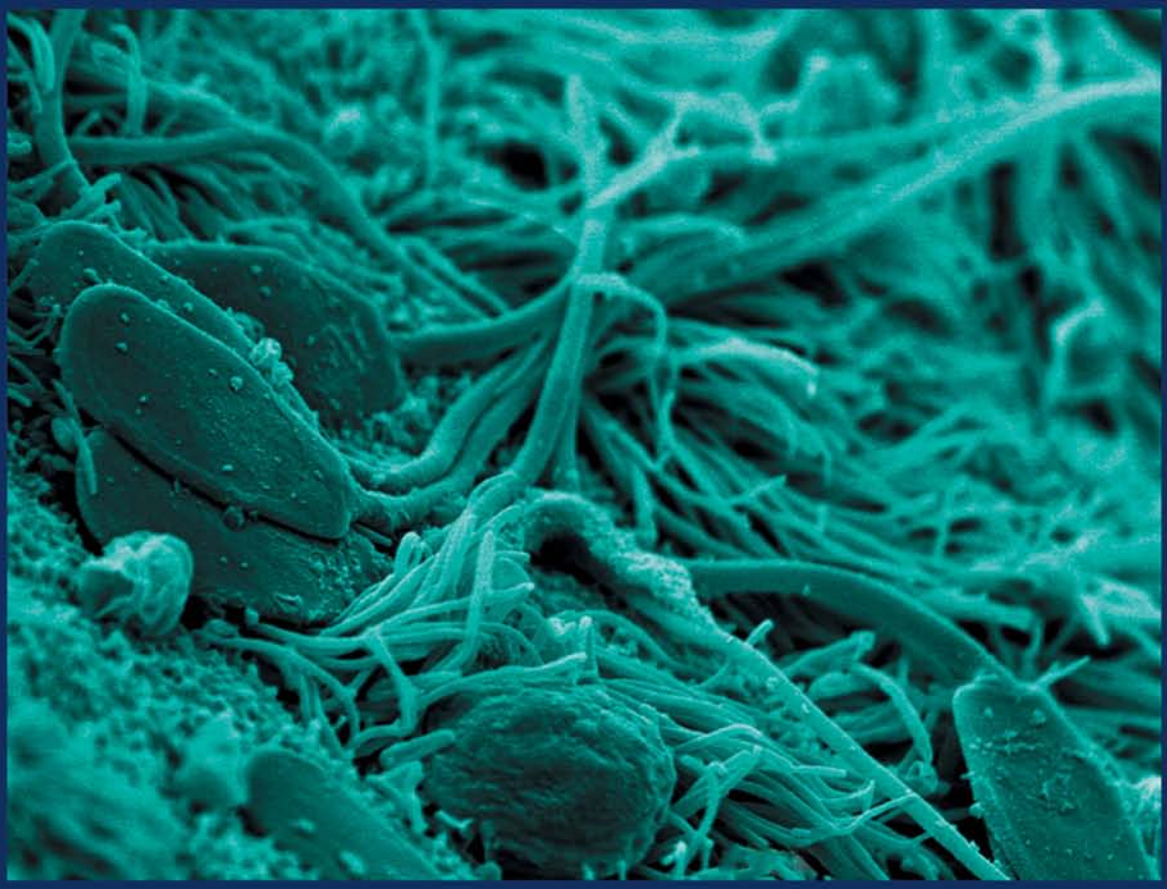
Control of Pig Reproduction VIII



Edited by
H. Rodriguez-Martinez,
J.L. Vallet and A.J. Ziecik

Nottingham
University Press

Control of Pig Reproduction VIII

Society of Reproduction and Fertility Volume 66

Proceedings of the Eighth International Conference on Pig Reproduction

Alberta, Canada
June 2009

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Nottingham
University Press

Nottingham University Press
Manor Farm, Church Lane, Thrumpton
Nottingham NG11 0AX, United Kingdom
www.nup.com

NOTTINGHAM

First published 2009
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British Library Cataloguing in Publication Data
Control of Pig Reproduction VIII
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ISBN 978-1-904761-39-6

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Cover image: Boar spermatozoa in the oviduct, SEM. H. Ekwall, SLU, Sweden
Typeset by Nottingham University Press, Nottingham
Printed and bound by H. Charlesworth and Co Ltd, UK

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Preface

The 8th International Conference on Pig Reproduction (ICPR) was held at the Banff Conference Centre in Banff, Alberta, Canada between May 31st and June 4th 2009. The conference continued the tradition begun in 1981 of holding meetings every four years to provide a forum to discuss the latest advances in pig reproductive biology and the application of this knowledge to improvements in pig fertility.

The outstanding success of the 8th ICPR was due to the tireless hard work, commitment and attention to detail of the local organising committee (LOC), chaired by Dr George Foxcroft. Their dedication ensured that the conference ran smoothly and was enjoyed by all delegates as a forum for stimulating scientific exchange within an environment that was conducive to fruitful interactions with colleagues old and new. The conference was attended by 151 delegates from 19 countries.

The scientific programme was developed by Dr Heriberto Rodriguez-Martinez, in consultation with the International Organising Committee. The programme of the 8th ICPR saw a deviation from previous meetings in that the morning sessions were devoted to state of the art lectures, while parallel sessions in the afternoon provided to opportunity to learn more of the latest developments in pig reproductive biology, or to consider how such discoveries could be used to practical advantage. In addition, some submitted abstracts describing particularly novel findings were selected to be expanded into longer papers and the authors invited to deliver an oral presentation at the meeting. This volume contains manuscripts accompanying the state-of-the art lectures, the parallel sessions and the expanded abstracts. In addition, 80 posters were presented at the meeting. The abstracts associated with those posters are not in this volume, but can be found at www/icpr2009.com. We thank the LOC for making this possible

Sincere thanks are due to Professor Heriberto Rodriguez-Martinez, Dr Jeff Vallet and Dr Adam Ziecik for editing the papers contained within this volume and for liaising with Nottingham University Press to ensure the timely publication of this book, which is published as a Society of Reproduction and Fertility (SRF) volume. We are confident that this volume will provide a valuable resource for all interested in pig reproduction and fertility.

Cheryl J. Ashworth
Chair, International Organising Committee

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The physiological roles of the boar ejaculate

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During ejaculation in the boar, sperm cohorts emitted in epididymal cauda fluid are sequentially exposed and resuspended in different mixtures of accessory sex gland secretion. This paper reviews the relevance of such unevenly composed fractions of seminal plasma (SP) *in vivo* on sperm transport and sperm function and how this knowledge could benefit boar semen processing for artificial insemination (AI). The firstly ejaculated spermatozoa (first 10 ml of the sperm-rich fraction, SRF [P1]) remain mainly exposed to epididymal cauda fluid and its specific proteins i.e. various lipocalins, including the fertility-related prostaglandin D synthase; than to prostatic and initial vesicular gland secretions. P1-spermatozoa are hence exposed to less bicarbonate, zinc or fructose and mainly to PSP-I spermadhesin; than if they were in the rest of the SRF and the post-SRF (P2). Since the P1-SP is less destabilizing for sperm membrane and chromatin, P1-spermatozoa sustain most *in vitro* procedures, including cryopreservation, the best. Moreover, ejaculated firstly, the P1-spermatozoa seem also those deposited by the boar as a vanguard cohort, thus becoming overrepresented in the oviductal sperm reservoir (SR). This vanguard SR-entry occurs before the endometrial signalling of SP components (as PSP-I/PSP-II and cytokines) causes a massive influx of the innate defensive PMNs to cleanse the uterus from eventual pathogens, superfluous spermatozoa and the allogeneic SP. The SP also conditions the mucosal immunity of the female genital tract, to tolerate the SR-spermatozoa and the semi-allogeneic conceptus. These *in vivo* gathered data can be extrapolated into procedures for handling boar spermatozoa *in vitro* for AI and other biotechnologies, including simplified cryopreservation.

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Introduction

Modern pig production is vertically integrated, with breeding done through AI. This first-line, easy, practical and highly effective biotechnology serves two major purposes (i) avoid spreading venereal (or otherwise semen-shed) diseases and, (ii) maximise dissemination of genetic material to large numbers of sows. With proper detection of oestrus (twice daily) and AI correctly performed (80-100 mL, cervical, 2-3 times/oestrus) with high-quality semen (> 2 x 10^9 motile spermatozoa), > 90% of farrowing rates and mean litter sizes of 12–14 piglets are reached, comparable with those achieved with natural mating (reviewed by Rodríguez-Martínez 2007a). With such results, it is not surprising that pig AI became an essential breeding tool, increasing worldwide from ~7% at the start of the 1980s (Reed 1985) to more than 80% 20 years later (Wagner & Thibier 2000). In the European Union (EU) ~84% of all sows/gilts are bred by AI (Feitsma, personal communication).

Improvements in the design of media, AI-catheters and moment of AI, have led to > 99% of all AIs done around the world being made with liquid semen (Wagner & Thibier 2000). Frozen-thawed (FT) semen is, however, rarely used (~1%, Thibier, personal communication), since cryopreservation is still considered "*quasi* experimental". However, breeding enterprises are interested in further developing cryopreservation, since FT-semen could be used in situations in which the widely used liquid semen can not, such as the international exchange of genetic lines without transporting livestock, the long-term conservation of superior genetic individuals in genetic resource banks, or the testing for presence of pathogens before use. Yet, boar spermatozoa show low cryosurvival (~40%) and a shortened lifespan among surviving spermatozoa. This obviously leads to lower farrowing rates and smaller litter sizes compared to liquid semen (reviewed by Rodríguez-Martínez 2007a). Moreover, cryopreservation procedures are cumbersome, time-consuming and yield few doses/ejaculate, all of which deter from its wider use.

Ongoing research aims to widen AI-use, by decreasing sperm numbers per AI-dose (increasing extension), designing novel media (chemically-defined) or quicker cryopreservation methods, and implementing alternative procedures for semen deposition (intra-uterine). Despite gains thus far (rev by Rodríguez-Martínez 2007a), innate characteristics of the boar ejaculate such as the specifics of the sperm structure and of the seminal plasma (SP), yet hinder full success. The SP is not just a sperm vehicle, but as relevant modulator for sperm function, for sperm transport post-breeding and as inductor of both innate and adaptive immunological responses by the female that would ensure reproductive success. Considering the current focus for novel semen extension and sperm treatments for AI, the present paper attempts to review aspects of the characteristics of the boar ejaculate in relation to sperm transport in the female genitalia *in vivo*, and how this knowledge could benefit boar semen processing for AI.

Ejaculation in the pig

Ejaculation is a highly coordinated physiological process involving neurological and muscular events that build two distinct phases; (i) emission (the formation and deposition of semen [spermatozoa and seminal fluid] in the urethra and (ii) the forthcoming ejection of the semen through the penis urethra (the so-called expulsion, or ejaculation proper). In boars, emission and ejaculation repeat as waves for 5-10 min, during which the complete ejaculate (250-300 ml in a mature boar) is sequentially verted into the female cervix lumen or it is manually collected *ex-corpore* into a recipient (Senger 2005). In the latter case, waves can be regarded by the operator as a sequence of three major fractions. These fractions are classically called *pre-sperm* (PSF, with a clear seminal fluid, some gel and a heavy degree of contamination of cell debris, urine and smegma from the preputium), *sperm-rich* (SRF, easily recognised by its creamy-white colour) and *post sperm-rich* (PSRF, that goes from greyish to watery

in aspect) and whose increasing amounts of "tapioca-like" flocula, signals the end of the ejaculation process, accompanied by a fading of the penile erection. Interestingly, a second ejaculation sequence can be manually stimulated with brief, firm, pulsating hand pressure applied to the penis, upon which a new, but smaller, SRF is often collectable (Mann & Lutwak-Mann 1981).

The ejaculate of the boar

The ejaculate is a sperm suspension (~ 50–90×10^9 in 9 month-old boars to ~ 70–110×10^9 when they reach maturity, i.e. after 12 months of age; Flowers 2008, Wallgren, unpublished results) in a SP composed of the mixture of the contents of the tails of the ductus epididymides and the secretions of the accessory sexual glands. The latter vary in content, volume and occurrence of excretion, building fractions of different composition. The PSF-SP contains mainly secretion of the urethral and bulbourethral glands, as well as of prostate; the SRF-SP is a blend SP where the emitted epididymal fluid in which spermatozoa (see Fig. 1) originally bathe, is diluted in vesicular gland and prostate secretions. Lastly, the PSRF-SP suspends few spermatozoa, being a fluid primarily derived from the increasing secretion of the vesicular glands, the prostate and, by the end of the ejaculation, the bulbourethral glands (Einarsson 1971, Mann & Lutwak-Mann 1981). The latter delivers a tapioca-like flocular secretion that coagulates in contact with the vesicular fluid the SP, as seen when the entire ejaculate is collected in an open recipient. The role of this process is, *in vivo*, to retain the ejaculate *in utero*, minimizing the transcervical backflow commonly seen at AI with liquid semen, where the gel component is consistently filtered away (Viring & Einarsson 1981). Spermatozoa are, therefore, ejaculated with a maximum peak concentration in the first portion of the SRF (i.e. the first 10–15 ml, called Portion 1 or P1, an easily collectable portion; Rodriguez-Martinez *et al.* 2005, Fig. 1), decreasing thereafter in numbers along this fraction to virtually disappear by the PSRF alongside with increasing secretion of the vesicular glands.

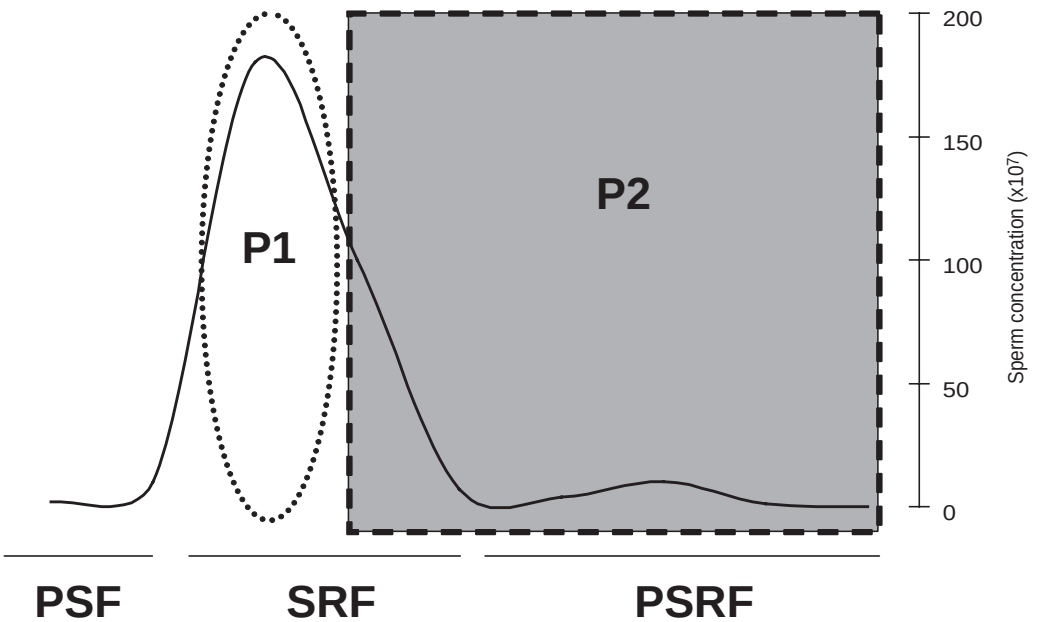


Fig. 1 Relative sperm concentrations (——) define ejaculate fractions in the boar ejaculate (PSF: pre-sperm fraction, SRF: sperm-rich fraction, PSRF: post sperm-rich fraction (includes gel component). Portions of the ejaculate can also be defined; P1: 1st 10 ml of the SRF, P2: the rest of the SRF and the PSRF.

The boar seminal plasma, a heterogenous fluid

Most studies of the boar SP have been done as bulk fluid (since this is the way the ejaculate has been sampled), but there are excellent studies of fractionated samples (Einarsson 1971, Lavon & Boursnell 1975), which have shown that, owing to its sequential formation, the SP contains many components, all of which interact with spermatozoa and the surrounding female environment. The PSF has mostly electrolytes (mainly Na and Cl); the SRF mainly proteins but also steroid hormones (Claus *et al*. 1987, Claus 1990), glycerophosphorylcholine, fructose, glucose, inositol, citrate, bicarbonate and zinc, while the PSRF has increasing amounts of proteins, bicarbonate, zinc, Na, Cl and sialic acid (summarised by Mann & Lutwak-Mann 1981).

The source of the steroid hormones found in the boar ejaculate varies. Most testosterone derives from the accessory sexual glands, while oestrogens are mainly (80–90%) introduced in semen by the epididymal contents (originally testicular; Claus 1990 and references therein). Oestrogens, which in the boar can reach $>10\,\mu$g/ejaculate, stimulate the myometrium directly or indirectly (through induction of endometrial $PGF_{2\alpha}$), as well as by influencing the release of LH (Claus 1990). Therefore, SP-oestrogens are considered important, along with the behavioural, neuronal release of oxytocin; for the coordinated, long-lasting uterine motility during oestrus which issues the rapid phase of sperm transport in the female (Langendijk *et al*. 2005) and, ultimately, fertility (Claus *et al*. 1989).

Proteins are a major component of the boar ejaculate (39.4 ± 13.45 mg/ml, Rodriguez-Matinez *et al*. 2005), 80-90% of vesicular gland origin and 75-90% of them belonging to the spermadhesin lectin family. This family comprises three members; the Alanine-Glutamine-Asparagine proteins AQN (-1 and -3), the Alanine-Tryptophan-Asparagine proteins [AWNs] and the Porcine Seminal Plasma proteins I and II [PSP-I and PSP-II] (Töpfer-Petersen *et al*. 1998). Spermadhesins are multifunctional 12-16 kDa glycoproteins whose biological activities depend on their sequence, grade of glycosylation or aggregation state, as well as their ability to bind heparin (the AQN-1, AQN-3 and AWN being grouped as heparin-binding proteins [HBPs]) or not (PSPs), as they attach to the sperm plasma membrane to various degrees from the testis to the ejaculate. Collectively, they have been related to multiple effects on spermatozoa including membrane stabilisation, capacitation, and sperm-oviduct or zona pellucida (ZP) interplay. HBPs seem to stabilise the supra-acrosomal plasmalemma prior to capacitation *in vivo* (Calvete *et al*. 1997). AWN-epitopes have been detected on boar spermatozoa bound *in vivo* to the ZP, strongly suggesting AWN is a *bona fide* sperm surface-associated lectin, mediating sperm-ZP interactions at fertilisation (Rodriguez-Martinez *et al*. 1998). *In vitro*, however, HBPs failed to promote sperm survival (Centurión *et al*. 2003), while PSPs, also binding to the sperm surface (Töpfer-Petersen *et al*. 1998), display protective action on highly-extended and processed spermatozoa (Caballero *et al*. 2004, 2006, 2008 and references therein). The PSP-I and PSP-II account for >50% of all SP-proteins, forming a non-heparin-binding heterodimer of glycosylated spermadhesins (Calvete *et al*. 2005) which depict immunostimulatory activities *in vitro* (Leshin *et al*. 1998) and *in vivo* (Rodríguez-Martínez *et al*. 2005). The various SP proteins originate from the testis, the epididymides and the sexual accessory glands (García *et al*. 2008), and their relative concentration vary among ejaculate fractions (see Fig. 2). Expectedly, the amounts of proteins increase 4-fold alongside the secretion of the vesicular glands, the relative concentrations thus being lowest P1, to increase (HBPs and, particularly the PSPs) towards the bulk of the PSRF (Rodríguez-Martínez *et al*. 2005), implying that the major proportion of spermatozoa is not immediately suspended in high amounts of SP-proteins *in vivo*, particularly not those firstly ejaculated.

Bicarbonate, an ion considered highly relevant for sperm motility, for induction of destabilisation changes in the plasma membrane and, ultimately, for *in vitro* and *in vivo* capacitation (reviewed by Rodríguez-Martínez 2007b), is present in the bulk boar ejaculate (20–23 mM/l). Concentration varies among fractions, from 14–17 mmol/l in the PSF and SRF, respectively, to the double i.e.

