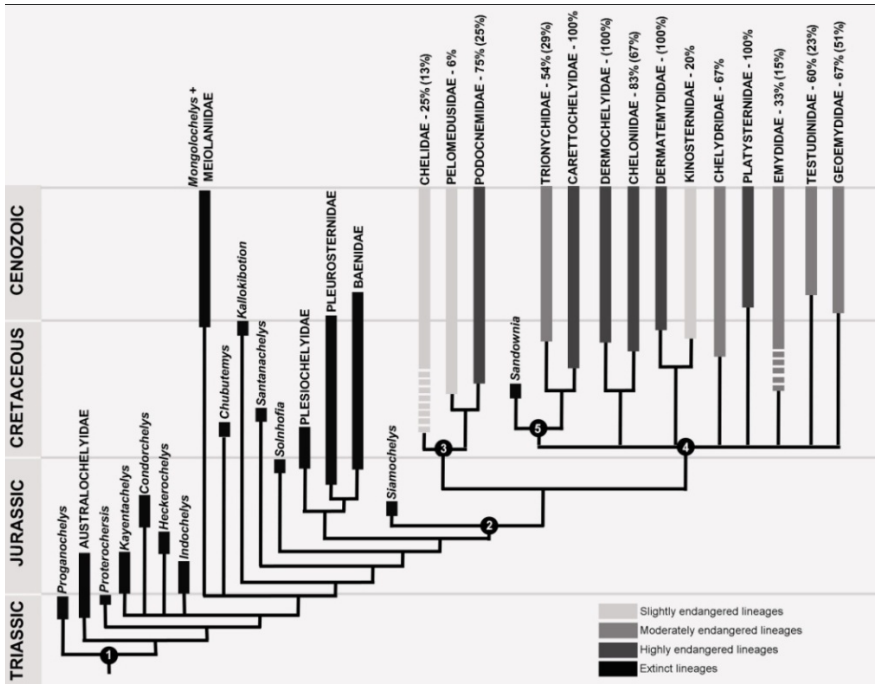

Figure 9. Evolutionary tree of turtles with phylogenetic relationships of stem taxa based on Sterli (2010). Phylogenetic relationships within Testudines (node 2) are not clarified because the lack of consensus among different authors (see text for details). The percentage of threatened species (vulnerable, endangered and critically endangered—see Table 1) is shown for each lineage. Percentages of endangered plus critically endangered species are within parenthesis. A quantification of the risk of lineage extinction is indicated based on Table 1: slightly endangered lineages (25% or less of species threatened), moderately endangered lineages (between 25% and 75% of species threatened), highly endangered lineages (75% or more of species threatened). Numbers in nodes mean: 1, Testudinata; 2, Testudines; 3, Pleurodira; 4, Cryptodira; 5, Trionychia.

Moreover, according to these authors, fossil record demonstrates that some clades (e.g. Pleurodira, Chelonioidea) were more diverse in the past and probably have suffered considerable extinction through geological time due to unknown phylogenetic factors promoting a greater risk of extinction for these lineages.

Nee & May (1997) noted that the importance assigned to a species is not necessarily proportional to the amount of evolutionary history it represents. For instance, conservation concern is often focused on their behavior, potential usefulness or their role in an ecosystem rather than simply on overall measures of evolutionary history. Moreover, from a systematic perspective, there has been

debate on what may be a greater loss: the demise of the sole survivor of an ancient lineage (for instance, the Central American river turtle, *Dermatemys mawii*) or the demise of a member of a rich species flock (for instance, the Roti Island snake-necked turtle, *Chelodina mccordi*) (Nee & May, 1997). Distinct taxa and 'living fossils' contribute disproportionately to overall biodiversity because the large amount of evolutionary history that may represent (Bowen, 1999). However, other authors have argued that these 'sole survivors' are a dead-end and conservation efforts should be focused on the species flock because its future evolutionary potential to restock biodiversity if a mass extinction episode occurs (Erwin, 1991). According to Bowen (1999), the leatherback sea turtle (*Dermochelys coriacea*) may illustrate the complexity of the decision making in conservation biology and that several perspectives (systematic, ecological and evolutionary) should be taken into account. This critically endangered species is the last survivor of a lineage that traces back to the Late Cretaceous or before. Thus it has a high relevance from the systematic point of view. In addition, it has a remarkable ecological and evolutionary significance because it is one of the few vertebrates that feeds on jellyfish (Scyphozoa) and has a suite of unique morphological and physiological adaptations allowing it to forage in freezing waters (Bowen, 1999).

This study demonstrates that the future of turtles is uncertain if no effective conservation measures are carried out. Turtles represent an exclusive group of reptiles and amniotes because their peculiar body plane and long evolutionary history, traced back since at least 225 million years ago, that justifies conservation efforts. Moreover, most endangered species of turtles inhabit aquatic environments. Their conservation may guarantee the conservation of fragile habitats such as rivers and wetlands that frequently suffer the impact of human activities.

ACKNOWLEDGMENT

Authors agree colleages of the Mesozoic Research Team (Institut Català de Paleontologia) and the Centre de Recuperació d'Amfibis i Rèptils de Catalunya (CRARC) for their support in research activities. This study has been funded by the project CGL2008-06533-C03-01/BTE (Ministerio de Ciencia e Innovación, Spain). J.M. has a grant of the Juan de la Cierva Program (JCI-2007-187-1593).

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In: Turtles: Anatomy, Ecology and Conservation ISBN: 978-1-61470-554-3
Eds: M. J. Cosgrove and S. A. Roe

Chapter 5

HEMATOLOGY OF THE LOGGERHEAD TURTLE, *CARETTA CARETTA*

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ABSTRACT

The study of the hematological characteristics of loggerhead turtles can be very helpful in the rescue and rehabilitation of this endangered species. Normal hemocellular and biochemical values are the basis for the evaluation of a subject's health status. The identification of the blood cells through cytochemical or immunocytochemical methods is the first step to obtain a correct classification of the cells. The following blood cell types were found: lymphocytes, monocytes, heterophils (the counterparts of mammalian neutrophils), eosinophils, basophils (rarely observed), erythrocytes along with some of their precursors, and thrombocytes. Lymphocytes and monocytes are easily distinguishable, as they are similar to the mammalian counterparts, whereas heterophils (which are the equivalent of mammalian neutrophils) and eosinophils both contain eosinophilic granules in their cytoplasm, making their identification difficult. The thrombocytes are nucleated cells, whose shape is variable in dry film smears of circulating blood, either round or oval; they take part in the hemostatic process, as suggested by their

tendency to form aggregates. The erythrocytes are also nucleated cells, showing an ellipsoidal and flattened configuration. In all specimens of this marine reptile, both healthy and unhealthy, the mature erythrocytes contain one characteristic inclusion in their cytoplasm, which was recognized as a Heinz body, formed by the precipitation of hemoglobin. The loggerhead's hemoglobin was studied in depth and revealed some specific characteristics, one of which is that under physiologic conditions it precipitates due to its instability.

While no data are available about the erythropoiesis in the primary organs, circulating erythropoiesis was revealed to occur in the loggerhead's blood, just as it also occurs at this site in other non-mammalian vertebrates.

Loggerhead blood infestation by spirorchids was reported by Wolke *et al.* (1982), while Di Santi *et al.* (2010) found two specimens of loggerheads hemoparasitized by *Theileria*.

MAIN BODY

The loggerhead *Caretta caretta* is an endangered species, according to the International Union for Conservation of Nature and Natural Resources, due to human activities disturbing or destroying marine environment and turtle nesting beaches throughout the world. In the last decades, sea turtle populations have been drastically reduced by hunting for meat, eggs and hide, incidental capture in fishing nets or by hooks, especially those used in longline fishing practices, uncontrolled coastal development, vehicular traffic on beaches and sea pollution.

The first step in the evaluation of the health of any animal, besides the veterinary examination, consists of blood tests which are aimed at establishing its hematological and biochemical profile. As blood is a special tissue, which comes in contact and interacts with all other tissues, the study of its components can lead to vital information regarding the subject’s health status. In recent times, hematological and biochemical data have been collected to define the normal, healthy values of marine turtles for scientific reasons *per se* as well as for evaluating the data of turtles that have been more and more frequently injured due to human activities. Internationally, many centers are currently active in the research and conservation of this and other sea turtle species as well as caring for sick and injured specimens. Since all blood parameters may vary along with the environmental temperature, geographical distribution of the population, age, sex and physiological condition of the

specimen, as well as the capture technique and the treatment of the blood sample, complete baseline ranges of blood parameters of healthy *Caretta caretta* from all geographical areas are still lacking. An assessment of reference intervals for the blood parameters of this sea turtle is clearly needed.