>30 mmol/l, in the PSRF (Rodríguez-Martínez *et al*. 1990b). Bicarbonate levels are lowest in the P1 (~13 mmol/l) (Saravia *et al*. unpublished, Fig. 3), e.g. the first cohort of ejaculated P1-spermatozoa does not bathe in a SP with high levels of bicarbonate.

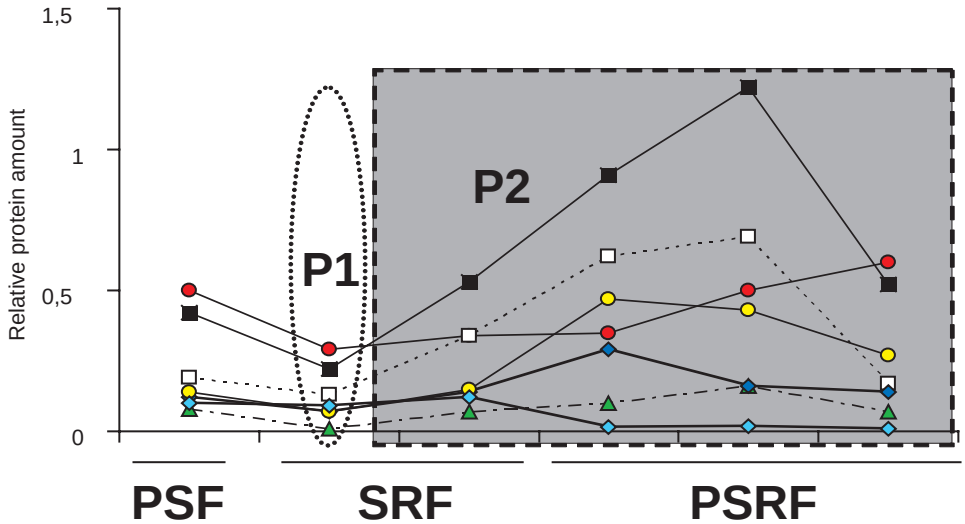


Fig. 2 Temporal relative amounts of seminal plasma proteins (●: PSP-I, ■: PSP-II, □: AQN-1, ▲: AQN-3, ◆: AWN-1, ○: AWN-2, ◇: inhibitor of acrosin/trypsin) in consecutive samples of the boar ejaculate (PSF: pre-sperm fraction, SRF: sperm-rich fraction, PSRF: post sperm-rich fraction (includes gel component)(modified from Rodriguez-Martinez *et al*. 2005).

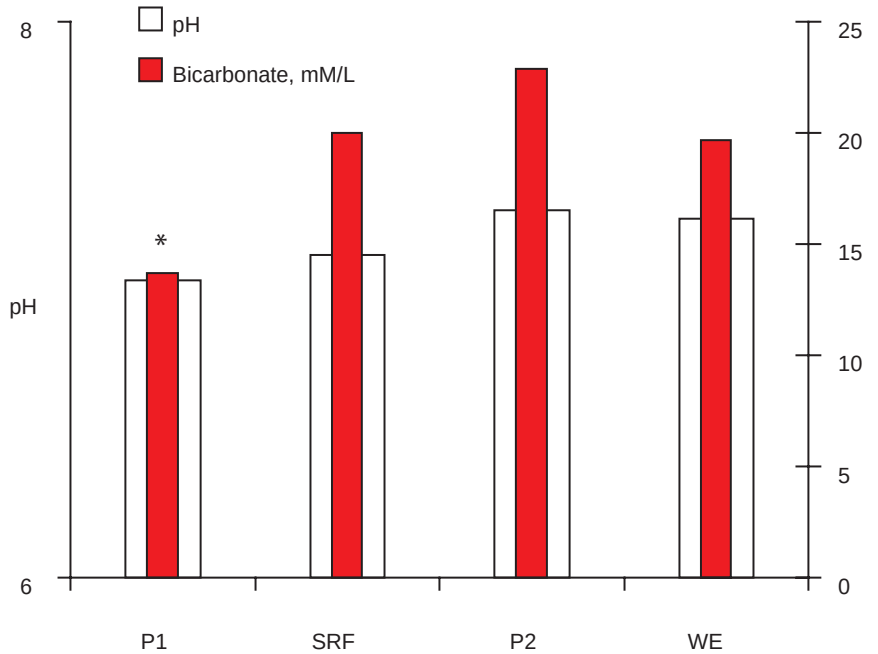


Fig. 3 Mean bicarbonate concentration (mM/L) and pH in different portions (P1 and P2), the sperm-rich fraction (SRF) of the ejaculate or the whole ejaculate (all fractions) of the boar (n = 20 boars; P1: 1st 10 ml of the SRF, P2: the rest of the SRF and the PSRF, WE: whole ejaculate, including PSF, SRF and PSRF. *: $P < 0.05$ (modified from Saravia *et al*., 2009b).

The cation zinc, unusually abundant in boar SP (Massanyi *et al*. 2003) is also of utmost importance for sperm function, and it is secreted in the SP alongside sperm and fructose emission, as shown in Fig. 4. Zinc stabilises, among other functions, sperm chromatin in human (Björndahl & Kvist 2003) and pigs (Kvist *et al*. 1987, Björndahl *et al*. 1990). *In vivo*, depletion of nuclear zinc by secretions decreases stability, being followed by a non-zinc, but disulphide-bridge dependent superestabilisation, which counteracts normal descondensation during fertilisation (Rodriguez-Martinez *et al*. 1990a). Such zinc-dependent stability can be challenged *in vitro*, by exposure to the detergent sodiumdodecylsulphate (SDS) and the zinc-chelating agent EDTA (a customary component of boar semen extenders, 6 mM) for 60 min at 60° C, which decondenses the sperm nucleus (see Fig. 4). As depicted, boar spermatozoa ejaculated in the later expulsed sperm-bearing portions (PSRF, where zinc levels are significantly higher) become more superstabilised than those expelled in the first portions, particularly the P1, since they clearly diminished their capacity to decondense *in vitro* alongside ejaculation. However, the total SP-zinc concentration as such is not an indicator of the free zinc amount that equilibrates with the sperm zinc content. A concomitant increase in citrate, having three binding sites for zinc at slightly alkaline pH, or presence of zinc-binding proteins could result in a zinc-chelating environment (Kvist *et al*. 1990).

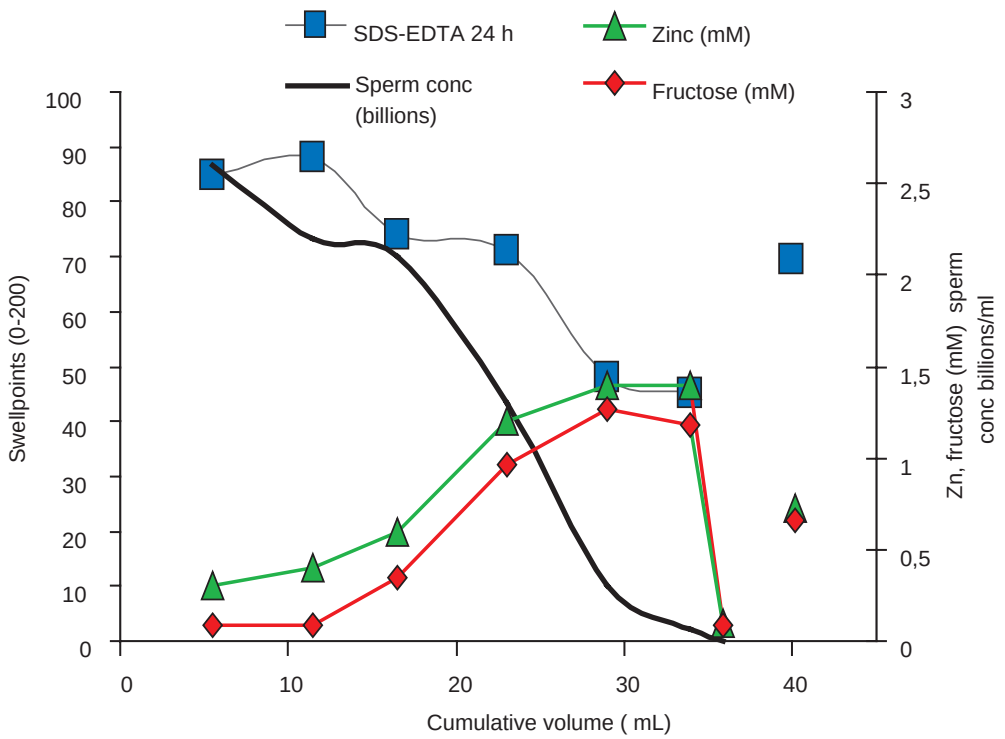


Fig. 4: Chromatin decondensation in vitro (of boar spermatozoa collected in 5 mL samples (n = 9) within the porcine sperm-rich fraction (SRF) and the post-sperm-rich fraction (PSRF) and an independently collected whole ejaculate (at 40). Zinc (mM), fructose (mM) and sperm numbers (x10^9/ml) are also depicted as markers. Decondensation was quantified as sperm nuclear swell-points* (i.e. the sum of % moderately decondensed sperm heads x 1 and the % grossly decondensed sperm heads x 2, maximal swellpoints are 200) after exposure to SDS-EDTA at 24h. (Kvist *et al*. unpublished).

The change from a zinc-dependent into a disulphide-dependent superstabilisation occurs upon storage of both cauda and ejaculated boar spermatozoa (Kvist *et al*., unpublished results) and enhanced by prior exposure to a zinc-chelating environment (Björndahl *et al*. 1990), as depicted in Fig. 5 for immature (from testis, rete testis, caput or corpus epididymides) or mature spermatozoa (from cauda epididymides, whole ejaculate and consecutive aliquots of the SRF [as A1-A4] and the PSRF [A5-A8]). Sperm chromatin decondensation in SDS-EDTA at 0 or 24 h post-collection was more readily seen in immature than in mature spermatozoa. While storage (up to 20 d) promotes the disulphide-bridge dependent chromatin superstabilisation in immature spermatozoa, mature spermatozoa can sustain short storage (24 h) without becoming superstabilised, provided they are not pre-exposed to zinc-chelating treatment (Björndahl *et al*. 1990). Zinc also seems to play a major role in maintaining the stability of the spermadhesin PSP-I/PSP-II heterodimer, an effect that can be reversed by EDTA or at acidic pH, as found in the sperm reservoir (SR), i.e. a 1–2 cm segment of the utero-tubal junction (UTJ) and the adjacent isthmus of each oviduct (Rodríguez-Martínez *et al*. 2005), suggesting the female also regulates the extent of its action (Campanero-Rhodes *et al*. 2005).

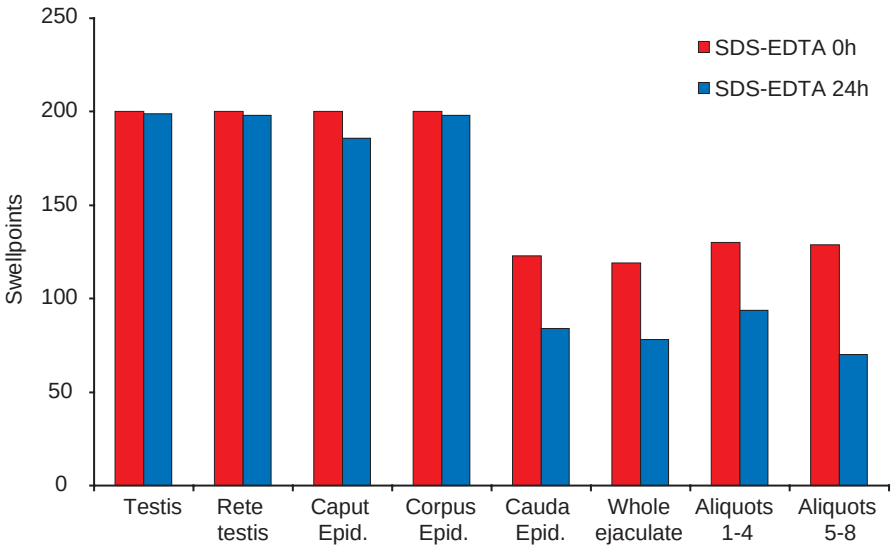


Fig. 5 Ability of the chromatin of boar spermatozoa, collected from various sources (testes, epididymides, whole ejaculate and consecutive aliquots of the SRF [Aliquots 1-4] and the PSRF [Aliquots 5-8]) to decondense in vitro (quantified as sperm nuclear swell-points*, i.e. the sum of % moderately decondensed sperm heads x 1 and the % grossly decondensed sperm heads x 2, maximal swellpoints are 200) after exposure to SDS-EDTA at 0 or 24h (Kvist et al. unpublished).

Distribution of the ejaculated spermatozoa in the female

During natural mating, the boar sequentially deposits the various fractions of the ejaculate in the cervical canal and elicits, by distending the uterine cavity, a stretching response from the myometrium. Both distention and SP-oestrogens, induce production/release of endometrial $PGF_{2\alpha}$ to, ultimately, provoke rhythmic, antiperistaltic (ad-ovarian) and peristaltic (ad-cervical) myometrial contractions. Antiperistalsis dominates the rapid phase of sperm transport through the

female internal genital tract and transports –within minutes– a small subpopulation of spermatozoa towards the SR (Rodríguez-Martínez *et al.* 2005). Ad-cervical peristaltis effectively mixes the uterine sperm suspension since the cervical canal is plugged by the ejaculated gel fraction. At AI with gel-free liquid semen, these contractions cause a large retrograde flow (up to 35-40% of the volume introduced, holding up to 20-25% of spermatozoa) within 30 min (Viring & Einarsson 1981, Steverink *et al.* 1998).

When semen is deposited (via mating or AI) pre-ovulation, enough spermatozoa (10^5 to 10^8) reach and colonise the functional tubal SR, to ensure successful fertilisation of all ovulated oocytes. Although this so-called 2nd phase of sperm transport mainly occurs between 5 and 60 min from insemination, the SR replenishment can take longer, depending more on sperm numbers inseminated than on the number of following matings or AIs (see review by Rodríguez-Martínez 2007b and references therein). The tubal SR is immunologically-privileged (Bergqvist *et al.* 2005), where sperm viability and fertilising capacity are preserved (Rodríguez-Martínez *et al.* 2001, Tienthai *et al.* 2004) for the entire preovulatory period. From the SR, restricted sperm numbers (10^2-10^3) are gradually, but apparently continuously released towards the presumed site of fertilisation at the ampullary-isthmic junction (AIJ), thus defining the 3rd phase of sperm transport, particularly when ovulation is approaching or has occurred (Rodríguez-Martínez 2007b). Not all spermatozoa are trapped in the SR, and trans-oviductal passage occurs during the preovulatory period (Viring 1980) but it is most evident post-ovulation, when spermatozoa do not need a long SR-storage. In either case, sperm numbers are importantly reduced (to hundreds) at the AIJ, a fact that strongly contributes to the physiological ratio (1:1) between spermatozoa and oocytes during fertilisation *in vivo* (Hunter & Rodríguez-Martínez 2004).

Which spermatozoa colonise the sperm reservoir?

Theoretically, anyone; provided they are potentially capable of interacting with the SR (Viring 1980). However, the sequentiality of the entry through the cervico-uterine lumen during mating has led to the hypothesis that the 1st ejaculated sperm sub-population (in P1) is, by reaching first the SR, overrepresented there. P1- and P2- (last portion of the SRF and the PSRF) spermatozoa were collected from fertile boars. The P1-spermatozoa were loaded with the fluorophore Hoechst 33252, while P2 spermatozoa were kept unstained until conventional cervical AI of equal sperm numbers ($\sim 10 \times 10^9$ spermatozoa) per portion was performed *ad modum* 12 h after onset of oestrus in weaned sows; either mixing P1 and P2 aliquots (control, $n = 5$) in a single AI flask (90–mL dose), or testing (a) a sequential order (P1-P2, Treatment A, $n = 5$) with P1 (10 mL) inseminated first, immediately followed by deposition of 80 ml of P2-semen or (b) an inverse order (P2-P1, Treatment B, $n = 5$). The sows were euthanized ~ 3 h later and the SRs flushed to recover the spermatozoa, which were accounted for as stained and unstained. While the number of spermatozoa flushed from the SRs did not differ between groups nor between boars (NS, ranging 0.9 to 2×10^9), the proportion of stained P1-spermatozoa significantly ($P < 0.05$) differed between groups, but not between boars. The highest proportion of P1-spermatozoa in the SRs (59.8 ± 5.66 %, means ± SEM) was found when a sequential order (P1-P2, Group TA) of insemination was issued (see Fig. 6). Reversing this order (Group TB, P2-P1) dramatically decreased the proportion to 15.6 ± 2.1%, much lower than when a mixed suspension (control) was inseminated at one time (36.9 ± 2.70%). The hypothesis tested proved valid; when spermatozoa were inseminated in the same order as ejaculated *in vivo,* they were overrepresented in the SR. It remains to be determined whether such proportionality is maintained at the site of fertilisation.

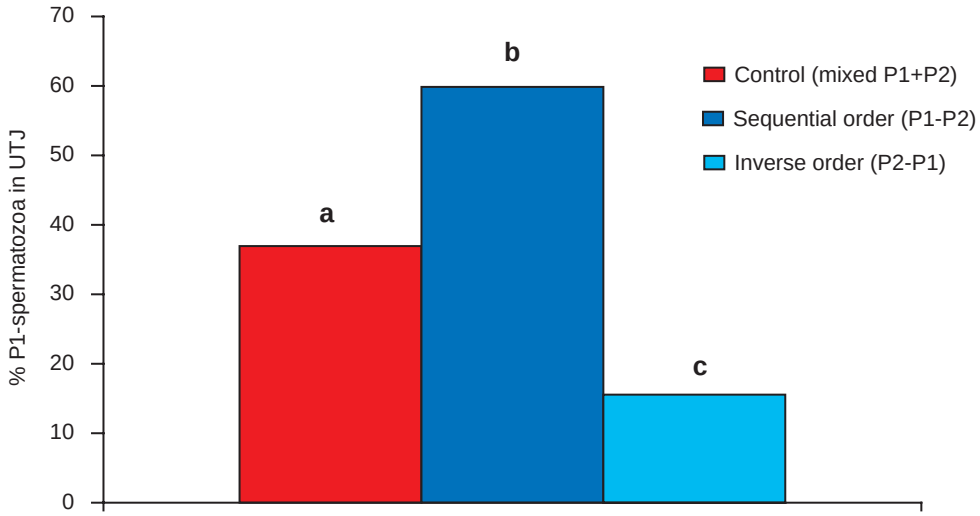


Fig. 6 Mean proportions of P1-spermatozoa present in the uterotubal junction (UTJ) of oestrous sows (n = 15, equally allotted) 3 h past-AI with a sequential order (P1-P2), an inverse order (P2-P1) or a mixture (P1 + P2, control) of ejaculate portions (P1: 1st 10 ml of the SRF, P2: the rest of the SRF and the PSRF); a-c: different letters denote significant differences, $P < 0.05$ (Rodriguez-Martinez *et al.* unpublished).

What happens with the spermatozoa that do not enter the sperm reservoir?

While ~40% of the volume of the AI-dose is lost rather quickly (within 30 min) via vaginal reflux, this fluid only contains ~22-25% of the inseminated spermatozoa. The remaining ones have either entered the SR "sanctuary" or are still in the uterine lumen. The uterine cavity is, ~10 min after AI, invaded by inflammatory polymorphonuclear granulocytes (PMNs) which migrate from the lamina propia (i.e., subjacent to the lining epithelium, where they accumulate after extravasation, presumably a result of the high levels of oestrogens that dominate pro-oestrus in pigs), through the lining epithelium (Lovell & Getty 1968, Rodríguez-Martínez *et al.* 1990c), see Fig. 7a-c. Massive numbers luminal PMNs are first detected by 30 min (Lovell & Getty 1968), to sustain entry for the following 2–3 h (Viring & Einarsson 1981), surpassing the number of inseminated spermatozoa (Matthijs *et al.* 2003). This dramatic uterine PMN-influx is accompanied by accumulation of macrophages, granulocytes and lymphocytes in the endometrial stroma and, to a lesser extent, into the base of the lining epithelium (Rodríguez-Martínez *et al.* 1990c, Bischof *et al.* 1994, Kaeoket *et al.* 2003, Robertson 2007); a picture not seen in the oviduct, except for the mesothelial-covered infundibulum (Jiwakanon *et al.* 2006) and for the presence of lymphocyte-like cells in the base of the SR and the adjoining isthmus (Rodríguez-Martínez *et al.* 1990c). The resulting mass of leukocytes and sperm/SP-debris are, during this hourly period, eliminated from the lumen by continuous vaginal discharge but also by epithelial phagocytosis (Rodríguez-Martínez *et al.* 1990c) so that a new inseminate can enter a cleansed uterine lumen, free from redundant spermatozoa or semen-associated micro-organisms. The lumen must, moreover, be ready to host and nurture the semi-allogeneic early embryos when they enter the uterus by 48 h after ovulation.

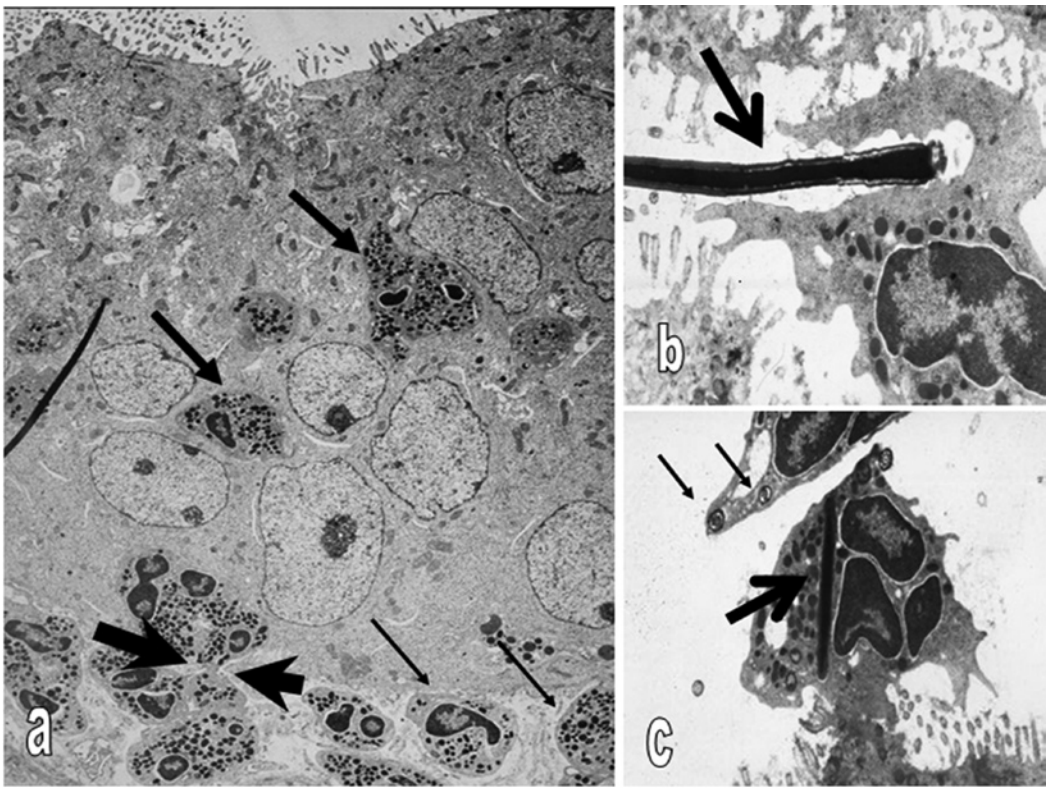


Fig. 7a-c: Entry of polymorphonuclear leukocytes (PMNs) into the uterine lining epithelium (a) and lumen (b,c) of oestrous sows, following AI of neat semen. The TEM-micrographs show the migration of PMNs from the lamina propria (a, thin arrows), through the basal membrane (a, between thick arrows) to the lining epithelium (a, medium-arrows) and the lumen (b,c) where they phagocytose individual spermatozoa (large arrows: sperm heads in b and c, small arrows: sperm tails in c)(micrographs H Ekwall, SLU).

Does the seminal plasma modulate the genital immunology of the female?

Or do the "*per natura*-antigenic" spermatozoa also play a role? While sows do not show changes in cellular immune reactivity after AI (Veselsky *et al*. 1981), gilts exposed to sperm and/or seminal antigens increased litter size in a following fertile mating (Murray & Grifo 1983, Flowers & Esbenshade 1993). AI with washed boar spermatozoa caused a greater influx of PMNs into the uterine cavity of gilts compared to SP or whole semen (Rozeboom *et al*. 1998, 1999), suggesting their chemotactic character (Rozeboom *et al*. 2001a). In contrast, AI of sperm-free SP increased by 5.4-fold the number of PMNs infiltrating the porcine uterine lumen (O´Leary *et al*. 2004).

Most data now suggest that the SP ensures reproductive success by promoting the survival of some spermatozoa, and conditioning the female immune response to tolerate paternal antigens. SP initiates –by interaction with the luminal epithelium- a cascade of downstream immunological effects, including the advancement of ovulation (Waberski *et al*. 2006), the production of progesterone (O´Leary *et al*. 2006) and other defence/immune responses. SP upregulates MHC class II and interleukin-2 (IL-2) receptor expression (Bischof *et al*. 1994), induces the transitory expression of pro-inflammatory (PMN-attractants) soluble cytokines (as

IL-1) and cyclo-oxygenase-2 (O´Leary *et al*. 2004) and the expression of interleukin-6 (IL-6), granulocyte-macrophage colony-stimulating factor (GM-CSF), and the monocyte attractant protein-1 (MCP-1) which, towards early pregnancy, leads to a transition of leukocyte phenotypes, PMNs being replaced by macrophages and dendritic cells (Robertson 2007). Boar SP also contains high levels of the immunosuppressive transforming growth factor-β (TGF-β, Robertson *et al*. 2002), a very potent, multifunctional cytokine group which, in the pig, regulates T-cell differentiation to reach a state of functional, adaptative immune tolerance to male antigens by the female (O´Leary *et al*. 2004, Robertson 2007). This would explain why priming to seminal antigens improves fertility in pigs in later oestrous events (Murray & Grifo 1983, Flowers & Esbenshade 1993, Rozeboom *et al*. 2000). Moreover, inter-boar differences in cytokine SP-content might lead to different adaptation levels by the females (Robertson 2007), thus suggesting the SP-induction of maternal tolerance might relate to the differences in embryo survival often observed among sires (e.g. innate fertility).

SP-infusion studies have, however, lead to -at first sight- paradoxical results, probably owing to differences in animal categories, methods and experimental design used. For instance, SP either induced (Bischof *et al*. 1994, O´Leary *et al*. 2004, Rodríguez-Martínez *et al*. 2005) or attenuated (Veselsky *et al*. 1991, Rozeboom *et al*. 1999, 2001b, Taylor *et al*. 2008, 2009) the inflammatory response of the pig endometrium during mating or AI. It is possible that we are simply facing several steps, firstly a transitory inflammatory response initiated by the SP and a secondary recruitment of antigen-presenting cells (macrophages and dendritic cells), pre-requisite for the generation of paternal antigen-specific T-cells (Schuberth *et al*. 2008).

SP-spermadhesins such as the PSP-I/PSP-II have shown capacity to bind to (Yang *et al*. 1998), and to enhance pig lymphocyte proliferation (Leshin *et al*. 1998). We tested, therefore, whether pig HBPs and PSPs, isolated from the SP of SRF samples collected from mature, fertile boars (Calvete *et al*. 1996, Rodriguez-Martinez *et al*. 2005) could recruit PMNs and different lymphocyte subsets into the lining epithelium of the pig uterus *in vivo*. Consecutive biopsies were taken (2-120 min) under narcosis and treated histologically and by immunohistochemistry (IHC) using mAbs against CD2, CD4 or CD8 lymphocyte subsets. Compared to controls (saline-infused uteri), exposure to the PSP-I/PSP-II heterodimer significantly ($P < 0.05$) induced the migration of PMNs (Log_{10}) to the surface epithelium, within 10 min of infusion (Fig. 8), a recruitment that was sustained over the experimental period, becoming 5-fold by 30 min and 7-fold higher from 60 min onwards ($P < 0.001$). PMNs were detected in the uterine lumen by 30 min and thereafter. The infusion of a similar dose of HBPs had no significant effect (NS). Regarding the IHC (Fig. 9), saline infusion did not significantly increase the number of CD2, CD4 nor CD8 positive (+) cells in the epithelium or the lamina propria over time (NS). CD2+ cell numbers were only significantly increased (4 to 7-fold from 10 min onwards) by infusion of PSP while no significant effect of any treatment was seen on CD4+ cells. CD8+ cell numbers increased significantly (3-fold) only after 60 min of the infusion of PSP. A 1–2 fold higher amount of CD8+ cells was seen during the entire period following the infusion of HBPs (NS). In sum, the heterodimer PSP-I/PSP-II induced an increase in the numbers of some uterine lymphocytes (such as CD2+ [Tk, NK, cytokine-rearing cluster] and CD8+ [cytolytic] cells, Gerner *et al*. 2009) *in vivo*. Numbers increased earlier for CD2+ (from 10 min onwards) than for CD8+ (60 min), suggesting that this immunostimulatory effect could be of primary nature. However, albeit being done *in vivo*, and registering changes over time (2–120 min), this experiment was done under narcosis, on few animals and testing single CD-subsets rather than combinations, which would have provided a more accurate quantification of the processes. On the other hand, PSP-I/PSP-II was confirmed as leukocyte chemoattractant in pigs, confirming previous *in vitro* (Leshin *et al*. 1998) and *in vivo* studies (Rodriguez-Martinez *et al*. 2005). AI of SP-PSPs in conscious oestrus sows, at doses 5-fold lower (3 mg/ml, 100-ml dose) than those present in the

boar ejaculate, led to a substantial (6-fold higher than in controls) recruitment of PMNs to the uterine cavity (35.4 ±12.56 vs 5.83 ±4.62 million PMN/ml, mean ±SD, $P < 0.001$). It remains to study, however, whether any portion of the ejaculate can induce this PMN entry.

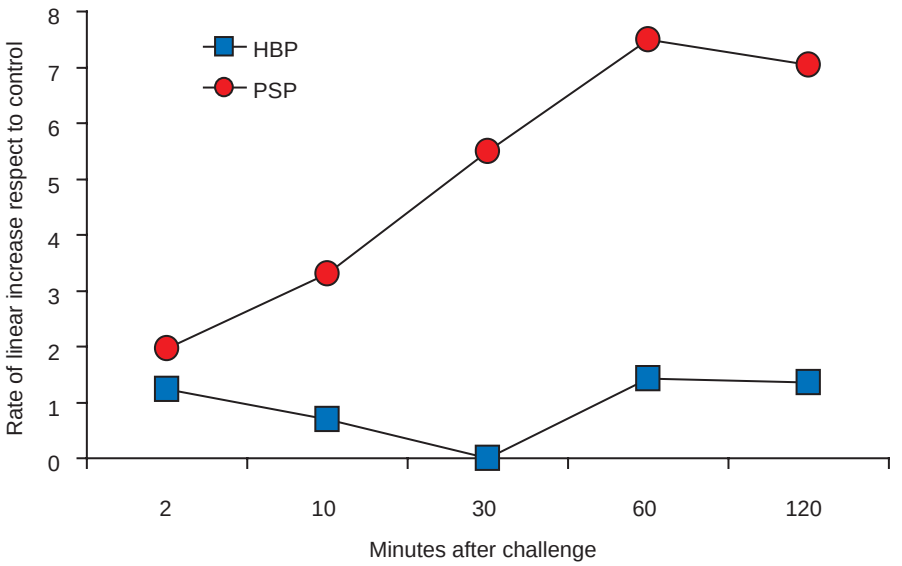


Fig. 8 Rate of linear increase rate for PMN recruitment (compared to control i.e. sham, saline infused uterine horn) to the uterine surface epithelium in oestrous sows (n = 6) at various times (2-120 min) after intrauterine infusion of porcine SP-HBP (HBP) or SP-PSPI/PSP-II (PSP) spermadhesins (3 mg/ml, 100-mL dose). The identity and purity of the protein preparations were assessed by N-terminal sequence analysis and Matrix-Assisted Laser Desorption/ Ionization Time-of-Flight (MALDI-TOF) mass spectrometry while amino acid analysis was used to quantify the amount of either protein (which averaged 15 mg/ml). Sows were, under narcosis, unilaterally infused with spermadhesins, respectively saline and endometria removed for biopsy at various intervals. The tissues were fixed (2% paraformaldehyde) and processed for histology and manual counting of PMNs at x400 using an ocular reticulum on coded slides. The relative number of cells (Log^{10}) was quantified in treatment vs control tissues at each time interval (Rodriguez-Martinez *et al*., unpublished).

Why are there sperm subpopulations in the boar ejaculate?

Many studies, including our own, have described the presence of sperm subpopulations in the boar ejaculate either by chromatin stability, morphology, motility or membrane resistance variables (Rodriguez-Martinez *et al*. 1987, Cremades *et al*. 2005, Peña *et al*. 2005, Saravia *et al*. 2007b and references therein, Druart *et al*. 2009) or even by fertilizing capacity *in vitro* (Xu *et al*. 1996, 1998; Zhu *et al*. 2000). These sperm subpopulations are apparently distributed either along the entire ejaculate, or within major fractions, such as the SRF. We therefore focused, from 2001, on portions of the SRF and PSRF, the so-named P1 and P2, seeking for differences in resilience between spermatozoa fortuitously ejaculated in one of these portions. Spermatozoa bathing in P1 cope better with different handling, such as storage at room temperature, cooling, or freezing-thawing, than P2-spermatozoa (Sellés *et al*. 2001, Peña *et al*. 2006, Saravia *et al*. 2007a, 2009a and references therein). Obviously, these differences would not be specifically allotted to differences among spermatozoa, since spermatozoa collected in the entire SRF (*as praxis*) are able to sustain handling as well. We have now established that it is the SP from

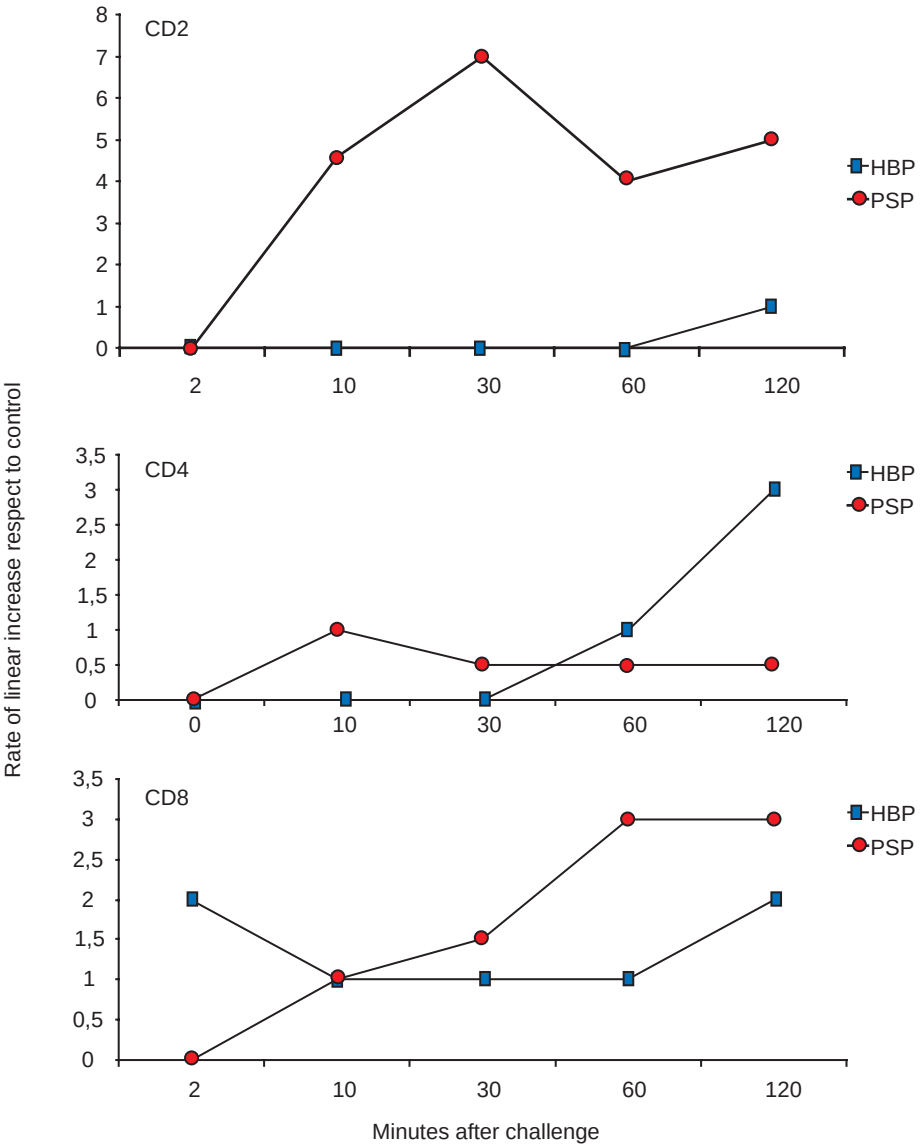


Fig. 9 Rate of linear increase rate for lymphocyte subsets (CD2, CD4 and CD8) recruitment (compared to control) to the uterine surface epithelium in oestrous sows (n = 6) (2-120 min) after intrauterine infusion of porcine SP-HBP (HBP) or SP-PSPI/PSP-II (PSP) spermadhesins (3 mg/mL, 100-ml dose). For description of the identity and purity of the protein preparations and general procedures see Fig. 8. Immunohistochemistry (IHC) using microwave-effected antigen retrieval was done with a standard avitin-biotin immunoperoxidase technique and primary mouse mAbs (VRMD, Pullman, WA, USA). Counting of IHC-marked T-cells in the tissue for biopsy was done at ×400 using an ocular reticle (tissue area: 0.0625 mm2), on coded slides. Particular attention was taken to the lining epithelium and the subjacent lamina propia. The relative number of cells (Log^{10}) was quantified in treatment tissues against control tissues, at each time interval (Rodriguez-Martinez *et al.*, unpublished).

these ejaculate portions (P1-SP or P2-SP) that differently influences sperm kinematics of those fortuitously P1- or P2-contained spermatozoa from individual boars, primarily or secondarily exposed (i.e. following cleansing and re-exposure) to pooled P1-SP or P2-SP from the same males during 60 min. Spermatozoa were subjected to controlled cooling and thawing in MiniFlatPacks™ (MFPs) and examined for motility (CASA) at selected stages of processing. A higher proportion of P1 spermatozoa than of P2 spermatozoa incubated in their native SP portion were motile from collection to post-thawing. When P1-spermatozoa were cleansed from their original SP and re-exposed to pooled P2-SP, sperm kinematics deteriorated from extension to thawing. By contrast, cleansed P2 spermatozoa increased motility to P1 levels, especially after thawing (Fig. 10) when re-exposed to pooled P1-SP. This influence of SP on sperm kinematics was not sire-dependent and presumably related to different concentrations or either SP proteins or other factors in the different SP-portions (Rodríguez-Martínez *et al.* 2008, Saravia *et al.* 2009a).