As in other poikilotherms, loggerhead hemogram values are influenced by the environmental temperature, so that the season and the geographical region consistently affect the rate of hemopoiesis. As might be anticipated, hemopoiesis is enhanced in warm seasons as opposed to the cold ones (Glomski and Pica, 2006*).* Erythropoiesis, specifically, is subject to seasonal fIuctuations: environmental temperature affects red cell production in all poikilotherms, so that the most active erythroid proliferation and maturation occur in warm seasons. In poikilotherms, the erythropoietic processes can be expected to be dormant in winter. In spring, with the awakening of subdued physiological functions, various percentages of erythroblasts appear in the circulating blood. Thus the relative level of erythroblasts in the circulating blood count can be used as an indicator of concurrent activity of erythropoietic bone marrow. Consistent with these considerations, the temperature of ocean waters is lower than that of inland seas and consequently the major erythrogramic values (RBC count, hematocrit and hemoglobin concentration in the blood of loggerhead turtles) in these *loci* have been identified as lower than their counterparts residing in the warmer inland seas, such as the Mediterranean Sea. Since sea turtles cannot regulate their body temperature, if the water temperature drops too quickly, they can become *cold-stunned*. This mainly happens to juvenile specimens, which should move to warmer waters before the temperature drops. Thus frequently, in cold seasons sea turtles are found *stranded* on the beaches or just offshore. They are lethargic and their immune system is depressed, consequently, infections can spread throughout their bodies. A case report about the recovery from a widely distributed and deep skin necrosis in a *cold-stunned* loggerhead specimen has been presented by Occhiello *et al.* (2009).

Biochemical blood and hemocellular values also vary according to the age of the specimens. Bradley *et al.* (1998) conducted monthly morphologic evaluations of the blood cells and hemograms of 10 Cape Romaine, South Carolina-collected loggerheads for the first year of these turtles' life. Lithium heparin was used as the anticoagulant for the hemogram. Smears obtained from un-anticoagulated blood were used to observe and describe the circulating blood cells: heterophils, eosinophils, basophils, lymphocytes, monocytes, immature red blood cells, hematogones (extruded nuclei) and

thrombocytes. Monitoring of hemogram values revealed an increase of the white cell counts, mean hemoglobin concentration (MCH) and mean corpuscular hemoglobin concentration (MCHC). Kakizoe *et al.* (2007) evaluated the changes in hematological characteristics and plasma chemistry values during the first three years of life of five juvenile loggerhead turtles, taken from the same clutch and grown in an indoor artificial nesting beach. The RBC count was stable during the whole period, while the WBC count showed variability in the first months, reaching the maximum value in the 7th month of life, which was approximately double of that obtained in adult females. In regard to WBC differential counts, the percentage of heterophils decreased and the percentage of lymphocytes increased until to the 10th month of life and remained stable for the rest of the observation period. Monocytes were observed after the 3rd month of life and their percentage fluctuated thereafter. Basophils were rarely observed. The authors did not observe or describe eosinophils. All biochemical values analyzed by the authors showed a wide variability throughout the three-year investigation. While many clinical findings and blood parameters showed significant differences between foraging and nesting turtles, as observed by Deem *et al.* (2009) in a comparative study over 83 loggerheads collected along the coast of Georgia (USA), parameters such as hemoglobin concentration, PCV, MCHC and plasma Fe^{2+} were stable during nesting season in 29 healthy loggerheads from Arabian Sea in the nesting season (Alkindi & Mahmoud, 2002).

Stamper *et al.* (2005) used body condition and hematological parameters to compare the health status of 42 migratory and 15 resident juvenile loggerhead sea turtles in North Carolina, and to also assess whether a relationship between the carapace barnacle load and the health status of the turtle could be established. A total of 18 migratory and 15 resident turtles were examined for barnacle epibiota load. Significant differences were found between hematological and biochemical values in migratory versus residential specimens, probably due to physiological activities correlatable to migration. An association between turtle weight and hematocrit was described and rationalized on the basis that longer dives were performed by larger turtles, thereby necessitating a higher hematocrit for a greater supply of oxygen. Unexpectedly, there was no correlation between carapace barnacle load and hematological values. The authors hypothesized that this result (i.e. the lack of an identifiable difference) may have been due to the limited number of specimens or to the limitation of the analysis of barnacle load.

The capture techniques may affect blood values as seen by Harms *et al.* (2003). They evaluated the effects of two capture techniques for 16 specimens of sea turtles, i.e., by trawl and by pound nets, in North Carolina, South Carolina and Georgia. In the first case, turtles were forcedly submerged for ≤ 30 minutes, while in the second one they were free to surface and to feed at will. Both capture systems caused alterations of many parameters, such as blood gas, acid-base and lactate status. However, more significant perturbations were induced in trawl-captured turtles.

In regard to blood withdrawal, in reptile plasma is preferred over serum because of the variability of the time for clot formation and, consequently, the significant changes in the chemical composition of the sample that may occur in the interval (Bolten *et al.*, 1992). In fact, the comparison between analysis of plasma and serum samples from 23 dually-analyzed specimens led to different results. Plasma samples anticoagulated with lithium heparin or sodium heparin showed no differences in concentration for each analyte. Furthermore, a remarkable source of variability is due to the different biochemical autoanalyzers, as the values obtained by using two different autoanalyzers varied more between themselves than among the results derived from samplings processed following three different post-phlebotomy methods of handling the aspirate (lithium or sodium heparin, or no anticoagulant). Sodium or lithium heparin are preferred as anticoagulants, as reptilian blood may be hemolyzed by EDTA (Jacobson, 1987; Grumbles *et al.*, 1990). A variability in the results for hematocrit and concentration or enzymatic activities of many biochemical analytes was similarly observed by WoIf *et al.* (2008) while testing five different analyzers with blood collected from 22 captive juvenile sea turtles, 18 of which were loggerheads. Moreover, for biochemical analysis, it is important to separate plasma from heparinized whole blood as soon as possible or at least within 24 hours post-collection, otherwise biochemical analyte values may be affected (Eisenhawer *et al.*, 2008).

Casal and Orós (2007) provided a morphological description of circulating blood cells of *Caretta caretta* (erythrocytes, heterophils, eosinophils, basophils, lymphocytes, monocytes and thrombocytes) and also studied their cytochemical characteristics in 35 juvenile loggerhead sea turtles. Some of these workers' cytochemical observations, with the exception of the basophil, differed from those reported for other sea turtle species. Moreover, Casal *et al.* (2007) evaluated the ultrastructural characteristics of the circulating blood cells (except basophils) in 15 juvenile specimens of *Caretta caretta.* A

peculiarity in the red blood cells was observed by Eiras *et al.* (2000), who found intracytoplasmic, mostly single inclusions in the mature erythrocytes of 20 juvenile loggerheads from Madeira, Portugal. The identity of the above inclusions was not definitively established, but the authors theorized they could be viral or rickettsial. More recently, Basile *et al.* (2011, a) identified these intraerythrocytic inclusions as Heinz bodies formed through the precipitation of loggerhead's unstable hemoglobin, which had been previously studied and sequenced by Petruzzelli *et al.* (1996).

In addition to the aforementioned investigations of the blood picture of the loggerhead turtle, many other studies have been conducted during the last decade to establish its hematological and biochemical parameters in specimens collected on a worldwide basis. In some instances however, the investigations embraced a small number of subjects. Plasma protein fractions were analyzed electrophoretically by Gicking *et al.* (2004), in a group of 41 healthy, wild loggerheads (juvenile and adult, male and female) from Florida Bay, USA. In 29 of the 41 turtles, four protein fractions were determined: albumin, alpha globulin, beta globulin and gamma globulin; 11 of the 41 specimens showed a beta-gamma bridging and just one turtle of 41 presented an additional prealbumin fraction Pires *et al.* (2006) determined hemogram and plasma protein values in eight captive loggerheads from Bahia, Brasil. Jacobson *et al.* (2007) collected data from specimens of *Caretta caretta* and *Chelonia mydas* (green turtle) entering the Port St Lucie Power Plant canal system in Florida, US, in order to monitor the wild populations of these species and to determine the normal hematocrit values, several plasma biochemical parameters and protein electrophoretogram fractions. Some hematological values of loggerheads from the Atlantic ocean were determined (Pires *et al.*, 2006; Jacobson *et al.*, 2007; Casal *et al.*, 2009; Casal & Orós, 2009; Deem *et al.*, 2009; Osborne *et al.*, 2010), some from the Pacific Ocean specimens (Flint *et al.*, 2010) and some from the Mediterranean sea population (Gelli *et al.*, 2009; Fazio *et al.*, 2010; Basile *et al.*, 2011, b). Hemocellular and plasma protein electrophoretogram fraction values collected by the aforementioned authors are reported in Table 1, biochemical values are summarized in Table 2. Values in tables 1 and 2 are mean values, medians or reference intervals, according to the original data presented by the authors. In case of studies including two or more groups of turtles or subsets, the lowest and highest values were reported.

Table 1. Hemogram values and electrophoretogram fractions in the loggerhead turtles (See References)

Hematological Parameters	Bolten et al 1992	Alkindi and Mahmoud 2002	Gicking et al 2004	Stamper et al 2005	Pires et al 2006	Jacobson et al 2007	Kakizoe et al 2007	Eisenhawer et al 2008
RBC (10^3/µL)				380-880	275		325-429	
Hb (g/dL)		8.87			8.65			
Hct (%)		31.78		28-32	33.12		16.0-26.2	
MCV (fL)					1214			
MCH (pg)					317			
MCHC (%)		27.94			26			
WBC (10^3/µL)				14.3-15.8	3.656		4.7-10.2	
Het (%)				27-60	59.4		35.8-73.3	
Eos(%)				2-6	10.4			
Bas (%)				0	0.13		0.00-0.17	
Lym (%)				36-65	29.3		24.6-58.6	
Mon (%)					0.9		0.0-2.2	
Azurophils (%)				3-4				
Het (10^3/µL)				3.48-6.33	2.16		2.508-5.120	
Eos (10^3/µL)				0.23-0.88	0.367			
Bas (10^3/µL)				0.00	0.003			
Lym (10^3/µL)				4.29-9.92	1.111		1.321-5.340	
Mon (10^3/µL)					0.019			
Azurophils (10^3/µL)				0.75-1.52				
TBC (10^3/µL)					10.968			
Total protein (g/L)	36-41		43	36-40	65	26.0-40.8	15.6-33.3	0.047-0.048
Pre-albumin (g/L)						0.0-0.4		
Albumin (g/L)	6-11		10	11-23		5,7-11.2	5.6-13.7	0.015
Globulins (g/L)				22-28				0.032-0.033
Alpha (g/L)			4.8			2.0-6.8		
Alpha-1 (g/L)								
Alpha-2 (g/L)								
Beta (g/L)			8.0			6.9-36.1		
Gamma (g/L)			19.4			9.6-17.6		
A:G ratio			0.33			0.24-0.51		

Table 1. (continued)

Hematological Parameters	Wolf et al 2008	Casal et al 2009	Casal and Orós 2009	Deem et al 2009	Gelli et al 2009	Fazio et al 2010	Flint et al 2010	Osborne et al 2010	Basile et al 2011
RBC (10^3/µL)		94-187	189	520					410-570
Hb (g/dL)									8.0-14.2
Hct (%)	24.5-28.0	28-40	29	32			13.9-47.3		23-34
MCV (fL)									487-723
MCH (pg)									170-261
MCHC (%)									34-42
WBC (10^3/µL)		1.6-5.9	5.8	9.005			2.63-50.91		17.5-23.8
Het (%)			78.9						41.0-62.4
Eos(%)			3.2						1.9-2.6
Bas (%)			0.0						0
Lym (%)			16.9						32.4-56.6
Mon (%)			1.3						0.2-2.6
Azurophils (%)									
Het (10^3/µL)		1.1-4-6		3.677			0.18-48.95		
Eos (10^3/µL)		0.2-0.3		1.152			0.09-3.45		
Bas (10^3/µL)		0.000001		0					
Lym (10^3/µL)		0.3-1.0		2.725			0.55-33.94		
Mon (10^3/µL)		0.01-0.07		0.960			0.05-3.22		
Azurophils (10^3/µL)				0					
TBC (10^3/µL)		42.6-44.3	43.8				0.07-17.49		2.0-4.9
Total protein (g/L)	31-35	24-41	28	37	42.8	41.9	29.0-72.2	33	
Pre-albumin (g/L)				0.0					
Albumin (g/L)	5-15	11-17	11	7.9	10.7	7.5	7.5-15.9	9.9	
Globulins (g/L)		13-24	17	29			21.2-59.0		
Alpha (g/L)								4.6	
Alpha-1 (g/L)				1.4					
Alpha-2 (g/L)				1.2					
Beta (g/L)				9.9				0.84	
Gamma (g/L)				15.7				0.97	
A:G ratio								0.43	

Table 2. Biochemical values in the loggerhead turtle (See References)

Hematochemical Parameters	Bolten et al 1992	Alkindi and Mahmoud 2002	Stamper et al 2005	Jacobson et al 2007	Kakizoe et al 2007	Eisenhawer et al 2008	Wolf et al 2008
Glucose (mmol/L)	4.42-4.45		4.39 -5.53	4.06-6.17	6.77-9.16	8.72-9.33	5.89-6.86
Cholesterol (mmol/L)	2.50-2.74			1.37-5.90	2.68-5.00	3.62-3.75	
Triglycerides (mmol/L)	0.91-0.98				0.27-1.68		
Urea (mmol/L)							
BUN (mmol/L)	17.89-18.73		22.5-28.57	10.22-34.32	0.015-0.051	52.94-54.77	37.97-43.99
Uric Acid (mmol/L)	0.041-0.065		0.02-0.04	0.030-0.083	0.008-0.057	0.048-0.059	0.024-0.036
Creatinine (µmol/L)	17.68-26.52				38.90-100.19		
Total Bilirubin (µmol/L)	0.34-5.13				2.05-8.21		
AST (U/L)	170-187		129-206	149-345	75.0-150.6	342-352	229-264
ALT (U/L)	2.0-12.2				1.2-6.6		
GGT (U/L)				8-31	1.2-6.0	16-24	
Amylase (U/L)					226.2-1036.2		
Lipase (U/L)							
CPK (U/L)				319-1742	590-2,927	1,022-1,419	299.5-972.0
LDH (U/L)	90-128		120-227		58.2-176.4		65-206
ALP (U/L)	10.5-14.0		9.0-23.0	7-22	833-5,041	36-38	20-45
Sodium (mmol/L)	155.9-162.0		151.0-158.0	148-168	143.20-154.40	155-161	148-156
Potassium (mmol/L)	4.0-4.2		3.30-4.50	3.5-7.9	3.20-4.48	4.0-4.3	3.8-4.4
Chloride (mmol/L)	116.5-119.3		112-118	103-128	103.00-120.60	119-126	118-123
Calcium (mmol/L)	1.57-1.72		1.55-2.20	1.07-1.82	1.49-2.12	1.72-1.85	1,35-1,77
Phosphorus (mmol/L)	2.56-2.62		2.03-2.26	1.92-3.04	1.98-5.42	2.85-2.94	2.18-2.59
Magnesium (mmol/L)			2.17-2.26	1.28-2.55		1.8-2.2	
Iron (µmol/L)	5.73	13,620					
Carbon dioxide (mmol/L)	21.0-23.7						
Anion gap			10.8-17.5				

Table 2. (continued)

Hematochemical Parameters	Casal et al 2009	Casal and Orós 2009	Deem et al 2009	Gelli et al 2009	Fazio et al 2010	Flint et al 2010	Basile et al 2011
Glucose (mmol/L)	3.3-7.2	7.10	5.88	6.06		4.3-8.9	5.38-9.10
Cholesterol (mmol/L)	3.6-8.7	3.69	1.94	1.99			1.92-3.74
Triglycerides (mmol/L)	1.3-7.4	0.85	0.62	0.60			
Urea (mmol/L)	7.2-36.3			6,87	10.34	21.7-48.4	
BUN (mmol/L)		36.1	29.63				12.78-73.02
Uric Acid (mmol/L)	0.06 - 0.10	0.06	0.041	346,000		0.017-0.157	0.059-0.159
Creatinine (µmol/L)	31.8-39.7	26.5	26.52	3,540,000	44.20	14.5-41.2	
Total Bilirubin (µmol/L)	3.4-17.2	3.42		850,000		0.9-2.9	3.42-6.84
AST (U/L)	123-194	202.2	165	468	37.15	78.7-274.6	44-184
ALT (U/L)	11-24	14.1	16	13.32	98.75		6
GGT (U/L)			9.0	1.11			
Amylase (U/L)			263				
Lipase (U/L)			1.0				
CPK (U/L)			534	3,704		157.4-2,211.1	
LDH (U/L)	<100-310	101.2	572	461.32		29.4-391.8	
ALP (U/L)	67-103	64.7		59.53		10.5-132.9	
Sodium (mmol/L)			156			141-158	
Potassium (mmol/L)			5.1			3.2-5.7	
Chloride (mmol/L)			130			107-125	
Calcium (mmol/L)	2.0-3.1	2.43	1.85	1.58		1.2-2.0	1.67-2.17
Phosphorus (mmol/L)			2.07	2.59		1.6-3.2	
Magnesium (mmol/L)						1.7-4.5	1.48-2.22
Iron (µmol/L)							
Carbon dioxide (mmol/L)			15				
Anion gap							