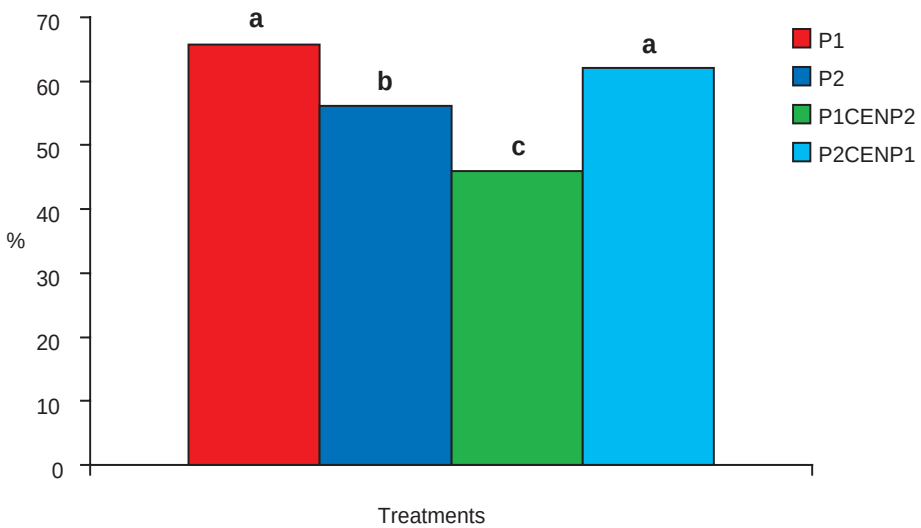


Fig. 10 Cryosurvival (% motility post-thawing) of boar spermatozoa either held in their "native" seminal plasma (P1-SP or P2-SP) for 60 min at room temperature or cleansed by centrifugation and incubated for 60 min t room temperature in the "other SP", as P1CENSP2 (spermatozoa from P1 cleansed and exposed to SP from P2) or P2CENSP1 (spermatozoa from P2 cleansed by centrifugation and exposed to SP from P1) (n = 25; P1: 1st 10 ml of the SRF, P2: the rest of the SRF and the PSRF), a-c: different letters denote significant differences, $P < 0.05$ (modified from Saravia *et al.* 2009a).

What are the differences between the P1-SP and P2-SP? Several, many of these already enumerated, such as they differ in zinc and bicarbonate amounts, the latter being 2-fold lower in P1-SP than in P2-SP (Fig. 3). In relation to this, the pH of P1-SP was 3 pH units lower than that in P2-SP. The ion bicarbonate plays important roles in sperm physiology, both by maintaining intracellular pH (ipH) and the homeostasis of the cell, and by modulating sperm motility and membrane stability through its effects on the sperm adenylyl cyclase (Henning *et al.* 2008). It is responsible for the initiation of motility at ejaculation (Rodríguez-Martínez 1991), and it is also considered the main effector of changes within the lipid bilayer of the sperm plasma membrane that are associated with sperm capacitation *in vivo* in the pig (Tienthai *et al.*, 2004). A lower concentration of bicarbonate (and lower pH) in P1-SP would modulate the ipH of the surrounding spermatozoa and thus its kinematics (Gatti *et al.* 1993, Saravia *et al.* 2009a).

P1-SP and P2-SP significantly differ also in their contents of total protein (the P1-SP having only 17-18% of the total, Rodriguez-Martinez *et al*. 2005). In addition, the P1-SP and P2-SP have clearly distinct protein profiles, the P2-SP having the highest concentration of spermadhesins, in particular glycoforms of PSP-I/PSP-II heterodimer of high mass (Rodriguez-Martinez *et al*. 2005). Using one- and two-dimensional SDS-PAGE electrophoresis to separate proteins in P1 and P2, followed by in-gel enzymatic digestion, mass fingerprinting and collision-induced dissociation tandem mass spectrometry (CID- MS/MS) for peptide sequencing, we have recently confirmed that the P2-SP mostly contained spermadhesins, while the P1-SP contained, besides PSP-I, actin and proteins of the Lipocalin family, namely the lipocalin-type Prostaglandin D-synthase (L-PGD-S), Epididymal Secretory Protein-1 (ESP-1) and Lipocalin (Table 1)(Calvete *et al*. unpublished). Lipocalins are multifunctional proteins, involved in retinol transport, pheromone-binding and transport and prostaglandin synthesis (Flower 1996, Marchese *et al*. 1998). As major epididymal proteins secreted in the caput segment, both EPS-1 and L-PGD-S are relevant for sperm maturation (Fouchecourt *et al*. 2002, Leone *et al*. 2002) and sperm quality (Chen *et al*. 2007). L-PGD-S, primarily involved in transporting retinoids and other lipophilic ligands, binds to the sperm apical ridge being strongly related to sire fertility, including pigs (Gerena *et al*. 1998, Fouchecourt *et al*. 2002, Flowers 1995).

Table 1. Identification of protein spots in boar seminal plasma portions P1 (first 10 mL of the SRF) and P2 (the rest of the SRF and the PSRF), separated by one (1D) and two (2D) dimensional SDS-PAGE electrophoresis followed by in-gel enzymatic digestion, mass fingerprinting and collision-induced dissociation tandem mass spectrometry (CID- MS/MS) to sequence selected peptide ions (Calvete *et al.*, unpublished).

1D SDS-PAGE		2D SDS-Page		Peptide ion		MS/MS-derived sequence	Protein
Ejaculate portion				m/z	z		
P1	P2	P1	P2				
1		1		488.4	2	AGFAGDDAPR	Actin [Q6QAQ1]
2				675.3	2	GFTEDGIVFLPR	Lipocalin-type Prostaglandin D synthase [Q765P8]
3				569.3	2	(214.2)TVVATDYR	Unknown
4				744.7	2	(258.1)CTYFCDXPR	Unknown
		2		584.6	2	GTPXANGDXAXK	Lipocalin [~XP_001917526]
		3, 4		508.4	2	SGINCPIQK	Epididymal Secretory Protein-1 [O97763]
		5-8	1-7	524.3	2	LDYHACGGR	PSP-I [P35495]
			8	528.3	2	GSDDCGGFLK	AQN-3 [P24020]
			9	508.8	2	INGPDECGR	PSP-II [P35496]
			10, 11	925.8	3	ASFHIYYYADPEGPLPFPYFER	AWN [P26776]

Is there any relation between ejaculate characteristics and fertility?

The obvious answer would be yes. However, to determine which characteristic is most relevant and, particularly, if it could be measured *in vitro* remains elusive (Popwell & Flowers 2004) since we are unable to relate results of sperm function to the fertility of pigs, which is not binary as in bovine, but combines pregnancy/farrowing and prolificacy (litter size). Moreover, by inseminating excessive sperm numbers, we mask the relations between measurements of sperm attributes (measured singly [motility, membrane integrity etc] or collectively [ZP-binding, IVF etc], Rodriguez-Martinez 2007a, Foxcroft *et al*. 2008).

As already enumerated, the SP plays major roles, at the level of the spermatozoa they cover and interact with, and for the signalling they excert towards the female genital tract, which has

consequences beyond our current understanding. Seminal antigens have proven beneficial for fertility improvement *in vivo*, with substantial variation among boars (Murray & Grifo 1983, Flowers & Esbenshade 1993, Rozeboom *et al*. 2000), suggesting that the SP of a boar differs somehow from that of another boar. A logical immediate difference is a variation in the type and the relative amounts of SP-proteins, of which some have been correlated to *in vivo* fertility and thus collectively named "fertility-asociated", such as the L-PGD-S (Flowers 1995, 1997, review by Foxcroft *et al*. 2008), clearly present in the P1-SP (Table 1). However, when these proteins have been tested *in vivo* or *in vitro*, results have varied, once more probably caused by the excessive sperm numbers used. Another major question remains; which is the mode of action of these SP-proteins? One tempting hypothesis has been already launched (Robertson 2007), where established differences in SP cytokine contents would lead to different degrees of maternal tolerance by the female and thus attain differences in embryo survival, leading to variation in fertility and, particularly, prolificacy between boars. It is hoped that this line of research will be followed.

Can we use this knowledge for boar semen processing or porcine AI?

Porcine SP from the SRF influences sperm physiology (reviewed by Rodríguez-Martínez *et al*. 2008). On the other hand, SP from solely the PSRF, with a higher amount of bulbourethral gland fluid reduced semen fertility (Iwamoto *et al*. 1992), presumably by the relatively increasing presence of AQN-3 in this portion (Iwamoto *et al*. 1995, see Table 1). Removal of SP (by extension in a buffer and further centrifugation/re-extension) is, therefore, customary during conventional cryopreservation of boar semen. However, SP-exposure is not solely negative. Addition of bulk SP to boar semen was initially considered to ameliorate the effects of sperm processing (from extension to sex-sorting) on sperm survival and the fertility after AI, mostly using empirical approaches (review by Caballero *et al*. 2008 and references therein). Addition of bulk SP (12.5% v/v) to semen for AI attenuated endometrial post-breeding reactions *in vitro* (Rozeboom *et al*. 2001b, Taylor *et al*. 2009) and *in vivo* (Rozeboom *et al*. 1999, 2000), but individuals still reacted when foreign proteins (such as BSA) were present (Taylor *et al*. 2009). Bulk SP-addition (~10%) of bulk SP increased sperm longevity post-thaw (Einarsson & Viring 1973), apparently by preserving membrane stability (Vadnais *et al*. 2005), as it occurs after flow cytometry for sex-sorting (review by de Graaf *et al*. 2008). AI-fertility could be improved adding 25-30% v/v of SP (Crabo & Einarsson 1971, Larsson & Einarsson 1976) but not when ~10% SP was added (Abad *et al*. 2007, Kirkwood *et al*. 2008). Positive effects (motility, viability and *in vitro* fertilising capacity) of adding exogenous SP (5% v/v) to semen during cooling were only seen when the supplement SP was derived from boars judged to have good semen freezability, irrespective of the total SP protein amount or profile (Hernández *et al*. 2007). This evident variation in results when using SP-supplementation is not surprising, since SP-addition was between 5% and 100% v/v, the SP used was either from the whole ejaculate (all fractions), different fractions, or solely from the SRF, from individual boars or from pooled sources, disregarding the large variability in SP-composition seen among sires, owing to age, breed and most likely having a genetic background (Roca *et al*. 2006). Exposure of highly-extended spermatozoa to low doses (1.5 mg/ml) of well identified SP-proteins gave different results. HBP-spermadhesins, which play major roles during sperm transport and fertilisation (Calvete *et al*. 1997, Rodríguez-Martínez *et al*. 1998), were not beneficial for sperm viability *in vitro* (Centurión *et al*. 2003). PSPs, on the other hand, provided substantial protection and maintained fertilising capacity (Caballero *et al*. 2004, 2006, 2008 and references therein). However, such benefits of PSPs could not be shown when handling high sperm concentrations

(as praxis when handling boar semen for AI) nor during cryopreservation (Hernández *et al*. 2007), probably because the spermatozoa had already been in contact with higher amounts of the SRF-SP after collection.

SRF-boar spermatozoa incubated at room temperature in their own SP became more resistant to cold shock (Pursel *et al*. 1973) and had, when pre-incubated for 3-20 h, higher post-thaw survival and fertilising capacity (Eriksson *et al*. 2001). Such incubation post-ejaculation with their surrounding SP is, therefore, nowadays customary in our laboratory, during the temperature decrease from 35–30ºC to room temperature, before first extension with BTS for 30-60 min (Saravia *et al*. 2007a, 2009a).

Removal of the vesicular glands did not affect either the freezability or the fertility of frozen-thawed boar spermatozoa (Moore & Hibbitt 1977), possibly by abolishing the documented induction of chromatin superstabilisation (Kvist *et al*. 1987), or the reduction of sperm binding to the oviduct (Summers & Pena 2008) caused by the vesicular gland fluid. Considering this would place the P1-spermatozoa in advantage, we recently attempted to simplify the customary freezing protocol for boar semen. We used solely P1-spermatozoa, holding them in their "native" SP for 30 min, extended with Lactose-EggYolk (LEY) before cooling to +5°C within 1.5 h, prior to being mixed with LEY+glycerol and orvus-es-paste (LEYGO), packed into MFP, and customarily frozen. The entire procedure, here named "simplified freezing (SF)" lasted 3.5 h compared to the "conventional freezing (CF)" that was used as control procedure, which lasted 8 h (Saravia 2008, Saravia *et al*. 2009b). As controls, spermatozoa from the SRF were compared to P1-spermatozoa. The P1-SF-processed semen showed similar proportions of sperm motility (and kinematics), plasma membrane and acrosome intactness post-thaw, to the SRF-semen customarily frozen (SRF-CF). Mean post-thaw sperm motility ranged from 56% to 69%, the highest percentages being among the P1-SF. Interestingly, there was barely any variation between sires or within-sire for P1-derived variables, in contrast to SRF, independent of the handling method (CF or SF). The reason behind a maintained P1-sperm survival after this shorter freezing process is yet unknown, but the particular milieu of the P1-SP (protein levels and types, lower bicarbonate, zinc and fructose levels etc), together with the use of the cryobiologically well-suited MFP are highly relevant. We are now awaiting for intended fertility trials to determine whether the fertilizing capacity of the processed semen was also preserved by the P1-SP as most *in vitro* results indicate.

Acknowledgments

The excellent technical support of the late Åsa Jansson is posthumously acknowledged. Funding has been provided by FORMAS and the Swedish Farmers' Foundation for Agricultural Research (SLF), Stockholm, Sweden; GERM (04543/07), MCN (AGL 2008-04127) and Séneca Foundation, Murcia; and BFU2007-61563, MCI, Madrid, Spain.

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Molecular kinetics of proteins at the surface of porcine sperm before and during fertilization

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Fertilization is a decisive moment in life and enables the combination of the DNA from two gametes to ultimately form a new organism. The sperm surface, especially the head area, has distinguishable subdomains that are involved in distinct fertilization processes. It is known that the sperm head surface undergoes constant remodelling during epididymal maturation and migration in the male and female genital tract. But intriguingly, the identity, origin and spatial ordering of proteins at the sperm surface that are involved in mammalian fertilization are essentially unknown. This review deals with sperm surface protein modifications that are under somatic cell control. As soon as the sperm is released from the seminiferous tubules it is subjected to these modifications. These surface reorganisations continue until the sperm reside in the fallopian tube where they meet the oocyte and may fertilize it. Most likely, a selective process allows only functionally mature and intact sperm to optimally interact and fertilize the oocyte. Recent data suggest that even the perivitelline fluid is involved in sperm surface remodelling as it contains factors which could facilitate the first penetrating sperm to fertilize the oocyte. In this contribution, the kinetics of proteins at the sperm surface will be overviewed. Better understanding of this would help to design strategies to improve male fertility or to devise novel contraceptives.

Introduction

Although it is still not clear how sperm fertilize the oocyte, the general consensus is that only functionally mature sperm can fertilizes the oocyte and this is somehow accomplished at the surface of the sperm head (Yanagimachi 1994). The sperm is a highly polarized cell with a minimum of cytosol and organelles (Eddy & O'Brien 1994). The sperm head consists of the nucleus that houses the male haploid genome which is highly condensed together with protamines in the late haploid phase of spermatogenesis, and a large secretory granule called the acrosome which is oriented over the anterior area of the sperm nucleus. At the distal part of the sperm head the flagellum sprouts. In the mid-piece of this flagellum mitochondria are spiralled around the microtubules of the flagellum. In the tail part, specific cytoskeletal elements surround the microtubules of the flagellum. The surfaces of the sperm head, mid-piece and the tail parts of the sperm are heterogeneous (Phelps *et al.* 1988, Gadella *et al.* 1995) and reflect the polar distributed organelles that lie under the surface. In particular, the sperm head surface is heterogeneous and at least three subdomains can be distinguished which have separate

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functions in the fertilization process. In general, the sperm has lost many somatic cell features and doesn't house an endoplasmic reticulum, Golgi, lysosomes or peroxisomes. Moreover, the loss of ribosomes disables the sperm's capability to express genes (both transcription as well as translation processes are silenced (Boerke *et al*. 2007)). Furthermore, due to the removal of almost the entire cytoplasm in the testis (Eddy & O'Brien 1994) and during epididymal maturation (Dacheux *et al*. 2005) the sperm has a typical ordering of the remaining organelles and cytoskeletal elements and probably this polar ordering is reflecting into the lateral domains ordering of the sperm's surface (Gadella *et al*. 2008).

Function of sperm membrane domains at fertilization

The subdomains of the sperm head area have diversified functions in the series of processes that are involved in fertilization. The apical ridge area of the sperm head specifically recognizes and binds to the zona pellucida (ZP, van Gestel *et al*. 2007); a larger area of the sperm head surface (the pre-equatorial domain) is involved in the acrosome reaction which results in the release of acrosome components required for ZP-penetration (Yanagimachi 1994, Flesch & Gadella 2000). The equatorial segment of the sperm head remains intact after the acrosome reaction and is the specific area that recognizes and fuses with the oolemma (the oocyte's plasma membrane) in order to fertilize the oocyte (Vjugina & Evans 2008). Although the plasma membrane at the mid-piece and tail of sperm are also heterogeneous, the functions of these surface specialisations are not yet understood (Kan & Pinto-da Silva 1987). It is possible that they are involved in the organisation of optimal sperm motility characteristics. The sperm surface protein organisation is rather complex and, especially in the sperm head, the surface is subjected to constant dynamical changes evoked by the series of changing environments in the male and female genital tract or during sperm handling as is overviewed in the next section.

Sperm surface kinetics

The domained surface of sperm is already apparent in testicular sperm (Eddy & O'Brien 1994), however, the molecular dynamics, involved in the establishment of surface specialisation upon spermatogenesis, is largely unknown. Generally, the polar organization of the extracellular matrix components the cytoskeleton and the cell organelles of the sperm are involved in its heterogeneous surface. In mature spermatids the amount of cytosol is minimal and indeed the observed surface domains mirror the organisation of the acrosome, the post-equatorial nucleus the mitochondria and the fibrous sheath, respectively. Moreover, once liberated in the lumen of the seminiferous tubule, the sperm will start its travel through the male and female genital tract and will meet a sequence of different environments. During this voyage, surface remodelling takes place most likely at any site within the two genital tracts. These constant changes start with stabilization of sperm in the male genital tract and is probably accomplished upon epididymal maturation (Gatti *et al*. 2004, Dacheux *et al*. 2005) and by re- and decoating events induced by the accessory fluids combined at ejaculation (Gwathmey *et al*. 2006, Girouard *et al*. 2008). Beyond surface rearrangements epididymal maturation also results in the removal of cytoplasmic droplets (the cytosolic remnant of the bridges between spermatids) and the acquisition of sperm motility. In the pig, the most important contributions to seminal fluid originate from the seminal vesicles and the bulbo-urethral gland (see Fig. 1). After their deposition in the female genital tract, the reorganization process continues to ensure its further journey in and prepare the sperm surface for its fertilization task. The removal of extracellular glycoprotein coating (release of decapacitation factors) and further remodelling by (cervical)

uterine and oviduct secretions activate the sperm to meet the oocyte (*in vivo* capacitation) (Suarez & Pacey 2006, Rodriguez-Martinez 2007; see Fig. 2). Surface reorganizations are also induced by the interaction of sperm with cumulus cells and remaining follicular fluid components that surround and impregnate the ZP (Getpook & Wirotkarun 2007, Gil *et al*. 2008) as well as in the peri-vitelline space (i.e. the fluid filled space between the ZP and the oolemma) (Barraud-Lange *et al*. 2007a,b). All these changing environments may cause surface remodelling of the sperm and thus may influence its potential to fertilize the oocyte.

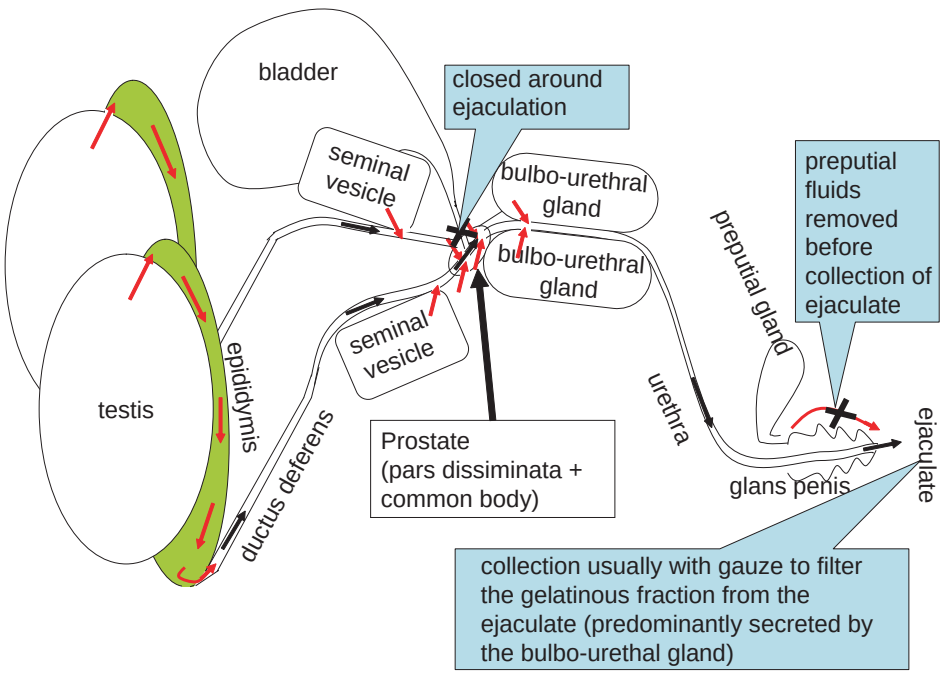


Fig. 1 An overview of the boar reproductive organs. Sperm that leave the testis mature in the epididymis then migrate via the ductus deferens and are mixed with secretions of the indicated accessory sex glands before entering the urethra. Red arrows indicate where the secretions are added to the migrating sperm. The transport of sperm is indicated with black arrows. The epididymis is indicated in green as this is the site where sperm are interacting with epithelia.