MORPHOLOGY AND CYTOCHEMICAL CHARACTERISTICS OF BLOOD CELLS

The identification of loggerhead's leukocytes is still a matter of debate due to the occurrence of rather similar eosinophilic cytoplasmic granulation in both heterophils and eosinophils, which can lead to misinterpretation of the differential count**.** Controversial results have been reported by various authors depending on the morphological or cytochemical methods employed to classify white blood cells in the differential count, so they often cannot be

compared. Unlike the granulocytes, lymphocytes and monocytes are easily distinguishable, as they are similar to their mammalian counterparts. Casal & Orós (2007) have provided morphological and cytochemical assessment of the loggerhead's white blood cells. The predominant lymphocytes are small and round with a similarly shaped nucleus that occupies the majority of the cytoplasm (nucleus: cytoplasm ratio 0.6) and which is also characterized by its dense, solid aggregation of chromatin (Fig 1b). The cytoplasm is basophilic, moderately granular and yields a positive NB-esterase reaction (in the absence of sodium fluoride). The monocytes are round cells, 33% larger than the lymphocytes, and maintain an eccentrically located, kidney-shaped nucleus whose chromatin pattern is less dense than that of the lymphocytes (Fig 1c). The cytoplasm is slightly basophilic, sometimes vacuolated, and it is weakly stainable with NBE (without fluoride). The nucleus:cytoplasm ratio is smaller than that of lymphocytes (0.5). Casal and Orós (2007) found rare basophils displaying the typical feature of mammalian counterparts such as intensely basophilic granules overlying the nucleus; they were stainable only with Toluidine Blue. The loggerhead's heterophils are large, round cells that present a round and central or oval and eccentric nucleus, containing clumped chromatin and a prominent amount of slightly eosinophilic cytoplasm full of weakly eosinophilic granulation (Fig 1b,c). They are stainable by acid phospatase (ACP) with and without tartrate, by peroxidase (PER), by chloroacetate esterase (CAE), Sudan Black B (SBB) and slightly stained by the periodic acid Schiff reaction (PAS). Eosinophils (Fig 1b) display a round configuration with a generally eccentrically placed round or oval nucleus containing clumped chromatin. The abundant cytoplasm is weakly basophilic (a characteristic feature of the eosinophils of at least some other species) and contains a few to a modest number of round eosinophilic granules stainable by ACP (with and without tartrate), NBE (with sodium fluoride), CAE and PAS and moderately by SBB.

The thrombocytes (Fig 1b), which take part in the hemostatic process, are complete, intact cells, morphologically different but functionally analogous to the mammalian platelets. This is revealed by their aggregation into small clumps observed in smears made from fresh, unanticoagulated blood. They were described as round or oval-shaped cells (Casal & Orós, 2007). In the oval cells the nucleus was ovoid and the transparent cytoplasm was accumulated at the two poles of the cell. In the case of the round forms, a round nucleus was surrounded by a thin rim of cytoplasm.

The erythrocyte of loggerhead turtles has the same fundamental structure that is universally maintained by the other poikilothermic vertebrates. It is a flattened, biconvex, permanently nucleated ovoid cell (ranging from 17-19 μm x 9-10 μm in size), whose configuration is recognized to facilitate passive transport in flowing plasma and offers a large surface area that enhances oxygen exchange (Fig 1a). This turtle's red cell, however, does present some species-related attributes comparable to those observed in the red cells of constituents of other *taxa*. These include an overall shape, dimensions, numerical quantity per volume unit of blood and amount of hemoglobin in a given cell which in sum characterize the erythrocytes of this marine species and other members of this general group. Thus marine turtles have comparatively large erythrocytes, approximately 400-700 fL in volume with a wide oval shape as opposed to terrestrial reptiles such as *Iguana iguana*, the common green iguana, which maintains smaller (~300 fL) narrower red cells which, in addition, have a greater cellular length to width ratio. This latter aspect confirms that the loggerhead's erythrocytes are not only larger but also proportionally wider than those of the iguana. lt is considered that the voluminous red cell of the turtle coupled with its large amount of hemoglobin is less efficient in rapid exchange of oxygen than a smaller erythrocyte with a proportionately equivalent of hemoglobin because of the (mathematically documentable) relatively greater cell surface area per hemoglobin content of the lesser-sized cell. The paradigm presented by the turtle's erythrocyte yields a slower, prolonged release of oxygen that would be of benefit for an air-breathing species that engages in long dives.

The ultrastructural characteristics of the circulating blood cells of loggerhead turtles (n=15) were investigated and detailed by Casal *et al.* (2007). Heterophils, eosinophils, lymphocytes, monocytes and thrombocytes were clearly identified in this study, while the basophils were so rarely observed that their number was insufficient for analysis. These workers reported that all the leukocytes are generally equivalent to their counterparts from other species of sea turtles. An exception was observed in the eosinophils which tended to be uniform in size, unlike the eosinophils of the green turtle, *Chelonia mydas* (Work *et al*., 1998), and also do not present any crystalline structure in their granules. The crystalloid center is a hallmark of the eosinophil granules in other species. The ultrastructure of eosinophil reveals that this cell is spherical and maintains a round or oval-shaped with variable amounts of heterochromatim. Some eosinophils have multiple small sized or one large electron-lucent cytoplasmic vacuole; as would be anticipated

mitochondria, endoplasmic reticulum and Golgi complexes are routinely identified.

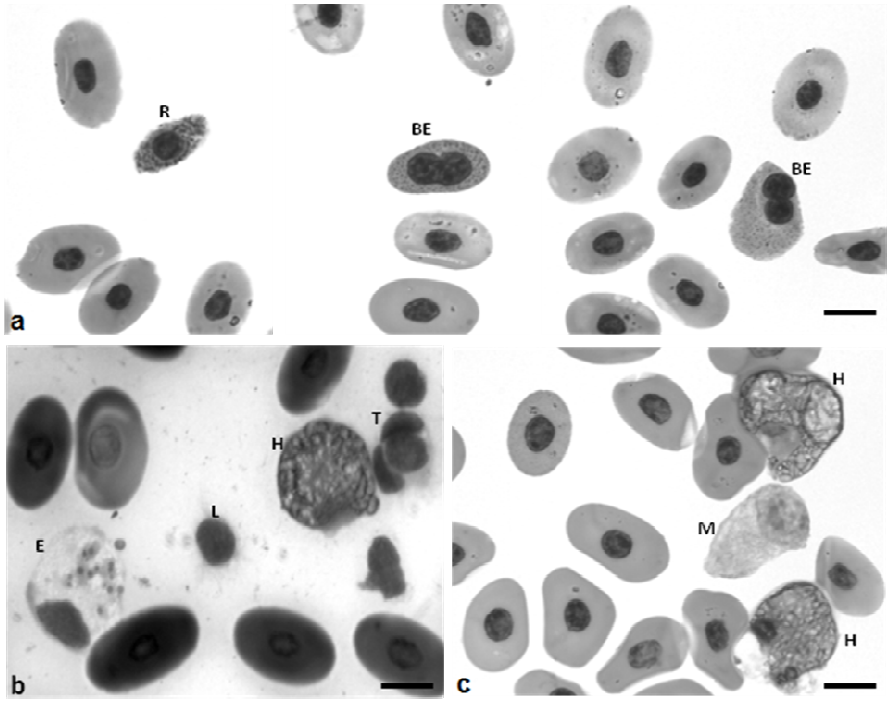

Figure 1. a. Circulating erythropoiesis in the loggerhead's blood: a reticulocyte (R) and two basophilic erythroblasts (BE) among mature erythrocytes (scale bar = 8 µm). b. Eosinophilic granulocyte (E), lymphocyte (L), heterophilic granulocyte (H), thrombocytes (T) (scale bar = 8 µm). c. Monocyte (M) and two heterophils (H) (scale bar = 7 µm).

Heterophils are round in shape and have an eccentrically placed spherical, heterochromatin-rich nucleus. Numerous electron-dense, round or elongated granules are dispersed throughout its abundant cytoplasm. A limited population of variably electron-dense, pleomorphic granules is also present. Organelles typically presented in the cytoplasm of an animal's cell (e.g., mitochondria) are identifiable. On the basis of the foregoing characterization the heterophils of the loggerhead resemble those of the fellow marine turtle *Chelonia mydas*, the green turtle. They are, however, unlike the heterophils of some other reptiles which concurrently present two apparently distinct

populations of heterophils (different levels of maturity?) in their circulation. One version of this granulocyte has homogenous electron-dense granules in its cytoplasm, while the other has granules some of which are electron dense while the others do not display this morphologic feature. The lymphocytes morphologically correspond to those of the green turtle and other reptiles with their irregularly round shape and spherical, often-indented nucleus containing a prominent mass of dense heterochromatin that fills most of the nucleoplasmic space. The nucleus is encircled by a halo of cytoplasm containing some small electron-dense granules accompanied by a few polyribosomes, mitochondria and sparse endoplasmic reticulum. The monocytes of the loggerhead turtle and the rarely described monocytes of the green turtle have similar ultrastructure. Loggerhead's monocytes are round or fusiform and their spherical nucleus contains a modest amount of heterochromatin. Some small electron-dense granules, a prominent Golgi complex and representatives of typical organelles such as mitochondria are present in the cytoplasm. Thrombocytes are usually oval shaped and sometimes round. The nuclei of oval shaped thrombocytes are correspondingly ovoid but the heterochromatin of these cells is not centrally located in the nucleus. Small granules of variable electron-density are observable in the cytoplasm together with canalicular structures (perhaps plasmalemmal infoldings as obtained in mammalian platelets) which are a common feature of reptilian thrombocytes.

INCLUSION BODIES OF ERYTHROCYTES

The erythrocytes, which are permanently nucleated cells, have an oval nucleus with dense heterochromatin. A certain electron-dense cytoplasmic inclusion is identifiable in some erythrocytes. Its origin has been a matter of debate and hypotheses have proposed that it could be viral or rickettsial in nature or a degenerating organelle. It has finally been concluded that it is a Heinz body derived from the precipitation of the loggerhead's unstable hemoglobin. In a series of studies of healthy and unhealthy, captive and wild free-living, juvenile and mature *Caretta caretta* conducted on circulating blood of specimens housed at the Sea Turtle Rescue and Rehabilitation Center of the Zoological Station Anton Dohrn (Naples, Italy) this specific inclusion was identified in a variable number of circulating erythrocytes in each of the assessed turtles (Basile *et al.*, 2011). It was characterized in MGG-stained dry

film blood smears as spherical in configuration, approximately 1-3 µm in diameter and somewhat dark blue in color. The inclusions were only seen in mature erythrocytes and were generally located along the major axis of the cell between the nucleus and plasmalemma. One inclusion body was identified in a given cell. In one evaluation of six juvenile and nine adult subjects, 3-82% of the erythrocytes presented this cytoplasmic incorporation. Heinz bodies are well documented in inframammalian and mammalian vertebrates. Tinctorial attributes of the Heinz bodies in the loggerhead include positive staining by brilliant cresyl blue, toluidine blue and the periodic acid-Schiff reaction. These loggerhead's Heinz bodies are Feulgen reaction-negative thereby indicating that they are not DNA. The etiology of the development of Heinz bodies in the loggerhead remains undetermined. Since they have been seen in healthy turtles, including a few captive healthy representatives that have been maintained in ideal normal environments for 2-4 years and also in sick turtles, it is apparent that they are not life threatening or disabling. The hemoglobin of the loggerhead has been theorized to be less stable than that of other species' hemoglobin. Its degradation under normal circumstances in a presumed long-lived cell or after possible exposure to pollution may consequently lead to the development of Heinz bodies. The removal of the Heinz body from the turtle's erythrocyte without destroying the latter has been thought to occur through a process known as "pitting", that is the elimination of only the inclusion body from the cytoplasm of the erythrocyte, so that the cell temporarily takes the shape of a teardrop (dacryocyte) and then reverts back to its original shape (Basile *et al.*, 2011, a).

ERYTHROPOIESIS

Circulating erythropoiesis or at least maturation of very immature erythroblasts is identifiable in the blood of the loggerhead. The youngest recognizable immature precursor of the erythrocyte in this *locus* is the basophilic erythroblast. It is a spherical cell with a centrally located round nucleus surrounded by a relatively narrow band of very basophilic cytoplasm.

With continued maturation the cell evolves into an intermediate form termed the acidophilic erythroblast, which presents a flattened ellipsoidal configuration. The cytoplasm is acidophilic due to the accumulation of hemoglobin, while the nucleus is oval and the chromatin is not as compact as that of a mature red cell. The reticulocyte is a further developed cell and is the

immediate antecedent of the mature erythrocyte. It is identified by the detection of a small amount of residual cytoplasmic RNA by supravital staining with brilliant cresyl blue.

HEMOPARASITIZATION IN LOGGERHEAD TURTLES

Reptilian erythrocytes, like those of the pisces and amphibians, are susceptible to intracellular parasitization. The inhabitance of a parasite in a red cell is frequently an unexpected or incidental finding during the examination of a blood film stained with MGG or comparable Romanowsky type dye. This apparently holds true for the erythrocytes of *Caretta caretta*. A benign infestation by the intraerythrocytic protozoan *Theileria sp*. has been recently detected for the first time in two specimens of Mediterranean loggerhead turtles. The vector of the parasite was thought to be the leech *Ozobranchus margoi*. The hemogramic data of these two turtles suggested that the infestation had an undetectable or minimal impact on the erythrocytic and leukocytic parameters or apparent health of the hosts (Di Santi *et al.*, 2010). Various other members of the Phylum Apicomplexa have been detected in loggerhead blood and are capable of invading leukocytes and erythrocytes. The previously noted *Theileria* is a protozoan that invades both types of cells. When erythrocytes are hosts for parasites, the affected red cells along with their inhabitants may be subjected to erythrophagocytosis (Jaensh & Raidal, 2006). In most infestations by apicomplexans, the parasitizations are well tolerated and the host vertebrates do not present clinical signs of disease. Diagnosis of infection is based on the recognition of the trophozoite, multinucleate schizont, or gametocyte inhabiting red cells (Telford, 1984; Barnard & Upton, 1994).

Since loggerheads are ectotherms their body temperature is more subjected to fluctuations than that of homeotherms, such as mammals and birds, thus making it easier for pathogens to grow and proliferate (Alfaro *et al.*, 2006). In sea turtle diseases, bacteria act both as primary pathogens and as secondary invaders, when the host's immune system is weak. The bacteria found in turtles are commonly isolated in localized infections but also play a key role in epizootics which lead to the spreading of bacteria in the host's blood (bacteraemia) and consequent septicaemia (Cooper, 1983). Infestation by three species of spirorchids (*Carettacola*, *Hapalotrema*, and *Neospirorchis*) was detected in the heart and blood vessels of mainly sub-adult loggerhead sea

turtles. This infestation causes severe debilitation and mortality (Wolke *et al.*, 1982). As the current information about infestations and infections in sea turtles is incomplete, more research about turtle parasites and hemoparasites are still needed to fully understand the interactions between turtles and pathogens.

Although many studies have been carried out to date and others are in progress, the hematology of this sea turtle still remains a fascinating subject and one of which only a few aspects are definitively comprehended and clarified. At least four major areas require further investigation.

Baseline and complete values of the hematologic parameters have to be established for the world wide, diversely distributed populations of the loggerhead turtle. This would be very helpful in sea turtle rescue and conduction of rehabilitation programs.

In regard to leukocytes, no data are available about the physiological roles of lymphocytes in the loggerhead immune system, so it would be very interesting to know whether B and T-lymphocytes are detectable in sea turtle blood and which duties they carry out.

A precise, accurate technique to routinely morphologically distinguish between the eosinophils and heterophils is needed for the performance of leukocyte differential counts.

Finally, data regarding loggerhead hemopoiesis are unavailable. This species is classified as endangered and thereby needs intensive evaluation. Any investigation of its hemopoiesis requires studies of the tissues in which this occurs. The maturational process and kinetics of the leukocytes and the thrombocytes cannot be studied in the peripheral blood since the progenitors of these cells are not normally present in circulation. In the case of the erythrocyte, although reticulocytes and some erythroblasts are identifiable in the circulation, a complete understanding of erythropoiesis requires examination of erythroid proliferation in the hemopoietic tissues.

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In: Turtles: Anatomy, Ecology and Conservation ISBN: 978-1-61470-554-3
Eds: M. J. Cosgrove and S. A. Roe

Chapter 6

RESPONSES OF FRESHWATER TURTLES TO DROUGHT: THE PAST, PRESENT AND IMPLICATIONS FOR FUTURE CLIMATE CHANGE IN AUSTRALIA*

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ABSTRACT

Australia was not always arid, and the freshwater turtles that have survived to this day are either relicts residing in river systems that have themselves had a history or continuity through the drying of the continent, or they are species that have adapted in some way to meet the challenges of drought. In this chapter, we report on how Australian freshwater turtles cope with periodic loss of habitat through drought at a range of spatial and temporal scales, and consider how they might fare under changing climates predicted to occur through global warming. Global warming is not occurring in isolation of other environmental changes at a landscape scale, and we look at what interactions there might

* A version of this chapter was also published in Meltdown: Climate Change, Natural Disasters and other Catastrophes - Fears and Concerns for the Future, edited by Kathryn Gow, published by Nova Science Publishers, Inc. It was submitted for appropriate modifications in an effort to encourage wider dissemination of research.

be between climate change and the increasing demands of agriculture, industry and our cities for water, in presenting challenges for our unique freshwater turtle fauna.