The possible mechanisms whereby the sperm surface is altered were reviewed earlier (Gadella 2008) and are summarized in Fig 3. It is very difficult to study sperm surface alterations *in situ*. However, for many mammalian species, including human, specific sperm handling and incubation media have been optimised for efficient *in vitro* fertilization purposes. In general, mammalian sperm are activated in a medium that compares with the oviduct in that it contains several capacitation factors, such as high concentrations of bicarbonate, free calcium ions and lipoproteins such as albumin (Flesch & Gadella 2000). In some species, specific glycoconjugates (Mahmoud & Parrish 1996) or phosphodiesterase inhibitors are added for extra sperm activation (Barkay *et al*. 1984). All strategies are designed to evoke capacitation *in vitro*. This implies that the researcher can observe the relevant sperm surface reorganisation primed under *in vitro* conditions for fertilization. The membrane composition as well as the ordering of membrane components can be compared with control conditions (media without capacitation factors) or with the membrane ordering of sperm at collection time. Sperm can be

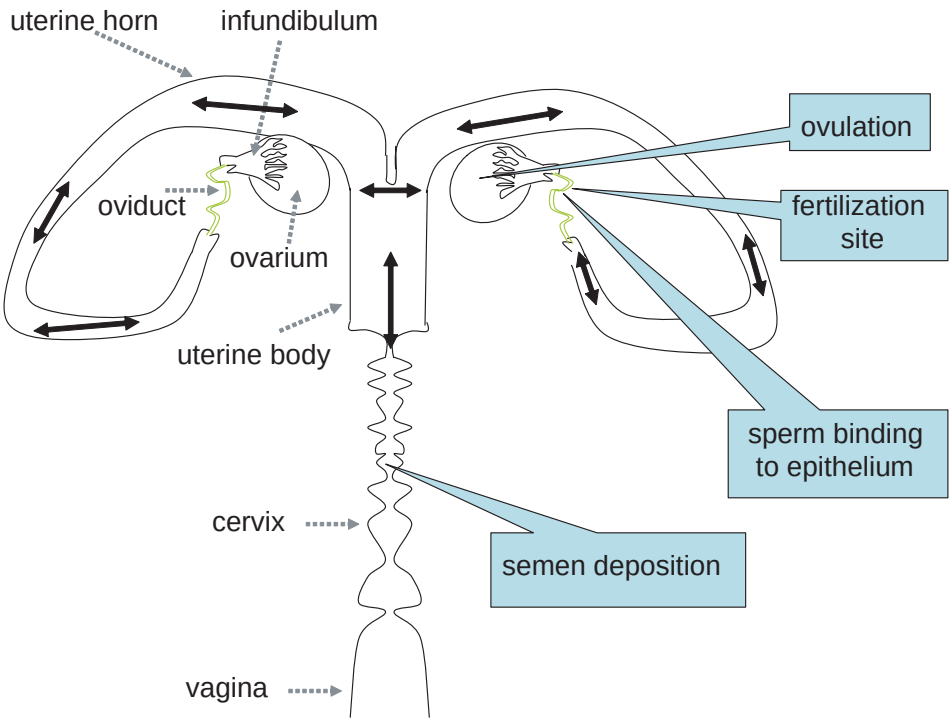


Fig. 2 An overview of the sow reproductive organs. The area where sperm interact with the epithelia is indicated in green (oviduct). Note that the entire female genital tract has been described to be involved in secretory activity. The quantity and composition of the epithelial secretions vary at different sites of the female genital tract and are influenced by the stage of the ovulatory cycle of the sow.

collected from boars where the preputial fluid is removed before collection and the gelatinous fraction is removed by filtering through gauze (see Fig. 1). The collected sperm are washed through a discontinuous density gradient to remove aberrant sperm and non-sperm particles, seminal plasma and factors delaying sperm capacitation (Harrison *et al.* 1996). The pelletted cells have, after being resuspended into *in vitro* capacitation media, been extensively studied for the surface reordering of membrane proteins and lipids in the sperm head (for reviews see Flesch & Gadella 2000, Gadella & Visconti 2006). Most relevant for fertilization is that sperm surface proteins that are entrapped into small lipid ordered domains (lipid rafts) are clustered into the area that is specifically involved in sperm zona binding (van Gestel *et al.* 2005) as well as in the docking of the sperm plasma membrane to the outer acrosomal membrane. It is necessary to stress the importance of the sperm surface reordering and changes in composition of membrane components by diverse extracellular factors. The induced lateral redistribution of membrane components appears to also be instrumental for the assembly of a functional sperm protein complex involved in sperm-zona binding as well as for the zona-induction of the acrosome reaction (Fig. 4; Ackermann *et al.* 2008, Tsai *et al.* 2007, van Gestel *et al.* 2007). Therefore, beyond the composition of sperm surface proteins, one needs to study how these proteins are organized and whether they are functionally complex for their physiological role in fertilization. Moreover, the relatively simply defined *in vitro* capacitation methods probably do not provide all information about sperm surface reorganisation *in utero* or in the oviduct where hormones and other bioactive non-protein components probably regulate sperm physiology

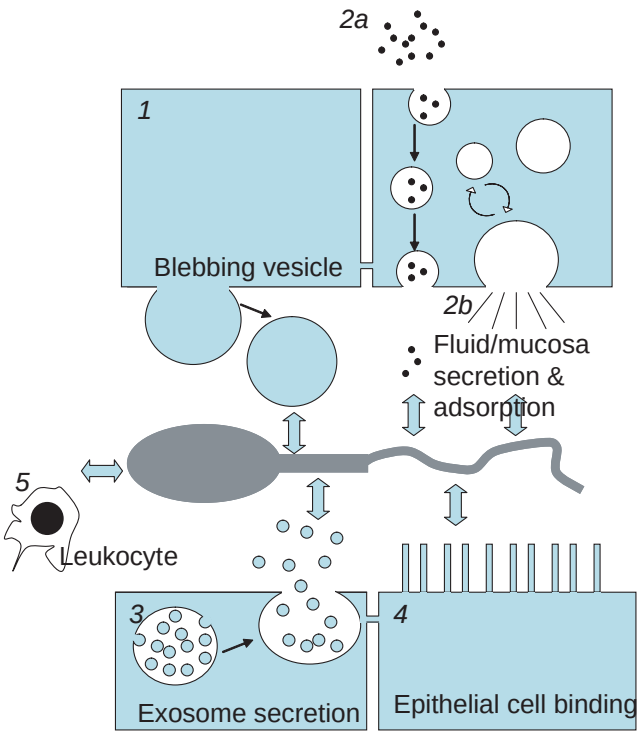


Fig. 3 Hypothetical scheme of possible interactions of male and female genital tract components with the sperm surface. 1. From the diverse epithelia of the male and female genital tract, blebbing vesicles containing cytosol might be released into the genital fluids. Such vesicles may interact and exchange surface components with sperm. It is highly unlikely that such vesicles fuse with the sperm as this would dramatically increase the volume of sperm (which has been reduced maximally in order to obtain an ergonomically designed cell optimally suited for fertilization). Blebbing of vesicles has been demonstrated in the ductus epididymides and the epithelia of the vesicular gland and the prostate of the boar (Rodriguez-Martinez personal communication). The eventual formation of bleb vesicles in uterus and oviductal epithelia is probably an artefact induced by removing these parts from the pig. 2a. Serum components can be released into the genital fluids by transcytosis (Cooper *et al.* 1988). Interestingly, lipoprotein particles may invade the surroundings of sperm and may facilitate exchange of larger particles and the sperm surface. 2b. Fluid phase secretion and adsorption of either fluid or mucosa may directly alter the ECM of sperm. 3. Apocrine secretion of exosomes has been suggested to alter the sperm surface and sperm functioning. Exosomes have been demonstrated to be secreted by the epididymis (epididymosomes) by the prostate (prostasomes) and the uterus (uterosomes) (Gatti et al. 2005, Thimon *et al.* 2008, Griffiths *et al.* 2008). Interestingly, exosomes may provide sperm with tetraspanins, a group of membrane proteins involved in tethering of proteins into protein complexes. Recently the addition of CD9 onto the sperm surface by membrane particles has been described to occur even when sperm reaches the perivitellin space (Barraud-Lange *et al.* 2007 a,b). 4. Sperm interacts with ciliated epithelial cells this has been observed in the epididymis and the oviduct (Gatti *et al.* 2004, Sostaric *et al.* 2008), and probably has a physiological role during *in situ* capacitation. Sperm interactions with other ciliated epithelial cells of the female and male genital tract have not been studied extensively. It is possible that such interactions are important for sperm surface remodelling and for sperm physiology. 5. Semen entering the uterus evokes immunological responses (Schuberth *et al.* 2008) such as the migration of leukocytes into the uterine fluid (Taylor *et al.* 2008) which may affect the surface of sperm but most likely is involved in cleaning the uterine fluid from deteriorated sperm (Woelders & Matthijs 2001). Probably the sperm fraction involved in fertilization occupies the lower parts of the oviduct (where no leukocyte infiltration takes place) at an earlier occasion than required for the responses in the uterus (Rodriguez-Martinez *et al.* 2005).

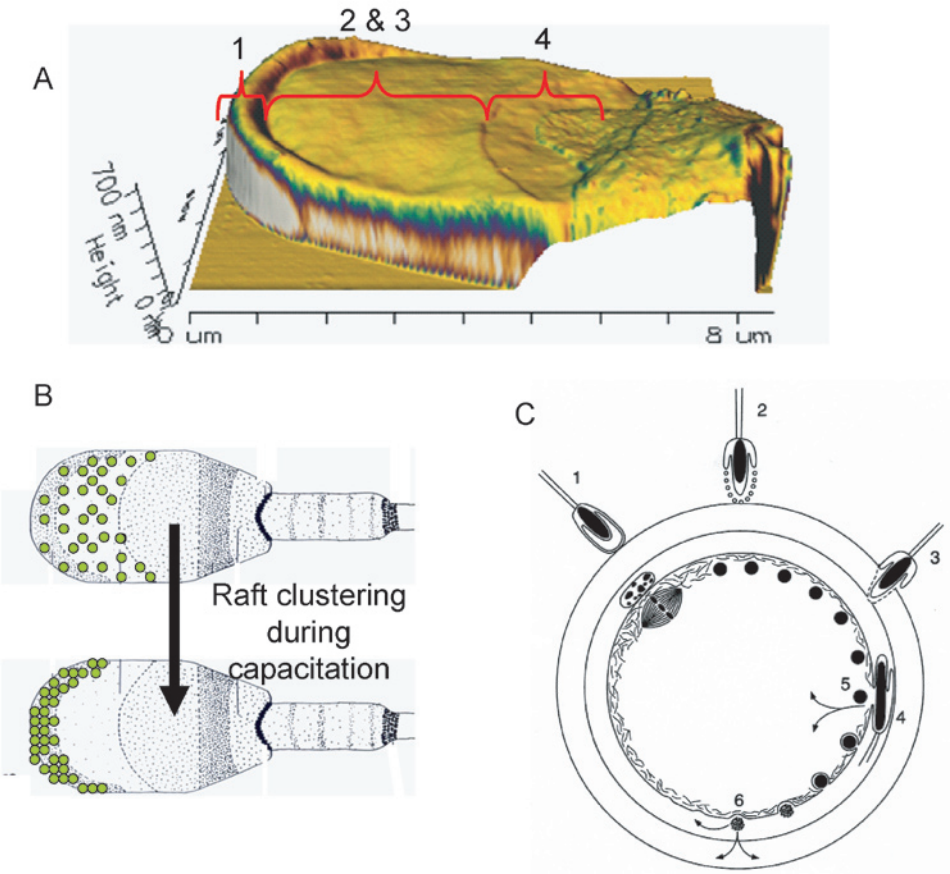


Fig. 4 Protein kinetics at the sperm surface. A. An atomic force microscopic surface view of a porcine sperm head. B. Lipid ordered microdomains at the sperm surface cluster into the apical ridge area of the porcine sperm during *in vitro* capacitation. C. The interactions that the sperm undertakes with the zona pellucida and the oocyte leading to fertilization. 1. zona binding, 2. the acrosome reaction, 3. zona drilling, 4. oolemma binding and fertilization, 5. activation of pronucleus formation and oocyte activation, 6. induction of a blockade for polyspermic fertilization. The numbers indicated in panel A refer to the specific sperm surface area where these interactions do take place.

differently. Nevertheless we have identified a number of proteins involved in zona binding and in sperm plasma membrane docking with the acrosome using *in vitro* capacitated sperm (see Fig. 5)

Origin and identification of sperm proteins involved in the cascade leading to *in vitro* fertilization

Testicular sperm, just released from the Sertoli cells into the lumen of the seminiferous tubules, are equipped with a number of proteins reportedly involved in ZP-binding. At its surface, the sperm has transmembrane proteins belonging to the ADAMs family, initially thought to be involved in the fertilization process and now reported to be involved in sperm-ZP binding.

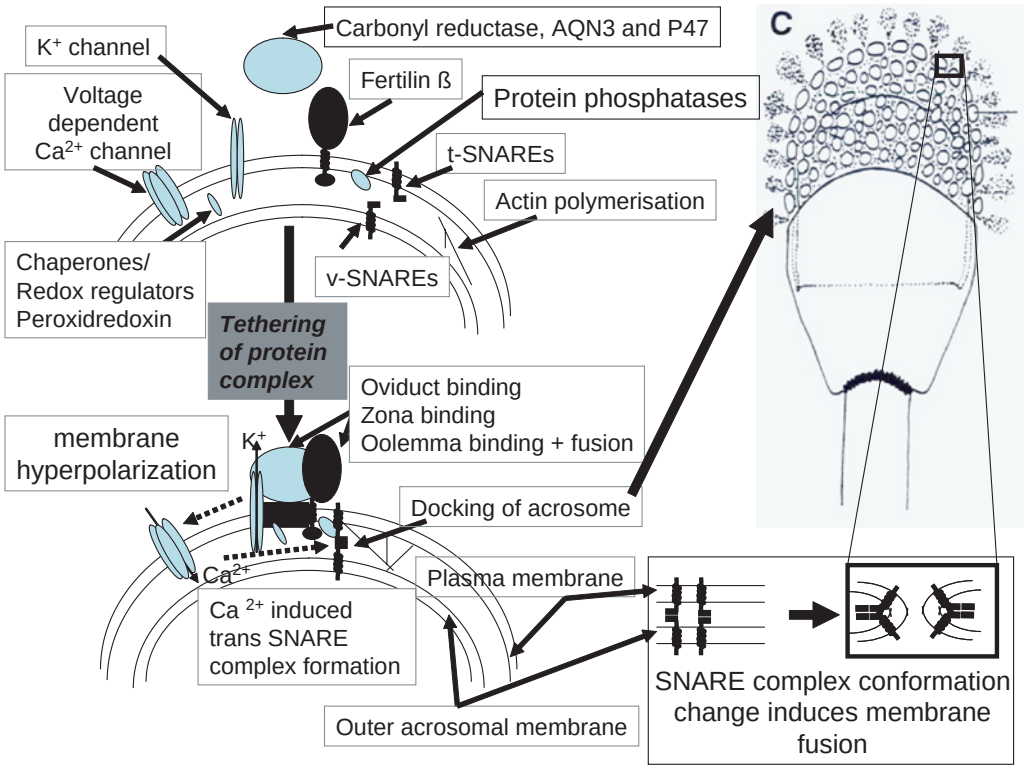


Fig. 5 Left: Summarizing scheme of a hypothetical sperm zona pellucida-binding complex formed during sperm capacitation by raft-induced protein clustering. This may result in a multifunctional protein complex known to play a role in diverse processes leading to fertilization. For explanation and identification of proteins see text. Note that this scheme is based on biochemical and proteomic approaches from epididymal and ejaculated sperm before and after IVF incubations. It is not clear how this picture compares to *in situ* fertilization where the sperm surface may have been functionally remodelled along the female genital tract. Upper right: Multiple membrane fusions involved in the acrosome reaction exclusive for the anterior part of the sperm head surface. Lower right: Molecular impression of trans SNARE fusion complexes that show a Ca^{2+} dependent configuration changes required for these membrane fusions (from Tsai & Gadella, unpublished observations).

ADAM-2, also named fertilin β, has such a function on boar sperm (van Gestel *et al.* 2007). Other testicular sperm proteins such as sperm lysosomal like protein (SLLP1, Herrero *et al.* 2005), and sperm acrosomal membrane proteins (SAMP14 and 32, Vjugina & Evans 2008) and Sp56 (Buffone *et al.* 2008) are involved in secondary ZP-binding as they are localized in the acrosome and only become exposed to the ZP structure after the induction of the acrosome reaction. Some secretory proteins like define CRISP are also involved in sperm-ZP adhesion, sperm oolemma binding or the fertilization fusion (Cohen *et al.* 2007, Da Ros *et al.* 2007, Busso *et al.* 2007a). Interestingly, CRISP 2 is of testicular origin but CRISP 1 and 4 originate from the epididymis (Busso *et al.* 2007b). The exact way in which CRISPs are associated to the sperm surface is not yet known, although CRISP 1 is one of the abundant proteins in epididymosomes (Thimon *et al.* 2008). Epididymosomes are also reported to influence the lipid composition of the sperm surface (Rejraji *et al.* 2006). Other proteins that have been shown to be added

to the sperm surface in the epididymis are P47 or SED1, known to have a role in oviduct and ZP-binding (Shur *et al*. 2006). In porcine, the spermadhesin AQN-3 and carbonyl reductase are also added to the sperm surface (Ekhlasi-Hundrieser *et al*. 2002). Inhibition of hamster carbonyl reductase activity caused a decreased affinity for the ZP while the sperm remained motile and intact (Montfort *et al*. 2002). Even under very stringent detergent conditions AQN-3 was still able to bind to the ZP (van Gestel *et al*. 2007). Proteomic analyses identified proteins that do not have a function directly in sperm ZP-binding but that are associated into a ZP-binding protein complex (van Gestel *et al*. 2007). Some of these proteins are involved in sperm signalling (such as protein phosphatases), while others are involved in the redox balance (peroxiredoxin 5). The latter include a potassium channel which might induce membrane hyperpolarization by K^+ efflux. This hyperpolarization may in turn open voltage dependent Ca^{2+} channels that enable Ca^{2+} dependent processes in the capacitating sperm. An interesting observation was that the major ZP-binding proteins listed above tend to aggregate in capacitating sperm (under IVF conditions) at the surface area involved in ZP-binding i.e. the apical head area (van Gestel *et al*. 2005, 2007) and that this coincided with the attraction of SNAREs (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) involved in the acrosome reaction (Tsai *et al*. 2007). The fact that both outer acrosomal SNAREs and plasma membrane SNAREs were observed after capacitation led to the assumption that the lipid ordered membrane aggregation is a preparative step for the acrosome reaction (as proposed in Fig 4). The identified ZP-interacting protein complex is thus not only involved in sperm-ZP binding but may link this event with preparative steps for the acrosome reaction.