Keywords: turtles, drought, climate change, habitat loss, freshwater fauna

INTRODUCTION

Australia has the lowest precipitation of any vegetated continent – only Greenland and Antarctica have less – but few people realize how dry Australia actually is. To put this in perspective, the average annual discharge from all of Australia's rivers is 237,582 gigaliters (Australian Bureau of Rural Sciences)[1] which is less than half that of the Mississippi River in North America. Consequently, much of the Australian continent is arid or semi-arid, and wetland habitats are small and poorly developed compared to other continents. As air and sea current patterns changed when Australia broke off from Antarctica, major episodes of progressive aridification occurred 30-35 Mya[2], 15 Mya and 2 Mya. The climate became not only drier, but also less predictable, and the biota that persisted had to contend with drought or periodic loss of free-standing water across much of the continent.

Drought is most simply defined as a departure below 'normal' precipitation (Dracup, Lee and Paulson, 1980; Wilhite and Glantz, 1985), although droughts can fall anywhere along a spectrum of climactic variability from long-term aridity at one extreme to annual cycles of wet and dry at the other. Aridity occurs where environmental water inputs are persistently insufficient to balance loss, whereas seasonally variable regions accrue a relatively temporary water deficit. In a sense, aridity can be considered as a long-term drought spanning millennia, whereas seasonally wet-dry climates cycle in and out of drought on a shorter term basis. The distinction between these scenarios is only a temporal one. Drought in the conventional sense falls somewhere in between, defined by water deficits spanning several years or decades compared to expectations derived over a period of historical record keeping, typically spanning decades or centuries.

[1] http://www.daff.gov.au/brs/social-sciences.

[2] Million years ago.

From the perspective of wildlife responses to drought, this temporal distinction is important to grasp. At either end of the drought continuum, water deficits (whether short or long term) can be a relatively predictable feature of the regional climate. That is not to say that annual fluctuations in the occurrence, amount, timing, and duration of wet and dry periods do not occur – but over the balance of several years the timing and duration of water shortage occurs in a predictable sequence that has persisted for several thousands or millions of years. Such protracted periods of relatively stable climactic patterns can shape biological communities through evolutionary responses so that morphology, physiology and behaviour are suitably matched to the conditions (Ligon and Peterson, 2002; Withers, 1993). However, an abrupt departure from the normal precipitation pattern that spans several years or decades (i.e., drought in the conventional sense) may cause the existing biological communities to struggle to keep pace with the changing climate, even if environmental changes are brought about by natural climactic cycles.

The impact of drought on wildlife should not be discussed without also considering the human dimensions. Human activities may result in the imposition or exacerbation of drought conditions, and our infrastructure in developed landscapes can alter the natural responses of wildlife to drought. For instance, anthropogenic global climate change threatens to increase aridity in several regions of Australia, as well as to increase stochasticity[3] in climate, which can influence the frequency, severity, and duration of extreme weather events such as droughts (Bates et al., 2008; IPCC, 2007). Human water resource development alters water flow and hydrological regimes, in a sense simulating and hastening the onset of drought conditions and attenuating seasonal cycles of wet and dry. Roads, feral predators, and other dimensions of human society may further hamper the ability of wildlife to respond to drought.

In this chapter, we introduce the Australian freshwater turtle fauna and explore how they cope (or in some cases, do not cope) with periodic loss of aquatic habitat through drought at a range of spatial and temporal scales, and consider how they might fare under changing climates predicted to occur through global warming. Global climate change is not occurring in isolation to other environmental alterations at a landscape level, and thus in assessing some of the challenges for Australia's unique freshwater turtle fauna, we examine what interactions there might be between climate change and the

[3] Stochasticity implies irregular and unpredictable variability.

increasing demands of agriculture, industry and cities for water and other natural resources.

THE FRESHWATER TURTLES OF CONTEMPORARY AUSTRALIA

There are no strictly terrestrial turtles or tortoises that inhabit Australia. Instead, the Australian turtle fauna is restricted to freshwaters and the surrounding ocean. In this chapter, we focus only on those that occupy the inland freshwater systems, which are dominated by species belonging to the family Chelidae. This family of side-necked turtles is restricted to the Australasian region and South America - not even fossil forms are found outside this range (Gaffney, 1991; Williams, 1953a, b) - so it is a group with clear Gondwanan origins. Their fossil record extends back to the late Cretaceous (de Broin, 1987) in South America and the Miocene in Australia (Gaffney, Archer and White, 1989). Among extant forms in Australia and New Guinea are the river turtles in the genera *Emydura* (5 species) and *Elseya* (7 species) of northern and eastern Australia and New Guinea, and *Rheodytes* (1 species) and *Elusor* (1 species) from east coastal rivers of Queensland. The critically endangered *Psuedemydura umbrina* resides in seasonally dry wetlands of south Western Australia. Australia is also renowned for its spectacular snake-necked turtles (12 species) that range in habitat from permanent waters (*Chelodina expansa*) to highly ephemeral[4] streams and ponds (*Chelodina steindachneri*). The pig-nosed turtle (*Carettochelys insculpta)* is the only species of Australian freshwater turtle outside of the family Chelidae, belonging to the family Carettochelydidae (the only extant species in this family) that lives in the major rivers and associated wetlands of northern Australia and southern New Guinea (Georges and Rose, 1993).

Not surprisingly, given this diversity, Australia's freshwater turtles occupy a wide range of habitats - from the flooded forests and extensive swamps of the Fly River and Kikori Delta in Papua New Guinea (Georges et al., 2006a, 2008) to the dryland rivers of Australia's arid interior (Georges et al., 2006b); from the tropical north to the snow country around Cooma; from dilute waters of the sandstone plateaus of Arnhem Land and the Kimberleys (Thomson et al., 2000), to the coastal floodplains of northern Australia and the saline or

[4] Ephemeral bodies of water tend to dry during drought and flood again following precipitation.

brackish coastal wetlands of the Styx River in Queensland and Lake Alexandria at the mouth of the Murray River in South Australia. However, Australia was not always arid (Quilty, 1994), and the freshwater turtles that have survived to this day are either relicts residing in watersheds that have themselves had a history of continuity through the drying of the continent, or they are species that have adapted in some way to meet the challenges of drought (Georges and Thomson, 2006). Below, we explore several ways that Australian turtles respond naturally to the drying of their aquatic habitat.

NATURAL RESPONSES TO HABITAT DRYING

Along with temperature, precipitation patterns that determine flooding and drying of surface waters are likely to be the most powerful drivers of freshwater turtle distributions and community structure[5] in Australia. Several of Australia's wetlands are under the influence of natural flood-drought cycles, and in these contexts, many turtles have adaptations to drought that allow them to successfully cope with habitat drying by moving to more permanent waters (escape in space) or by remaining in or near the dry waterbody in an inactive state to await the return of flooding (escape in time). However, several species do not regularly contend with the drying of their waterholes and may lack any evolved responses to cope successfully with drying.

Aestivation

In regions where water bodies seasonally or periodically dry, many Australian turtles have evolved specializations to survive extended periods of drought by entering a state of inactivity called aestivation (Burbidge 1981, Kennett and Christian 1994, Roe and Georges, 2007, 2008, Roe et al., 2008). The Western Swamp Turtle, *P. umbrina*, inhabits temporary swamps that flood during winter rains, but by late spring or early summer the swamps have dried and may remain so for 6-9 months (Burbidge, 1981). Several species of turtles in the genus *Chelodina* (*C. canni, C. longicollis, C. rugosa, C. steindachneri*) also inhabit wetlands that dry for several months. In some

[5] Community structure reflects the number and types of species in an area and their relative abundances.

extreme circumstances, *C. longicollis* can survive aestivation for 13–16 months (Stott 1987; Roe and Georges, 2007), while others (*C. steindachneri*) may occasionally need to survive for longer time periods out of water (Cann, 1998), although in this latter case no empirical work has yet confirmed this.

Aestivation is a strategy to survive the challenges of drought by reducing the body's energy and water demands. This may involve a suite of morphological, physiological, and behavioural traits. When drought occurs, turtles may respond by leaving the wetland and seeking refuge in naturally occurring holes in the ground, or by burrowing several centimeters under soil, leaf litter, woody debris, or other structures (Burbidge 1981; Chessman, 1983; Rees 2008; Roe and Georges, 2007, 2008). Alternatively, some burrow in wetland sediment where they remain entombed until rains return (Cann, 1998; Kennett and Christian 1994). Morphological adaptations for terrestrial aestivation are perhaps best demonstrated in *P. umbrina*, which has an extensively roofed skull and an expanded shell that allows for the complete withdrawal of their head and limbs to reduce water loss. Other turtles employ physiological specializations to further conserve energy and water. For instance, *C. rugosa* reduces metabolism by 72% during dry season aestivation (Kennett and Christian, 1994), while *C. longicollis* is slow to lose water to evaporation and can further conserve water by altering the composition of excreta (Chessman, 1984; Rogers, 1966; Roe et al., 2008). While these behavioral, morphological and physiological specializations highlight adaptations that can greatly extend survival during drought, turtles are nevertheless vulnerable at this time. Predation rates can be high (Fordham et al., 2006b), and if waters do not re-flood before energy stores are depleted, turtles will eventually die of starvation or dehydration (Roe and Georges, 2008, Roe et al., 2008). Thus aestivation is only a temporary means of increasing the probability of surviving drought, and the duration that a waterhole remains dry is a critical factor in whether turtles can persist in the area.

Movement to other Waterholes

As an alternative to aestivation, turtles may move to more permanent waterholes when drought strikes. While it is not uncommon to see turtles of several species emigrating from wetlands in Australia (i.e., when they cross roads, golf courses, lawns, or paddocks), this is one of the least well

understood aspects of turtle behaviour (Roe and Georges, 2007). We have studied overland movements in *C. longicollis* at Booderee National Park and in the suburbs of Canberra (Graham et al., 1996; Kennett et al. 1990; Rees, 2008; Roe and Georges, 2007). At both sites, drought cues mass emigrations from drying wetlands into nearby permanent waterbodies. These permanent waterbodies offer temporary refuge during drought, where turtles remain (sometimes for years) before staging a return to temporary wetlands following floods. Turtles regularly travel up to three kilometers overland to reach permanent water, but occasional movements of more than five kilometers overland have also been reported in *C. longicollis* (Parmenter, 1976; Roe, 2007). However, just as the temporal duration of dry conditions is a limiting factor for survival through aestivation, an ability to escape to freshwater drought refuges depends upon the availability of waterbodies in the immediate vicinity that are somehow more resilient to drought. Most freshwater turtles could presumably traverse short distances (i.e., a few hundred meters) overland, but when farther distances must be traversed or when the intervening terrain is especially harsh, few would be capable of making such travels. Even the most basic capacities for (and limitations to) overland travel remain largely unknown for the majority of Australia's freshwater turtles.

Life History Specializations to Drying

The onset and duration of habitat drying and the return of floods can vary predictably (i.e., seasonally) or unpredictably (i.e., stochastically). It is in those species, that have to contend with unpredictability in flood-drought cycles, that we see some of the most interesting life history and behavioural "solutions" for survival. *Chelodina rugosa* deals with unpredictability in the recession of wet season flooding by laying its eggs underwater where development is suspended until the ground dries (Kennett et al., 1993a, b). Development is then adjusted so that embryos can compensate for differing durations of inundation to hatch at the optimal time prior to the onset of the ensuing rainy season (Fordham et al., 2006a). *Carettochelys insculpta* overcomes unpredictability in the onset of the wet season for its hatchlings by late-term embryonic aestivation. Eggs develop rapidly in anticipation of the wet-season floods then enter a state of torpor within the eggshell (Webb et al., 1986). Oxygen deprivation brought about by torrential rain that floods the nest cavity brings the hatchlings out of their slumber and they hatch and make their

frenzied way into the rising floodwaters (Doody et al., 2001; Georges, 1992). Hatchlings of other species, like *C. longicollis*, may emerge from eggs but remain within the nest chamber until heavy rain softens the soil and allows their escape. *Chelodina longicollis* adults have a range of adaptations that make them well-suited for both overland travel to refugial waterbodies and for extended bouts of terrestrial aestivation. This flexibility allows them to cope with drought and other unpredictable occurrences of aquatic habitat loss in a variety of ways depending on the context (Roe and Georges, 2008).

Permanent Water Specialists

Permanent water specialists[6] represent the vast majority of turtle genera in Australia, and it is these that are least likely to tolerate the drying of their aquatic habitats for any length of time. Permanent waters include lakes, ponds, rivers, and streams that typically remain inundated or maintain water flow without drying. Permanent water specialists include *Emydura*, *Elseya*, *Rheodytes*, *Elusor*, and *Carettochelys*, but *C. expansa* and *C. oblonga* also rely on habitats that do not typically dry out.

Turtles that are rarely exposed to drying conditions typically lack adaptations that would allow them to successfully contend with habitat drying by evacuating or aestivating (Chessman, 1984; Christiansen and Bickham, 1989; Gibbons et al., 1983). However, permanent water specialists may occasionally enter areas that periodically dry, such as when river flows force them out of channels and into shallow backwaters or downstream waterholes that can quickly become isolated when flows subside. In these scenarios, turtles can become unexpectedly stranded by drying floodwaters and many perish as a result (see Figure 1).

Nevertheless, some of Australia's permanent water specialists successfully inhabit arid regions, with some of the most widely fluctuating water flows on earth, and where most water can vanish for long periods. The Cooper Creek turtle, *Emydura macquarii emmottii*, has a most interesting life history, as it manages to persist in areas where other turtles cannot. This species occupies the permanent waterholes of the Cooper Creek floodplain in central Australia. Precipitation over much of the Cooper catchment is exceptionally low

[6] Habitat specialists tend to occupy a narrow range of environmental conditions, whereas habitat generalists can occupy a wide range of conditions.

(approximately 330 mm per year, Australian Bureau of Meteorology,[7] but monsoonal rainfall in its headwaters results in intermittent and widespread flooding of this dryland river.

Photo: Queensland EPA Roma. Photo reproduced with permission.

Figure 1. When freshwater refuges with a long history of water permanence dry, permanent water specialists typically perish, such as in Lake Numalla, Queensland, where some 10,000 Murray turtles were estimated to have died.

An exceptionally flat landscape results in an extensive network of distributary channels, peppered with isolated and deeper waterholes. It is in these waterholes that this species resides between episodes of floods, often in exceptionally high population densities (Georges et al., 2006b). Should waterholes dry (which happens during the worst of our droughts) the turtles perish, but when the system floods once again others are able to reinvade from refugial waterholes that did not dry. This dynamic allows this river turtle to persist in Australia's arid centre. The scale of this landscape is immense – waterholes are separated by hundreds of kilometers of arid and semi-arid land, and the temporal scale of flooding is measured in years instead of months, attributes presumably unsuited to the drought survival strategies of any other Australian turtle.

[7] http://www.adl.brs.gov.au/water2010/pdf/monthly_reports/awap_3_report.pdf.

DROUGHT IMPACTS AND THE HUMAN DIMENSION

The human dimensions of drought relevant to freshwater turtles fall into two broad categories. The first is creation of conditions that either cause drought or produce drought-like conditions, and the second includes activities that limit the natural ability of turtles to respond to drought. To assess the impact of drought on turtles, we must understand how these anthropogenic dimensions interact to directly and indirectly influence their populations.

The most visible consequence of drought in freshwater systems occurs when a water body dries completely, and consequences are most severe when water bodies that typically serve as aquatic refugia also dry out. Surface waters can recede and eventually dry from decreased runoff from rainfall, inflow from rivers, or supply by groundwater. Though rainfall ultimately drives all three processes, our demands on water for irrigation, industry and municipal activities hasten the pace of drying and extends the temporal and spatial impact of drought into areas that were once more extensively flooded or served as permanent water refuges (Kingsford and Thomas, 1995; Kingsford, 2000). Reports of mass turtle mortality in drying wetlands of Australia are increasing (see Figures 1 and 2), though such reports have not yet made their way into the scientific literature, as they have in other countries (Christiansen and Bickham, 1989, Bodie and Semlitsch, 2000). Many turtles die of starvation or dehydration during or following wetland drying, but drought can indirectly cause mortality as well. Turtles emigrating from a drying waterbody are vulnerable to predators including the introduced European Fox (Spencer, 2002), or they may encounter vehicular traffic in developed areas resulting in mass mortality on roads in Australia (Chessman, 1988; Rees, 2008), see Figure 3) and elsewhere (Aresco, 2005; Ashley and Robinson, 1996). Exclusion fences can also entrap turtles on land where they eventually die of exposure to predators, high temperatures, or dehydration (Anonymous 1941; JHR and AG pers. obs.).

Feral pigs can even target and depredate turtles aestivating in the mud of dry wetlands, threatening the persistence of turtle populations in northern Australia (Fordham et al., 2006b, 2007). All of the above are examples of how human alterations to natural systems interact with drought to exacerbate impacts upon turtles.

Photo: John Roe.