In our approach, we only identified the major ZP-binding proteins of the sperm plasma membrane but did not consider the identification of minor proteins or hidden proteins (covered on 2 D IEF-SDS-PAGE gels by the glycosylated ZP-proteins). Therefore, we cannot exclude that we have missed sperm surface proteins involved in sperm-ZP interactions. Although we did not find any proteins originating from one of the accessory male sex glands (Fig. 1) it cannot be excluded that these glands also play a role in sperm surface modification during ejaculation. For instance, the possibility that prostasomes could fuse with sperm (Burden *et al*. 2006) is interesting but needs to be experimentally validated. In any case, no prostate-derived proteins have this far been described to be involved in sperm-ZP interaction although these fluids are known to have an influence on the sperm surface organisation and protein composition (Russell *et al*. 1984).

Surface modifications at the female genital tract

As mentioned above, far less is known about the contribution of the female genital tract to the ZP-binding processes in mammals (Fig. 2). Sperm reside for hours to days in the cervix, uterus and eventually the isthmic part of the oviduct (depending on species for time and deposition place). In pigs, the semen is normally deposited in the cervix. Although there are no data on the role of cervix epithelia and secretory products, it is well established that small numbers of sperm can be inseminated when deposition is directly in the uterine body (Behan & Watson 2006, Roberts & Bilkei 2005). Moreover, a very low dose of sperm can be used for deep intrauterine insemination (for review see Vazquez *et al*. 2005, 2008). A recent *ex vivo* study using a lectin competition binding assay in uterine segments established that binding of sperm to the uterine epithelium was carbohydrate-dependent (Taylor *et al*. 2008). However, the proteins involved in this binding are not identified and it is also not clear whether this binding is selective, thus allows only small numbers of sperm to migrate deeper in the uterus or that whether this binding allows modification of the sperm surface that is needed in later processes

leading to fertilization. The composition of uterine fluid and its effects on the sperm surface or sperm functioning has been completely neglected by most researchers. The only study on the effects of porcine uterine fluids on sperm reports on lipid modifications in sperm membranes (Evans *et al*. 1987). Porcine sperm contain non-genomic progesterone receptors at the plasma membrane (Jang & Li 2005) and most likely this hormone binding at the sperm surface is part of the *in vivo* sperm capacitation process. Murine uterine fluids have been shown to contain exosomes. Interestingly these particles called uterosomes contain the sperm adhesion molecule SPAM-1 and other GPI-linked proteins that can be exchanged with cauda epididymal sperm (Griffiths *et al*. 2008). It is possible that this exchange improves the sperm's capacity to fertilize the oocyte. However, this possibility is not yet established nor is the presence of uterosomes in the pig species. When semen enters the uterus, it elicits immunological responses which can be observed by a migration of leukocytes (predominantly polymorphonuclear neutrophils; PMNs) into the uterine fluid (Lovell & Getty 1960, Schuberth *et al*. 2008, Taylor *et al*. 2008). It is not clear whether this infiltration will affect sperm that later enter the oviduct but leukocytes clearly reduce the amount of sperm that will enter the oviduct by phagocytosis (Woelders & Matthijs 2001). It is also uncertain whether phagocytosis is selective (for aberrant sperm) or only unselectively depletes the amount of sperm that migrate further to the oviduct, which is free of leukocytes and the site where sperm are capacitated *in vivo* in order to fertilize the oocyte (Suarez 2008). In pigs the role of leukocyte infiltration on fertilization is questionable as the vanguard cohort of sperm that occupy the oviduct have been shown to reside and bind to the oviduct epithelia of the isthmic region within 30 minutes after insemination (Rodriguez-Martinez *et al*. 2005) which is long before the invasion of PMNs into the uterus.

A number of reports have shown that fluids from the oviduct stimulate sperm capacitation and induce hyperactivated sperm motility. One of the factors involved in this sperm activation is bicarbonate, which is also commonly used for IVF treatments (Rodriguez-Martinez 2007). Oviduct-specific glycoproteins (OSG) as well as osteopontin have been shown to support fertilization in the cow and are secreted by the oviduct (Killian 2004). A sperm binding glycoprotein from the oviductal fluid has recently been shown to induce porcine sperm capacitation (Teijeiro *et al*. 2008). The lower part of the oviduct is considered to function as the sperm activation site, making sperm capable to meet and fertilize the oocyte. In the isthmus, small numbers of sperm are bound and become capacitated *in vivo,* there, sperm await to be released at ovulation, to migrate to the upper part of the oviduct (the ampulla) and to fertilize the passing oocyte(s) (for review see Suarez 2008). To this end, the oviduct epithelia and fluids contain sperm binding factors as well as sperm releasing factors that are causing sperm adhesion and release in the correct timing around ovulation (for binding and release characteristics in the bovine oviduct see also Sostaric *et al*. 2008). Most likely, spermadhesins such as AQN-1 are involved in the formation of the oviductal sperm reservoir as they are involved in sperm binding to this specific epithelium (Ekhlasi-Hundrieser *et al*. 2005). Note that some spermadhesins (DQH, Manaskova *et al*. 2007, AQN-3, AWN, P47, Gadella 2009) are added to the sperm phase during epididymal sperm maturation, others (like PSP-I /PSP-II subunits) are partialy added in the testis (Garcia *et al*. 2009). Interestingly, recently oviduct-specific glycoproteins have been shown to modulate sperm-ZP interaction and to control the polyspermic fertilization rates in pigs (Hao *et al*. 2006, Coy *et al*. 2008). Polyspermy is a well recognised problem in pig IVF and prevention of this unwanted phenomenon may be accomplished by additions of oviductal fluid components to the IVF media. Oviduct epithelial annexins have been suggested to immobilize bovine sperm (by binding bovine sperm proteins BSP; Ignotz *et al*. 2007). Annexin A2 has also been proposed to be involved in sperm-oviduct binding in the sow (Teijero *et al*. 2009). In the bovine species this interaction is reversed by oviductal fluid factors such as catalase (Lapointe &

Sirard 1998). Catalases that secreted from oviduct may protect sperm from peroxidation damage as is demonstrated in the cow (Lapointe *et al.* 1998). The interplay of varying glycoproteins at the surface of sperm and oviduct epithelia or oviductal fluid as well as the varying amounts and composition of glycosidases probably orchestrate proper sperm activation just around ovulation in the pig (Carrasco *et al.* 2008, Töpfer-Petersen *et al.* 2008). However, the effect of oviduct and uterine proteins on sperm-ZP binding as well as their putative association to the sperm surface is not yet established. Of course it is possible that secreted products of the female genital tract enhance sperm-ZP binding and that more protein candidates from the female genital tract should be added to the surface of the ZP-interacting sperm. In this respect, also the ZP itself, besides being a binding target, may add proteins to the sperm surface. The cumulus cells and the ZP were impregnated with follicular fluid and remnants of this fluid probably will remain attached to the cumulus oocyte complex. For instance, different growth factors and extracellular matrix components have been involved in interactions of sperm to the cumulus oocyte complex (for review see Einspanier *et al.* 1999). Sperm that interact with these structures may respond to these fluid components like they do to extracted follicular fluid (hyperactivated motility, Getpook & Wirotkarun 2007, Gil *et al.* 2007). It should also be mentioned that the oviductal fluid has also been found to be supportive for early embryonic development of the fertilized pig oocyte (Hao *et al.* 2008, Lloyd *et al.* 2009).

Finally, an interesting observation has recently been made showing that membrane remodelling occurs after the acrosome reaction when sperm reach the perivitelline space but before the fertilization fusion. Within the perivitelline space, membrane fragments containing CD9 are added to the sperm surface (Barraud-Lange *et al.* 2007a,b). If correct, this would demonstrate the "bestowment principle" that may exist in mammalian reproduction as the oocyte facilitates the first incoming sperm in the perivitelline space to fertilize by transferring functional tethering proteins to the surface of sperm cells. It remains to be established whether such process also enables oviductal sperm to bind to the ZP. It may also be mentioned that sperm proteins involved in oolemmal binding and the fertilization fusion are reported for mouse and human sperm (Ellerman *et al.* 2006, Vjugina & Evans 2008) but data for boar sperm are scarce.

Conclusions

The continuous sperm surface remodelling that occurs during sperm transit from the rete testis towards the oviduct and possibly even within the peri-vitelline space and the physiological role of this surface kinetics is, to a large extent, *terra incognita*. The identification of different complex proteins systems within the male and female genital tract is promising; however, their role and function in events associated with sperm-oocyte interactions are still difficult to test (Boerke *et al.* 2008).

Wild-type and genotypically knock-out mice have already been used to validate the function of certain proteins proposed to play part in sperm ZP-binding, the acrosome reaction, oolemma binding as well as oolemma-sperm fusion (for an overview see Vjugina & Evans 2008). Such approaches delivered some valuable information on the potential impact of certain proteins involved in mammalian fertilization. However, the molecular intervention of transcription and translation in gametes is hampered by the fact that in sperm both processes are silenced and in the oocyte almost all mRNA is stored for post-fertilization translation. Therefore, it is possible to intervene with molecular processes involved in either gametogenesis or post-fertilization development rather than the molecular processes required for gamete interaction and fertilization. An example of this is a mutation in spermatogenic cells of the syntaxin2/

epimorphin gene. The protein translated from this gene plays, according to our research, a role in the acrosome reaction (Tsai *et al.* 2007) but the mutation causes a defect in the transition from spermatocyte to spermatids. Thus, a phenotypic knock-out of syntaxin 2 cannot be used to study the effect of this protein on fertilization simply because the knock-out phenotype fails to produce sperm (Akiyama *et al.* 2008). Furthermore, in many cases homologous genetic recombination applications have shown that knocking out the expression of phenotypic factors that were previously believed to be essential for fertilization were found to be dispensable to this process (Okabe & Cummins 2007). This could be partly explained by the fact that biological systems contain redundancies and compensatory mechanisms and both processes are believed to play a prominent role in the evolution of gamete interaction and thus in speciation (Turner & Hoekstra 2008, Herlyn & Zischler 2008). On the other hand, future outcome from genomic approaches devoted to study the molecular mechanisms involved in mammalian fertilization may also indicate that a substantial modification of classical fertilization models is required (Okabe & Cummins 2007).

The production of knock-out pigs is very expensive and time consuming but it is possible to isolate specific structures of the male or female genital tract from pigs. Gene specific silencing of protein translation is possible with interference RNA technology. In this way the specific role of proteins in fertilization and in sperm surface kinetics can be studied. The big problem is that the treatment of explants and cells cause dedifferentiation and alter their interaction with sperm (Sostaric *et al.* 2008).

Therefore, after leaving the testis, sperm are subjected to a series of events causing continuous sperm surface remodelling, with specific sperm surface protein kinetics, relevant for its final fertilization task. Difficulties in molecular intervention approaches as well as the difficulties to study sperm surface remodelling *in situ* are nowadays compensated by high throughput proteomic technologies that allow identification of low abundant proteins. In combination with off-gel full LC-MS/MS platforms, sperm surface isolation and purification technologies, isobaric tagging strategies for peptides (Ernoult *et al.* 2008, Zieske 2006) will enable us to cover the full sperm surface proteome (Gadella 2009) in the near future, when the full pig genome and its annotation will become accessible to the public (expected early 2010, Churcher, personal communication).

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Optimal characteristics of spermatozoa for semen technologies in pigs

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Despite the great potential of sperm technologies such as sperm cryopreservation and sperm sex sorting for the improvement of different aspects of swine production, artificial insemination with fresh or stored semen is currently the only sperm technology used at a commercial scale in the pig industry. The lower reproductive performance associated with the use of these sperm technologies is the reason for such limited use. Since optimal characteristics are required for successful application of frozen-thawed and sex-sorted boar spermatozoa, the present paper summarises the value of the current available methods for their functional assessment as well as the effects of these technologies on boar sperm functionality. In addition, strategies developed to reduce sperm damage and improve the yields of both sperm technologies in swine production are also reviewed with particular attention to the contributions of the authors.

Introduction

Over the past decade, considerable interest has been directed toward the development and improvement of assisted reproductive technologies (ARTs) for swine. Although some ARTs, such as embryo transfer, *in vitro* production of embryos, cloning and transgenic technology, have been successfully attempted, the current low level of efficiency limits their use in applied production systems. However, ARTs in which only spermatozoa are handled may be applicable for the pig industry in a short period of time (Martinez *et al*. 2005). Certain sperm technologies, such as cryopreservation of male gametes and sperm sorting for gender pre-selection, might be of great benefit to the swine industry for improving the efficiency of production. Nevertheless, the low reproductive performance associated with the application of these technologies limits their current use.

Many factors come into play in determining the success and spread of sperm technologies. One of these factors is the fertilising ability of the spermatozoa. Handling spermatozoa during technical procedures is usually associated with a decrease in fertilising ability.

Mammalian fertilisation is a complex process involving a precisely programmed set of events during which the spermatozoa acquire their fertilising ability through a series of molecular and cellular changes that are pre-requisite for successful interaction with a female gamete (Petrunkina *et al*. 2005, Waberski *et al*. 2005). To be able to carry out these tasks, the spermatozoa must maintain many physical and biochemical parameters that allow it to bind to the zona pellucida and penetrate into the oocyte cytoplasm. In addition, the spermatozoon must have an intact nucleus capable of proper de-condensation, nuclear reorganisation and genetic performance

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in order to maintain proper zygotic and embryonic development (Graham & Moce 2005). Spermatozoa are cells with a limited biosynthetic capability (Amann *et al*. 1993), and thus their function can be influenced dramatically by the external environment. Long periods of storage, cryopreservation or sperm sorting by flow cytometry can have a detrimental impact on its function and fertilising ability (Maxwell *et al*. 1998, Maxwell & Johnson 1999, Martinez *et al*. 2001, 2005, Roca *et al*. 2004, 2006, de Graaf *et al*. 2008).

In many cases, if not all, the ultimate objective for the practical application of sperm technologies is to obtain optimal reproductive performance. For this reason, special attention should be given to improve the functionality and fertilising ability of the treated spermatozoa.

Since optimal characteristics are required for successful application of frozen-thawed and sex-sorted boar spermatozoa, the present paper summarises the value of the current available methods for their functional assessment as well as the effects of these technologies on boar sperm functionality. In addition, strategies developed to reduce sperm damage and improve the yields of both sperm technologies in swine production are also reviewed with particular attention to the contributions of the authors.

Current procedures to evaluate optimal characteristics of boar spermatozoa

Sperm technologies such as cryopreservation or flow cytometric sex sorting require extensive *in vitro* handling, which eventually induces dramatic changes in sperm survivability. Extension, cooling, laser illumination, freezing and thawing, among others, not only cause cell death but also subtly damage most of the spermatozoa in the surviving population, leading to reduced sperm lifespan both *in vitro* and *in vivo* (Maxwell *et al*. 1998). These procedures involve different degrees of change in sperm function following physical and biochemical damage to sperm membranes, DNA, cellular signalling mechanisms, and later on are manifest in alterations to the fertilising capacity of these cells.

Spermatozoa in an ejaculate vary in the integrity of the attributes needed for successful fertilisation as well as in their capability to overcome all the events leading up to fertilisation (Rodriguez-Martinez 2007). Similarly, spermatozoa subjected to technologies such as cryopreservation or sex sorting respond differently to the treatment involved in these procedures. Such variation among cells within a given ejaculate complicates the use of laboratory evaluations as accurate estimation tools (Holt & Van Look 2004). Moreover, it may be assumed that standard seminal parameters like motility, morphology, and sperm concentration are insufficient to indicate subtle changes in sperm cells subjected to these treatments. Detection of such changes is currently an issue of great importance (Peña *et al*. 2007, Petrunkina *et al*. 2007). Therefore, functional assessment analysis should be used to determine minimal and early physical and biochemical damage along with failed metabolic activity in treated sperm samples.

These functional tests include sperm kinematics (assessed by computer-assisted analysis), evaluation of early changes in sperm membranes, evaluation of damage to the sperm chromatin structure, and DNA integrity and sperm-oocyte or sperm-oviduct interactions (Holt *et al*. 2007, Silva & Gadella 2006, Petrunkina *et al*. 2007).

Traditionally, computer-assisted sperm analysis (CASA) systems have been introduced for routine motility evaluations at the commercial level due to their ability to objectively identify sperm motility and motions parameters (Didion 2008). However, in common practice, the use of the mean values of motility descriptors simplifies the motility analysis but does not take into account internal variability, thus providing misleading information about sperm quality, which is not revealed by the usual statistical measures (Holt *et al*. 2007). New approaches to the

analysis of data provided by CASA systems involve the identification of sperm subpopulations by means of clustering algorithms. Consequently, these systems offer new insight into the true structure of sperm populations under different experimental conditions and therefore on the relationship between motility, sperm quality and, ultimately, fertility (Cremades *et al*. 2005; Holt *et al*. 2007. See Fig. 1). Recently, by means of CASA systems, it has been possible to find computerised systems to objectively estimate sperm head dimensions with a similar statistical approach. Computer-assisted sperm morphometry analysis (CASMA) can be used to distribute spermatozoa in morphometrically distinct subpopulations within the ejaculate and to disclose their relationship with sperm quality (Saravia *et al*. 2007).

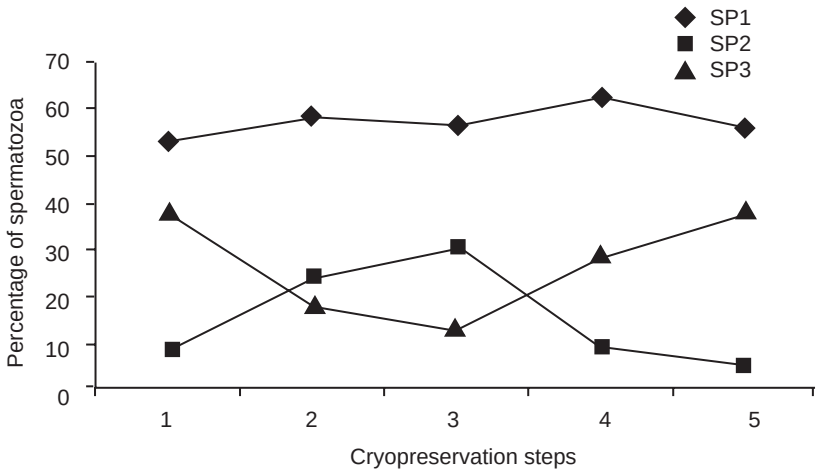


Fig. 1 Behaviour of different motile sperm populations (SP1: cells with progressive and vigorous movement; SP2: progressive cells only; SP3: vigorous cells only, hyperactive-like) identified within one ejaculate by using CASA and statistical analysis during a cryo-preservation procedure. The graphic shows how only those spermatozoa belonging to SP1 are able to maintain optimal motility levels during the freezing and thawing processes. The percentages of motile spermatozoa were recorded at five steps during a conventional boar semen cryo-preservation procedure: 1: Immediately after arrival of the BTS-extended sperm-rich ejaculate fraction (22ºC) at the Laboratory of Andrology. 2: After centrifugation and extension of the sperm pellet with LEY extender at 17ºC. 3: After adding the LEYGO extender at 5ºC. 4: After thawing and re-extension with BTS (1:1, vol/vol) and holding in a water-bath at 37ºC for 30 minutes. 5: After holding the re-extended thawed semen in a water bath at 37ºC for 150 minutes (modified from Cremades *et al*., 2005).

During recent years, special attention has been paid to plasma membrane integrity since spermatozoa with disrupted plasma membranes are considered unable to fertilise. Plasma membrane integrity is traditionally assessed after staining cells with membrane-permeable dyes such as SYBR-14, combined with propidium iodide as a counter-stain (Silva & Gadella 2006). Although checking sperm viability is a widespread and highly useful technique for primary sperm quality screening, it cannot discern sub-lethal changes in the sperm cells.

Male gamete structures are extremely sensitive to technological procedures. During these processes, they can suffer a series of events similar to those taking place during sperm capacitation, or as more recently hypothesised, premature ageing or so-called apoptosis-like changes (Peña *et al*. 2009). In either case, spermatozoa affected by these destabilising events

undergo membrane changes that apparently lead to cell death (Peña *et al*. 2009). Some of these changes are linked to early membrane destabilisation and can be evaluated by determining the degree of lipid packing disorder with the lipid dye merocyanine-540 (Harrison & Gadella 2005, Guthrie & Welch 2005) or by investigating membrane asymmetry through changes in the position of certain phospholipids (using Annexin-V) such as phosphatidylserine, which may be trans-located from the inner to the outer leaflet of the plasma membrane after being disturbed (Peña *et al*. 2003). Changes in membrane permeability imply destabilisation of membranes and can be monitored using the impermeant nuclear dye YO-PRO-1. YO-PRO-1 is used to detect early changes in membrane permeability, alone or in combination with other stains (Peña *et al*. 2005).