Figure 2. Receding waters can concentrate turtles, resulting in high densities. Disease and competition for food increase under such conditions, killing many individuals even before the water body dries completely such as in Lake McKenzie, Jervis Bay Territory.

Photo: Damien Michael. Photo reproduced with permission.

Figure 3. Turtles forced to emigrate from drying waterbodies frequently cross roads in developed areas, often leading to mass mortality from vehicular collisions. This *Chelodina expansa* was killed on the Riverina Highway adjacent to the Wonga Wetlands and the Murray River.

Drought can also result in important changes to habitat heterogeneity with subsequent impacts on turtles. For instance, large river systems naturally include not only the deeper and permanent channel, but during floods the aquatic habitat expands to encompass adjacent floodplain wetlands (e.g., oxbows, billabongs, marshes, and sloughs). Such flooding events resupply these highly productive and important wetlands with water and nutrients and provide a conduit for turtles to travel into them (Doody et al., 2002; Georges et al., 2006). Low rainfall and high use of upstream water during drought reduces both the extent and duration of flooding in these adjacent wetlands such that, in Australia's most developed rivers, these habitats may no longer flood at all (Kingsford, 2000; Roshier et al., 2001).

Turtle communities in such systems are often spatially organized according to species habitat preferences (Chessman, 1988), and in Australia several species of turtles including *C. insculpta* (Doody et al., 2002). *C. longicollis* (Chessman, 1988), and *C. expansa* (D. Bower, University of Canberra, unpubl. data) make extensive use of these flooded backwaters. Not only would turtle community diversity decline should river floodplains fail to flood, but our work in Booderee National Park in the Jervis Bay Territory indicates that such drought-induced changes in hydrology can also occur in wetlands isolated from river flows, with subsequent effects on turtle growth, reproduction and regional abundance (Kennett and Georges, 1990, Roe and Georges, 2008).

The retention of water in reservoirs is a common way to meet societal water demands in times of drought, but ironically, this too can have negative effects on some turtles. For example, the Fitzroy turtle (*Rheodytes leukops*) inhabits shallow riffle zones (Tucker et al., 2001), a habitat affinity linked to its gill-like cloacal respiration and diet of aquatic invertebrate prey (Gordos, 1999; Priest, 1997). Dams that transform free flowing rivers to lake-like conditions or that otherwise alter natural flow regimes and increase sedimentation rates have degraded much riffle habitat in the Fitzroy River catchment, which has certainly contributed to the listing of *R. leukops* as a Vulnerable species (Georges, 1993). A change in turtle community diversity (typically a reduction in the number of species) often follows the construction of dams, with river (lentic) turtles being replaced by pond (lotic) turtles or species with more general habitat preferences (DonnerWright et al. 1999; Tucker et al., 2001). Our changes to natural hydrology patterns in aquatic systems are not limited to rivers, but also include modifications to still-water wetlands for increased water retention during drought (i.e., farm dams, Brock

et al., 1999). In this case, humans' influence on some species may be positive, as farm dams, irrigation channels and reservoirs for water retention in agricultural and pastoral regions now offer drought refuges. The proliferation of these artificial aquatic habitats has perhaps augmented populations of *C. longicollis* in dry regions (Rees, 2008) and may even have facilitated its expansion into areas that were previously too dry (Beck, 1991). However, the persistence of these "unnatural" wetlands and the benefits they offer aquatic turtles into the future is uncertain under changing climate scenarios.

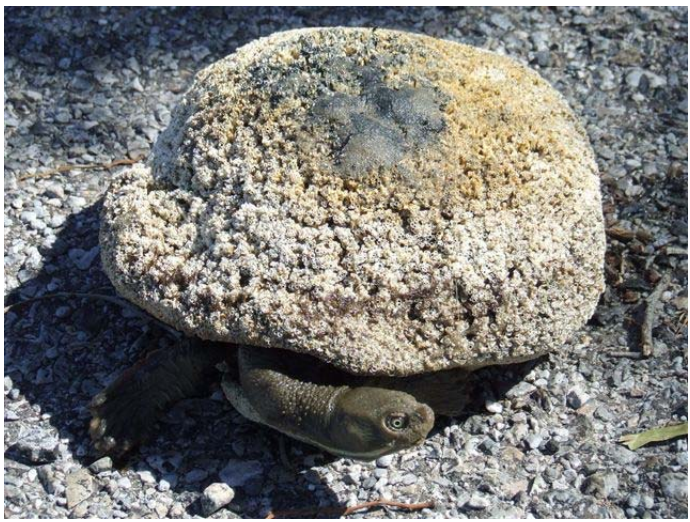

Photo: Sally Grundy. Photo reproduced with permission.

Figure 4. Increased salinization of freshwater habitats has led to the encrustation of turtles' shells by estuarine tube worms. In severe cases encrustation can impede turtle movements and limit the ability to retract their head and limbs, such as in this *Emydura* in Lake Alexandrina, South Australia.

The rising salinity of freshwaters is another major issue with which turtles must contend in Australia. Rising soil salinities brought about by land clearing, intensive irrigation and reduced rates of evapotranspiration[8] have allowed water tables to rise and contaminate freshwater systems (Lemly et al., 2000), but the impact on Australian turtles in these systems has not yet been examined. We know that in some cases increasing salinities have brought

8 Evapotranspiration is the movement of water from the ground to the atmosphere via evaporation and plant transpiration.

saline-tolerant taxa[9] into unwelcome contact with turtles, as in the case of turtle fouling by encrusting estuarine worms in Lake Alexandria, South Australia (see Figure 4). Many nominally freshwater turtles may successfully utilize saline environments (Dunson et al. 1986, Georges and Rose, 1993, Moll and Moll, 2004); however, the physiological, behavioral, and population level responses of Australian turtles to rapidly increasing salinities are in immediate need of assessment.

THE FUTURE OF AUSTRALIA'S TURTLES IN A CHANGING CLIMATE

Predictions for changes to Australia's climate include a reduction in precipitation across much of the continent, particularly in the southern temperate regions. This will result in expansion of arid and semi-arid zones that are less hospitable to turtles, and an increase in the frequency, duration and intensity of drought. The recent drought in south-eastern Australia, whether or not it is part of a natural cycle, serves as a harbinger of impending climate change and focuses our attention on what might be in store for the future. Turtles in the Murrumbidgee River and elsewhere have perished in waterholes not known to dry since European colonization. The Murray-Darling River now seldom flows into the sea, and the combination reduced water flows with marine incursion and increased salinization is presenting major challenges to the freshwater turtle communities of Australia's largest river system. In the south-west, *P. umbrina*, one of the rarest turtles in the world is already on the brink of extinction, being restricted to a few small ephemeral swamps. Any further reduction in precipitation will require continual human intervention to maintain this species in the wild (Kuchling and Dejose, 1988, Kuchling et al., 1991).

If there is any reason to be optimistic, it is that this unfolding crisis has been experienced to some degree by the Australian biota before, and those species that are with us today are there because they have survived the changes. The challenge will be to determine what humans have done to reduce the capacity of the biota to survive climate change as it has done in the past. In

[9] Saline-tolerant taxa are those animals that can successfully inhabit waters with higher concentrations of dissolved salts than in normal freshwater. Freshwater typically has dissolved salt concentrations below 0.5 parts per thousand.

the case of *P. umbrina*, the answer is clear. We have dramatically reduced the extent of available habitat through reclamation of swampland for urbanization and agriculture, rendering this species extremely vulnerable to climate change. In the Murray-Darling drainage and other watersheds that are currently experiencing greatly reduced flow, we need to know where the refugial areas were during past periods of drying so that we can protect their integrity during this drying event.

These refugia are areas in which the turtles and other aquatic biota persisted during extended periods of low precipitation, only to expand out to their current distributions when precipitation increased. On a shorter timescale, responses to drought may involve a dynamic of localized extinction and repopulation. What have we done - in terms of fragmenting our freshwater systems through habitat degradation, construction of weirs, dams and roads, and alteration of flow intensity, duration and timing - to disrupt this dynamic? These considerations apply at many geographic scales, from the Cooper Creek watershed in the arid centre to individual wetlands and wetland networks, such as at Booderee National Park in the temperate south-east.

CONCLUSION

Clearly, failure to address several issues, including the maintenance of adequate water during periods of reducing precipitation, the isolation of off-channel waterbodies in the lower Murray to address human demands for water quantity and quality, the construction of more dams and storage facilities without exploring alternatives, and our increasing take from aquifers with little regulation, are all responsible for environmental changes that are likely to have major (and potentially adverse) impacts on freshwater communities, of which turtles are an integral part.

Regardless of the ultimate cause of recent droughts in Australia and other parts of the world, the challenge before us is to improve water resource management in a way that allows for more efficient use of water to meet societal demands, while at the same time ensuring that the natural capacity for biota to respond to drought is maintained.

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