Cell volume is another informative method for the detection of functional membrane changes in live cell populations. The volume regulation capability of spermatozoa is of considerable importance for sperm survival during several processes, including cryopreservation (Petrunkina *et al* .2005, 2007).

Mitochondrial integrity and functionality are clearly related to sperm viability and motility, and they also play an important role in early sperm changes during technological treatments (Peña *et al*. 2009). Several dyes have been proposed to evaluate the functional status of these organelles, mostly measuring mitochondrial inner membrane potential, such as JC-1, Mitotracker Green or Mitotracker Deep Red (Guthrie & Welch 2006, Bussalleu *et al*. 2005, Hallap *et al*. 2005).

Many other analyses can be performed to investigate sperm functionality, such as measuring the intracellular calcium concentration (Caballero *et al*. 2009), stimulating protein tyrosine phosphorylation (Piehler *et al*. 2006), or searching for the presence of activated caspases (Moran *et al*. 2008), intracellular reactive oxygen species (Guthrie & Welch 2006) or other signalling pathways, all of them intimately related to detrimental changes in sperm functionality.

Several studies have shown that spermatozoa with normal function, including motility, viability, fertilisation rates and initial cleavage rates can have damaged DNA that will result in reduced fertility and an increase in early pregnancy loss (Bathgate 2008). Changes in sperm chromatin structure and DNA integrity have been widely related to infertility in several mammalian species, and it is recommended that chromatin integrity should be studied as an independent complementary parameter for a better assessment of sperm quality. Sperm chromatin structure assays (SCSA), comet assays and the sperm chromatic dispersion test are some of the techniques currently applicable to evaluate boar sperm DNA fragmentation (Fraser & Strzezek, 2005; Hernandez *et al*., 2006; Lopez-Fernandez *et al*., 2008)

In recent decades, laboratory assessments of either fresh or processed semen have evolved to become more detailed and have moved from the diagnosis of sperm attributes to determining the fertilising potential of the sample using *in vitro* tests such as zona pellucida (ZP) and oviductal epithelium binding assays, *in vitro* penetration assays or *in vitro* maturation/*in vitro* fertilisation/ *in vitro* production (IVM/IVF/IVP) systems (Rodriguez-Martinez 2003).

In 2005, Waberski *et al*. reviewed the procedures developed to evaluate the functional competence of boar spermatozoa by analyzing *in vitro* sperm-oocyte and sperm-oviduct interactions. In the former case, the ability of spermatozoa to interact with the ZP is evaluated by means of a sperm-zona binding assay, a sperm-zona penetration assay or an *in vitro* fertilisation assay, whereas in the latter case, the main aim is to assess multiple functions of the sperm plasma membrane that must be carried out before fertilisation.

Experiments conducted in our laboratory demonstrate that the evaluation of penetration rates and the average number of spermatozoa per penetrated oocyte using zona intact pig oocytes at the germinal vesicle stage (immature oocytes) in a homologous *in vitro* penetration (hIVP) assay provides a useful way to accurately predict male fertility (Martínez *et al*. 1993,

1996). This assay is simple and rapid as it avoids the *in vitro* maturation of oocytes. Other parameters such as pronuclear formation and early embryonic development can be obtained using IVM/IVF/IVP systems. However, the problems of polyspermic penetration of oocytes and the poor quality of the embryo culture medium remain in the porcine IVF system, resulting in low-efficiency production of viable embryos and making their use for the prediction of fertility difficult (Gil *et al*. 2005).

The sperm-oviductal epithelium binding assay is emerging as an important tool for evaluation of sperm functionality since it evaluates plasma membrane quality for the multiple functions necessary prior to the fertilisation process (Waberski *et al*. 2005). Since binding to the oviductal epithelium stabilises the spermatozoa and enhances their survival in a hostile environment, it is clear that the ability of sperm cells to bind to the oviduct is a very important attribute intimately related to fertilising ability (Petrunkina *et al*. 2007). Along this line, several studies performed using this assay have demonstrated that the binding index was found to be lower in sperm samples coming from sub-fertile sires compared to that of fertile sires (Petrunkina *et al*. 2007, Waberski *et al*. 2005).

How sperm technologies affect boar sperm fertilising ability

Artificial insemination (AI) could be considered the most relevant sperm technology used worldwide in the reproductive management of domestic mammals (Weitze 2000). The manipulation of boar semen for AI generally involves two main steps consisting of dilution and, in most cases, storage of extended spermatozoa. Although this protocol is less stressful for the spermatozoa, some detrimental effects on their fertilising ability may be induced, especially if long storage periods are required (Vazquez *et al*. 1998, de Ambrogi *et al*. 2005).

The situation is different when sophisticated sperm technologies are applied to the boar gametes. Special characteristics of the boar sperm plasma membrane composition determine the extreme sensitivity of spermatozoa to cooling and freeze-thaw processes as well as to procedures that involve high dilution rates such as sex sorting by flow cytometry (de Graaf *et al*. 2008, Vazquez *et al*. 2009). This peculiarity of the male pig gamete is likely one of the most important limiting factors in the commercial use of these technologies for swine production.

Cryopreservation of spermatozoa involves several steps (temperature reduction, cellular dehydration, freezing and thawing) that may compromise sperm viability and normal function (Medeiros *et al*. 2002). Detrimental effects of the cryopreservation procedure include damage to the sperm plasma membrane and other organelles as a result of osmotic stress, cold shock and intracellular ice formation (Guthrie & Welch 2005).

Over the years, great efforts have been dedicated to reducing the negative effects of freezing procedures on boar spermatozoa by introducing several improvements in sperm cryopreservation protocols (Eriksson *et al*. 2001, Carvajal *et al*. 2004, Saravia *et al*. 2005). As result of these investigations, sperm survival rates above 50% have been achieved along with promising fertility results (Roca *et al*. 2006).

Despite the improvements and the hopeful results, post-thaw viability, lifespan and fertility of cryopreserved boar spermatozoa are still reduced as a consequence of cellular injuries that arise during cryopreservation procedures (Medeiros *et al*. 2002) and remain at sub-optimal levels (Sancho *et al*. 2007). These facts together with the great variability in sperm freezability among boars and the large number of spermatozoa required per AI dose has discouraged the extensive use of frozen semen in commercial swine production (Johnson *et al*. 2000).

As noted above, the short lifespan of boar spermatozoa following cooling and freeze-thaw processes is one of the major drawbacks to the successful application of cryopreserved boar

spermatozoa in commercial AI programmes, and it explains the low fertility results obtained (Roca *et al*. 2006). Premature capacitation (Watson 2000) and/or premature ageing (Peña *et al*. 2009) suffered by post-thaw spermatozoa have been pointed out as the main cause for the reduction in sperm lifespan, and thereby in fertilising capability. In either case, the reduction has been related to alterations in the cholesterol content of sperm membranes as well as with excessive formation of reactive oxygen species (ROS) as a consequence of the cryopreservation technique (Roca *et al*. 2004).

As occurs in cryopreservation procedures, flow cytometric sorting of X- and Y- chromosome bearing boar spermatozoa involves several steps that can induce stress, damage or even kill spermatozoa. These steps include DNA staining, exposure to the UV-laser beam, high working pressures, high dilution rates in different extenders (which may involve changes in osmolarity and pH), centrifugation to concentrate the cells and in some cases the further stress of the cryopreservation procedure itself (Bathgate, 2008, Vazquez *et al*. 2008a, Garcia *et al*. 2007). Both the physical effect of the sorting procedure and the high dilution rate cause alterations in the functionality of sorted boar spermatozoa (Parrilla *et al*. 2005). However, the extensive dilution of spermatozoa (from approximately 800 x 10^6 in a sperm-rich fraction to a final concentration of sorted sperm of around 1 x10^6 according to Bathgate, 2008) has been identified as the main determinant factor in the reduction of sorted sperm functionality. This fact might be due to the removal of seminal plasma factors required for the maintenance of sperm plasma membrane integrity and sperm functionality (Maxwell & Johnson, 1999, Centurion *et al*. 2003, Caballero *et al*. 2004, 2008).

Therefore, as a result of all the manipulations needed for sperm sorting by flow cytometry, a stressed cell population with destabilised plasma membranes and showing capacitation-like changes is obtained (Maxwell & Johnson 1999). This status renders the spermatozoa immediately able to fertilise an oocyte whether under *in vivo* or *in vitro* conditions without the need to perform further capacitation treatments (de Graaf *et al*. 2008). Detrimental effects caused by this premature capacitation status include reduced viability, lower fertilising ability and compromised storage capability after sorting (Maxwell & Johnson 1999, Parrilla *et al*. 2005, see Figs 2 and 3). From a practical point of view, the reduction in the lifespan of sorted boar spermatozoa is an important drawback for the application of sexed semen in pig farming because, in most cases, great distances must be travelled between sperm-sorting laboratories and AI facilities (Vazquez *et al*. 2009).

Another important disadvantage of sorting technology when applied to AI in pigs is the reduction in fertility parameters with regard to farrowing rates and litter size (Vazquez *et al*. 2003, Garner 2006, Johnson *et al*. 2005). Whether this alteration in the reproductive parameters is a consequence of the low number of sorted sperm used – which may be unable to produce the minimum number of embryos necessary to maintain the pregnancy – or is due to alterations in sorted sperm DNA – resulting in the poor developmental potential of embryos – remains unclear. Further investigations into this phenomenon are clearly necessary (Bathgate 2008, Vazquez *et al*. 2009).

Strategies for optimising the characteristics of spermatozoa subjected to technological treatments

The handling of spermatozoa for sperm cryopreservation and sperm sexing by flow cytometry usually includes the concentration and the dilution of the spermatozoa, processes that produce a detrimental effect on the sperm cells. Consequently, new approaches are needed to improve these steps.

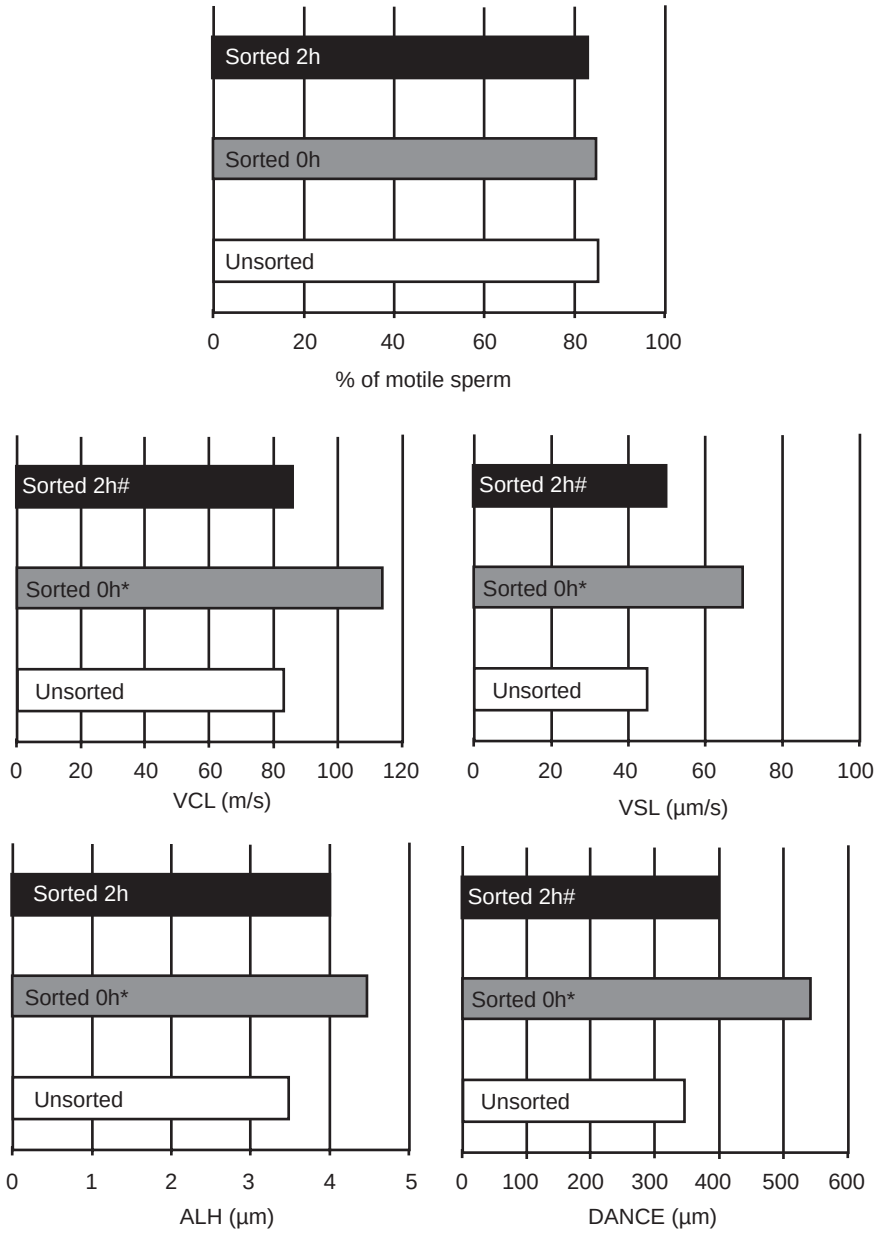


Fig.2 Changes in motion parameters in unsorted and sorted spermatozoa analyzed by CASA at 0 (sorted 0h) and 2 hours (sorted 2h) of storage after sorting in the presence of seminal plasma. The percentage of motile sperm was maintained at around 80% up to 2h of storage. Among the other variables explored, sperm velocity (VCL or VSL) and angularity parameters (ALH and Dance) increased significantly ($p < 0.05$) immediately after sorting (sorted 0h) only to return to unsorted sperm values after 2h of storage in the presence of seminal plasma. VCL: Curvilinear velocity; VSL: Straight-line velocity; ALH: Amplitude of lateral head displacement; Dance: Curvilinear velocity multiplied by amplitude of lateral head displacement. * $P < 0.05$ compared to the unsorted sample, # $P < 0.05$ compared to sorted 0h (modified from Parrilla *et al.*, 2005).

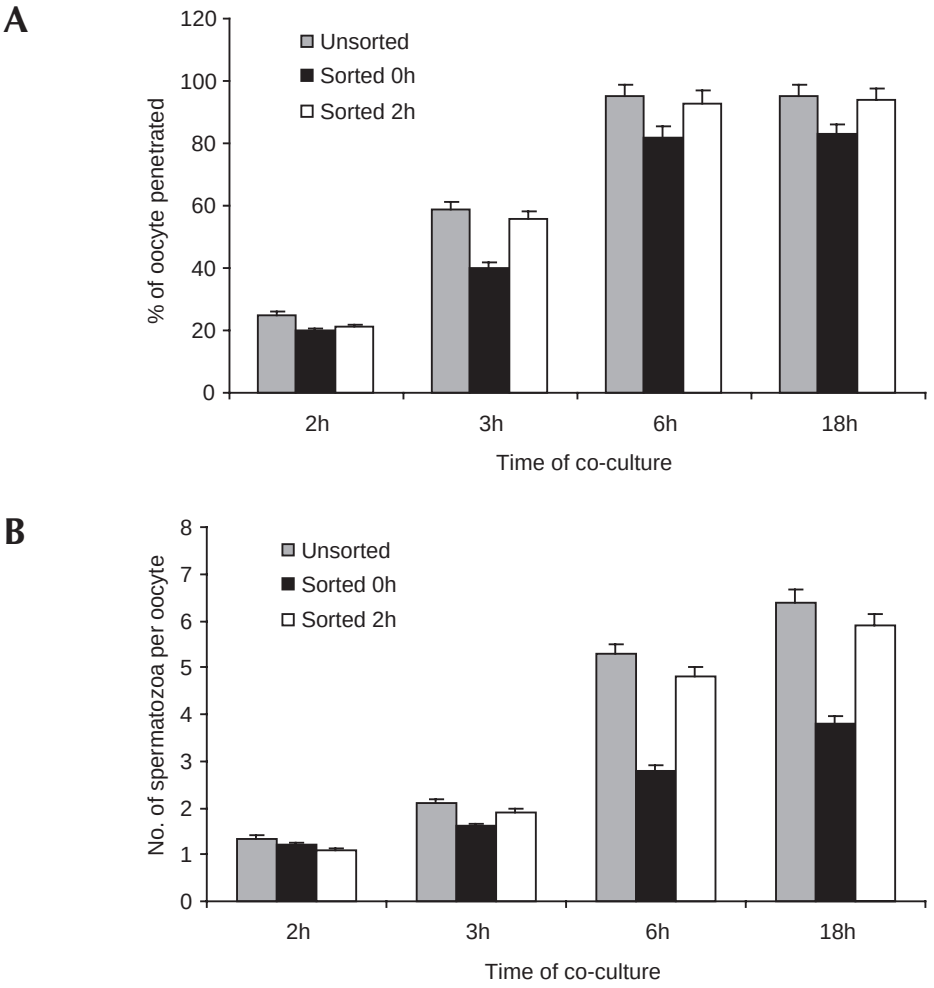


Fig. 3 Effects of flow sorting and flow sorting and storing in the presence of seminal plasma (SP) on time-course (2, 3, 6, 18 hours) penetration rates (A) and the number of spermatozoa per oocyte (B). Sorted 0h: Spermatozoa sorted and co-incubated just after sorting; Sorted 2h: Spermatozoa sorted and stored for 2h in presence of SP. The histogram shows how spermatozoa sorted and stored for 2h in the presence of SP had higher penetration rates and number of sperm per oocyte. This difference may be because the flow sorting procedure induces a scrambling in the plasma membrane components, affecting glycoproteins related to oocyte recognition and penetration. Incubation with SP for 2h might reorganize the plasma membrane, restore the fertilizing capability and increasing the percentage of penetrating spermatozoa. * indicates significant differences ($p < 0.05$) between histogram bars (modified from Parrilla *et al*., 2005).

Methodologies for sperm concentration

Although centrifugation is the usual procedure used for concentration of spermatozoa, it has been described as a process with detrimental effects on sperm cell membranes, reducing its

fertilising capacity (Katkov & Mazur 1998, Carvajal *et al*. 2004). Moreover, the induced damage appears to be worse when centrifugation is applied to "weak" spermatozoa, such as those that have been sex-sorted (Garcia *et al*. 2007). Sedimentation, as an alternative to centrifugation, can be use to concentrate boar spermatozoa, improving the *in vivo* fertilising ability of sex sorted boar spermatozoa (Garcia *et al*. 2007).

Implementation of additives to the sperm extenders

Improving the sperm environment through the use of an optimal medium is a principal strategy to overcome the low sperm quality obtained after application of sperm technologies. Addition of seminal plasma, certain proteins from the seminal plasma and antioxidant substances to the media surrounding the male reproductive cell have been used with cryopreserved and sex- sorted boar sperm with promising results (Maxwell & Johnson 1999, Roca *et al*. 2004, Centurión *et al* .2003, Garcia *et al*. 2007).

Seminal plasma

Seminal plasma (SP) is the biological fluid in which the spermatozoa are bathed. It is well known that SP contains factors that influence both the spermatozoa and the female genital tract (Mann & Lutwak-Mann 1982, Rozeboom *et al*. 2000). Most sperm technologies include the removal or extreme dilution of SP, a process that has been associated with a decrease in sperm quality and the acquisition of capacitation-like changes that lead to a shortening of sperm lifespan (Maxwell & Johnson 1999). Therefore, a common counter-measure to alleviate these detrimental effects has been the addition of a certain proportion of SP to sperm extenders (Maxwell & Johnson, 1997). However, there is considerable variability in the results reported in the literature regarding the effect of SP on frozen-thawed spermatozoa (Caballero *et al*. 2004, Maxwell *et al*. 2007, de Graaf *et al*. 2008). Initial research in sperm cryopreservation showed that pre-freezing spermatozoa in their own SP protected against cold shock (Pursel *et al*. 1973). On the other hand, Kawano *et al*. (2004) showed an improvement in sperm cryosurvival when SP was removed immediately after collection. Similar results have been reported regarding the addition of seminal plasma to post-thaw extenders with either improved membrane status (Vadnais *et al*. 2005) or diminished viability of the cryopreserved spermatozoa (Ericksson *et al*. 2005).

The application of SP to sex-sorted spermatozoa has been more straightforward. The addition of a certain proportion of SP (usually 10%) to the collection media of sex-sorted spermatozoa improves sperm viability (Maxwell & Johnson 1999). This protective effect is associated with a decrease in the capacitation-like changes observed in the sex-sorted spermatozoa (Maxwell & Johnson 1997, Parrilla *et al*. 2005. See Figs 2 and 3). Although the beneficial effect of SP on sex-sorted sperm is somewhat consistent, as demonstrated by its inclusion in the collection media of most modern boar sperm sorting protocols (Grossfeld *et al*. 2005), such an effect is, however, boar-dependent (Caballero *et al*. 2004). These differences are associated with the variability observed in the SP composition among males as well as between ejaculates or fractions of the same ejaculate (Caballero *et al*. 2004, Rodríguez-Martínez *et al*. 2005, 2008, Saravia *et al*. 2009). Furthermore, a similar donor-dependent effect has been observed regarding sperm freezability, where the addition of SP from boars with good freezability to freezing extenders improved sperm cryosurvival (Hernández *et al*. 2007). These differences are prompting researchers to study the effects of specific components of the SP, such as SP proteins.

Seminal plasma proteins

In boars, the bulk of SP proteins (>90%) belongs to the spermadhesin family, a group of 12–16 kDa glycoproteins that bind to the sperm surface. Depending on their binding capability, spermadhesins can be classified into heparin-binding (AQN-1, AQN-3, AWN) and non-heparin-binding spermadhesins (PSP-I/PSP-II heterodimer) (Töpfer-Petersen *et al*. 1998). The PSP-I/PSP-II heterodimer exerts a protective effect on highly diluted spermatozoa (Centurión *et al*. 2003). Based on immunolocalisation studies, PSP-I/PSP-II heterodimer has been seen to bind to the acrosomal cap of the spermatozoa. The binding of the heterodimer to the sperm surface seems to stabilise the plasma membrane of the spermatozoa (Caballero *et al*. 2006), delaying capacitation-like events, such as the increase in intracellular calcium and remodelling of the surface proteins of the sperm membrane (Caballero *et al*. 2006, 2009). This protective effect points to the PSP-I/PSP-II as a candidate additive for extending the viability of technologically treated spermatozoa. In this way, the PSP-I/PSP-II heterodimer has been tested as an additive for sex-sorted spermatozoa, showing a similar protective effect as that observed with SP (García *et al*. 2007). Experiments carried out in our laboratory using laparoscopic insemination demonstrated that the fertilising ability of sex-sorted spermatozoa collected in the presence of the PSP-I/PSP-II heterodimer increased when the PSP-I/PSP-II heterodimer was combined with sedimentation (instead of centrifugation) to concentrate the sperm sample (García *et al*. 2007).

Antioxidants

Sperm technologies often include procedures that are associated with the generation of ROS, which are related to defective sperm function (Muiño-Blanco *et al*. 2008). The use of antioxidants has been proposed to diminish the generation of ROS. Addition of catalase and superoxide dismutase reduced post-thaw ROS generation, improving sperm motility, viability and the ability of frozen-thawed spermatozoa to produce embryos *in vitro* (Roca *et al*. 2005). A beneficial effect of antioxidants was also seen with regard to the quality of flow-cytometrically sorted frozen-thawed bull spermatozoa (Klinc & Rath 2007), suggesting that the addition of antioxidants could be complementary for improving the fertilising ability of sex-sorted spermatozoa. Unfortunately, there are no references regarding the effects of antioxidant addition to the media used in sex sorting protocols for boar sperm.

Other strategies to optimise sperm treatment technology outputs

Application of sperm cryopreservation or sperm sex sorting in pigs generally implies the use of a small or very small number of weak spermatozoa. Together with the particular characteristics of the anatomy and reproductive physiology of the sow, this fact should be taken into account for the development and optimisation of protocols that allow the achievement of optimal fertility results after *in vivo* fertilisation.

Different strategies have been proposed in recent years to obtain optimal fertility results from insemination with a reduced number of boar sperm. These new procedures are mainly related to the site of semen deposition. In an attempt to deposit the spermatozoa as close as possible to the location of fertilisation by post-cervical insemination (intrauterine insemination, Watson & Behan 2002), deep intrauterine insemination (Martinez *et al*. 2001) and laparoscopic intra-oviductal insemination (García *et al*. 2007, Vazquez *et al*. 2008a, 2008b) procedures have been described and successfully used in combination with cryopreserved (Roca *et al*. 2003) and sex-sorted spermatozoa (Vazquez *et al*. 2003). In the case of sex-sorted spermatozoa, it

has also been demonstrated that, in addition to the insemination procedure, the effect of the time of insemination relative to ovulation has an important influence on the success of the insemination procedure (reviewed by Vazquez *et al*. 2008a, 2008b).

Concluding remarks

Although it is clear that optimal sperm characteristics are those that allow the spermatozoa to fertilise an oocyte with subsequent embryo development, better knowledge of the characteristics of spermatozoa subjected to different technologies and an accurate prediction of fertilising ability by the application of the most appropriate sperm assessment assays are essential for optimising the yields of these techniques.

Reduced viability, lifespan and low fertility after the application of boar spermatozoa subjected to different sperm technologies have been widely described by several authors. These are the main drawbacks to overcome before these technologies are ready for application at commercial level. Important achievements have been made in cryopreservation and sex sorting protocols by modifying sperm handling procedures as well as through the addition of different substances. The consequent improvements in sperm functionality have been described by several different laboratories.

Furthermore, it should not be forgotten that fertilisation is a process that not only depends on sperm factors but also largely affected by aspects of the AI procedure and the physiology and management of the female. AI methods that allow deposition of the spermatozoa as close as possible to the site of ovulation and also as close to the ovulation time as possible are currently the most powerful tools for optimising the *in vivo* fertility of boar spermatozoa that have been subjected to cryopreservation or flow sorting.

As a general conclusion, it should be noted that important progress has been made in sperm technologies and many promising results have been achieved, which will be helpful for introducing these technologies on to pig farms. However, further research efforts should be pursued in order to facilitate a fully successful introduction of these sperm technologies to the swine production industry.

Acknowledgements

The authors are grateful to A. Fazeli for helpful suggestions and for critically reading the manuscript. Financial support from CICYT (AGF2005-00760, AGL2008-04127) and SENECA (04543/GERM/07).

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Recent advances in boar semen cryopreservation

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Since 35 years ago boar semen has been frozen and used for artificial insemination (AI). However, fertility of cryopreserved porcine sperm has consistently been low as boar sperm are more sensitive to cellular stress imposed by changing osmotic balance, oxidative stress, low-temperature exposure, cryo-protectant intoxication etc. and are less able to compensate for these deficiencies at commercially applicable dosages. Additionally, differences in sperm freezability among individuals are well known. Here we review current advances on tests to screen sperm quality post-thaw, on ways of diminishing individual boar effects, on improvement of cryo-protection by novel extender components, on packaging and freezing protocols and freezing and thawing methods, and on the handling of sexed boar sperm. Major advances have been registered, which have improved cryo-survival and the capacity to process boar semen for commercial AI.

Introduction

The history of boar sperm freezing is characterized by three major events. About 60 years ago Chris Polge found by chance that glycerol was an effective cryo-protecting agent (CPA); Pursel & Johnson (1975) developed the Beltsville freezing and thawing procedure using a pellet method and Westendorf *et al*. (1975) started to freeze boar sperm in plastic straws. Since then important steps for freezing have been developed in laboratories around the world, but boar sperm cryo-survival has consistently been low. Throughout cryopreservation, sperm are exposed to cellular stress imposed by changing osmotic balance, oxidative stress, low-temperature exposure, and cryo-protectant intoxication. Apart from lethal changes due to cryo-injury, sub-lethal changes occur in sperm that take place at a molecular level, impacting on their cellular function and fertilizing capacity. Additionally, differences in sperm freezability among individuals are common. This review describes different perspectives and practical application of recent achievements in the improvement of the quality of frozen-thawed (FT-) boar semen.

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Integrity of frozen thawed boar sperm

Evaluation of frozen/thawed semen quality

Sperm kinematics assessed by computer-assisted sperm analysis (CASA) has great potential to give better insight into boar sperm function and heterogeneity. More than 30 different kinematic parameters related to linearity, velocity and lateral head displacement allow for detailed analysis of kinematic characteristics of single sperm. In addition to post thaw motility, the function of relevant sperm signal transduction systems, e.g. the activation of transduction systems (e.g. the activation of adenyl-cyclase) by short-term incubation with bicarbonate (Holt & Harrison 2002; Satake *et al*. 2006) can be measured with CASA systems. Despite this potential, CASA parameters were not found to be very sensitive at monitoring cooling-associated changes of sperm function (Saravia *et al*. 2007) or predicting fertility of FT- sperm (Rodriguez-Martinez 2007). One reason for this is related to the inability of some CASA systems to examine populations and at the same time focus on individual sperm. Means and standard deviation of sperm kinematics are inappropriate to detect small populations of rapidly and linearly moving sperm, which may be significant for fertilization (Holt *et al*. 2007).

The main damage to sperm due to cryo-injury occurs at the level of the plasma membrane and appears to be more closely related to fertility than sperm motility (Rodriguez-Martinez 2007). Following detection by fluorescence techniques, about 50% of boar sperm are commonly classified as "dead" after freezing/thawing. The additional detection of sub-lethal changes in plasma membrane function impairing sperm fertilizing capacity seems to be of high diagnostic relevance. Membrane characteristics of thawed sperm are similar in certain respects with sperm that are in advanced stages of capacitation. Although they do not correspond completely at the molecular level, this phenomenon led to the idea of "cryo-capacitation" (Watson 1995; Green & Watson 2001). Attempts to verify the occurrence of capacitating-like changes following cryo-preservation were made by detecting phospholipid disorders in plasma membranes using the lipophilic fluophore Merocyanine-540 (Harrison *et al*. 1996) and by induction of acrosome exocytosis using calcium ionophore A23187 in presence of bicarbonate as capacitation inducer (Guthrie & Welch 2005a). Compared to fresh semen, cryo-preserved sperm showed a reduced response to bicarbonate (Guthrie & Welch 2005a; Saravia *et al*. 2007). The presence of lipids and proteins of seminal plasma (SP) and/or egg yolk may alter the response to bicarbonate rather than the cooling process itself (Guthrie & Welch 2005b; Saravia *et al*. 2009b). Additionally, the sequential manner in which capacitational changes occur and contribute to a destabilization process leading to cell death (Petrunkina *et al*. 2007) may differ between fresh and FT- sperm. FITC-conjugated Annexin-V has been used to measure phospholipid scrambling by specifically binding to phosphatidylserine molecules that accumulate in the plasma membrane exoplasmic leaflet (Gadella & Harrison 2002). Cryo-preservation caused a significant increase in Annexin-V-positive staining in live sperm (Peña *et al*. 2003; Guthrie & Welch 2005b). It is suggested that increased Annexin-V-binding in thawed sperm results from plasma membrane damage incurred during freezing and thawing (Guthrie & Welch 2005b), rather than from expression of capacitation (Silva & Gadella 2006; Rodriguez-Martinez 2007).

Detection of early changes in the sperm plasma membrane at any of the main three domains is very important for cryo-storage protocols. During cryo-preservation the major osmotic gradient across the sperm membranes essentially changes with sperm undergoing volumetric changes as water and solute leave and enter the cells. This osmotic response can be potentially lethal to the sperm if it causes them to swell or shrink beyond their osmotic tolerance limits. Cell death occurs mainly during the thawing process, when the dehydrated sperm are exposed to severely hypo-osmotic conditions (Gilmore *et al*. 1996). Electronic volume measurements by

cell counter display the response of large numbers of sperm to osmotic challenges including the identification of subpopulations with different osmotic responsiveness. A relationship of volumetric parameters to fertility was established for frozen bull sperm (Petrunkina *et al*. 2001) and sperm binding to oviductal explants in vitro seems to be related to fertility of frozen semen (De Pauw *et al*. 2002; Khalil *et al*. 2006). However, a prediction of fertility remains questionable due to difficulties in standardizing the assay and loss of tissue differentiation during culture. Microscopic assessment of cryo-preserved boar sperm using a simplified hypo-osmotic swelling test gave comparable results to flow cytometrically analyzed plasma membrane integrity, despite measuring different membrane domains (Saravia *et al*. 2005) and is significantly related to fertility (Perez-Llano *et al*. 2001).

Oxidative stress during the freezing-thawing cycle due to excessive generation of reactive oxygen species (ROS) or deficiencies in the antioxidant defense system can induce serious damage to many biological macromolecules, such as proteins, lipids and mitochondrial DNA (mtDNA) as well as nuclear DNA (nDNA), which could lead to impaired biological properties and eventually to cell death (Aitken & Baker 2004). Pro-apoptotic changes in cryo-preserved sperm are characterized by increased Ca^{2+} concentration, disturbance in mitochondrial membrane potential, reduced ATP levels and the release of pro-apoptotic factors in the cytoplasm (Martin *et al*. 2004, 2005; Peña *et al*. 2009). Different methods used to assess nuclear DNA damage after freezing and thawing have so far given controversial results. DNA fragmentation as detected with single cell gel electrophoresis (comet assay) was significantly increased in FT- compared to fresh sperm and was affected by the individual boar (Fraser & Strzezek 2005), the presence and type of the cryo-protectant, and by seminal plasma (SP) (Fraser & Strzezek 2007b; Hu *et al*. 2008). In contrast, although the sperm chromatin structure assay (SCSA) did not reveal biologically significant changes due to sperm freezing/thawing in unselected boars (Saravia *et al*. 2009b), sires classified as "good freezers" were less susceptible to sperm chromatin denaturation than "bad freezers" (Hernández *et al*. 2006). Varying results among different studies may depend on the innate variation among boars used but, since most stud boars have been selected for sperm quality, such differences are minor and only appear in a small number of males (< 1/1,000) it is more likely that the tests need further standardization. DNA fragmentation assays as determined by single cell electrophoresis may be less accurate as it is based on single cell determination and cannot distinguish between dead and live sperm.

Boar-to-boar variation on sperm freezability

As for many other mammals (Holt *et al*. 2005, Loomis & Graham 2008), male-to-male variability in post-thaw sperm quality has been extensively demonstrated in pigs (Larsson & Einarsson 1976, Thurston *et al*. 2001, Medrano *et al*. 2002a, Saravia *et al*. 2005, Roca *et al*. 2006b). This individual variability is the primary factor explaining differences in sperm cryo-survival between boars (Roca *et al*. 2006a). However, since ejaculates within a given male most often respond reproducibly to the same cryo-preservation protocol, grouping of sires as "good", "moderate" or "poor" sperm freezers can be done, based on post-thaw sperm quality. Between 20 to 33% of stud sires are considered "poor" sperm freezers (Thurston *et al*. 2002, Roca *et al*. 2006a, 2006b, Hernández *et al*. 2007a), most likely genetically driven. Thurston *et al*. (2002) compared the genomic DNA of boars using the Amplified Fragment Length Polymorphism (AFLP) technique and identified different AFLP profiles between ''good'' and ''poor'' sperm freezers. Some of these profiles were related to post-thaw sperm quality and were, therefore, considered to represent significant markers of sperm cryo-sensitivity. The underlying mechanism(s) for the genetic differences related to cryo-preservation-induced sperm injuries are yet unknown but may represent differences in

sperm biochemical composition and physiology (Holt *et al*. 2005). Whether the polymorphism of testis- and epididymis-expressed candidate genes affecting sperm quality traits in fresh boar semen (Lin *et al*. 2006) are linked to cryo-preservability remains to be proven. In this context, Waterhouse *et al*. (2006) showed a relationship between long-chain polyunsaturated fatty acids in the plasma membrane of thawed sperm and boar-to-boar differences in sperm cryo-sensitivity. These inter-boar differences might equally be, however, related to variations in SP composition. Rath & Niemann (1997) observed that boar differences in post-thaw motility were only significant for ejaculated sperm, whereas epididymal sperm had a consistently higher post-thaw motility. More recently, Saravia *et al*. (2007) showed a significant improvement of sperm cryo-survival together with a substantial reduction of boar-to-boar variability on post-thaw sperm quality when cryo-preserving just the first 10 ml of the sperm-rich fraction of the ejaculate, a portion characterized by the highest sperm concentration alongside with low presence of sex gland secretions. Altogether, these results seem to indicate that the exposure of sperm to the SP during the ejaculation process could modify their cryo-sensitivity.

Minimization of the negative effects of boar-to-boar variability on post-thaw sperm quality within the commercial semen freezing industry should first be addressed towards identification of potentially "good" or "poor" sperm freezers. In absence of a simple blood-based genetic test and given the fact that sperm freezability is highly consistent within stud boars (Roca *et al*. 2006a), the potential sperm freezability of a given boar can be identified by means of sperm freezability tests (SFT), consisting of the cryo-preservation of 5-10 ml of just one ejaculate per boar. Experience from Spain over the past 12 years indicates that implementing a SFT can predict its relative sperm freezability potential. After performing the SFT-test, 112 boars (24.6%) showed $\leq 35\%$ viable and motile sperm after thawing and were considered as "poor" sperm freezers. Additionally, action should be taken towards the improvement of sperm cryo-survival of ejaculates collected from boars classified as "poor" sperm freezers. The ability to cryo-preserve ejaculates from "poor" sperm freezers remains important in the context of genetic resource banking and international exchange of genetic material (Holt *et al*. 2005). Such action could be undertaken by customizing the cryo-preservation protocol. An example is by individually-tailoring the freezing-thawing protocol, a concept suggested by Watson (1995) and recently applied by Hernández *et al*. (2007b) using a so called "split-ejaculate freezing test". Individual ejaculates were divided and cryo-preserved using 12 different protocols that differed with regard to cooling rate, glycerol concentration or warming rate. Significant interaction was found between ejaculate and cryo-preservation protocol, with the influence of glycerol concentration and warming rate being particularly relevant for post-thaw sperm quality. Such handling reduced the percentage of "poor" freezers from 26.4 to 7.5%. An alternative has been recently presented by cryo-preserving the first 10 ml of the sperm-rich fraction of each ejaculate (Rodriguez-Martinez *et al*. 2008; Saravia *et al*. 2009a, b).

Cryo-injury in boar sperm

The sperm membrane is regionally differentiated and displays different behaviour and interactions depending on structural regions. The acrosome (especially the equatorial segment), the post-acrosomal and mid-piece segment and, the principal and end tail segments, are all structurally and functionally crucial to sperm function. Cryo/thawing-protocols cause loss of selective permeability and integrity of these domains, mostly during rewarming (Medrano *et al*. 2002b). Inadequate ice formation and the subsequent osmotic stress induced during freezing and re-warming are the two major factors responsible for sperm cryo-injury (Watson 2000) and compromise sperm function due to alterations in the membrane constituents, particularly lipids and proteins. These phenomena might be associated with pro-apoptotic changes in sperm caused by reactive oxygen species (ROS) produced under stress conditions.

Protein tyrosine phosphorylation, which plays a crucial role in the regulation of cell proliferation and differentiation, is associated with capacitation-like changes in sperm, sometimes termed "cryo-capacitation" when induced by cryo-preservation (Urner & Sakkas 2003, Bravo *et al*. 2005). The level of phosphorylation of tyrosyl groups is regulated by the balance of protein tyrosine kinases (PTKS) and protein tyrosine phosphatases (PTPases). Phosphorylation of tyrosine residues in sperm varies among boars and at different stages of the cryo-preservation procedure according to the "Kortowska" method (Strzezek *et al*. 1985). Recently it has been demonstrated that fresh semen from boars with good freezability was characterized by a low content of phosphotyrosine residues in the extracted sperm proteins, whereas semen from boars with poor freezability exhibited a high content of phosphotyrosine (Wysocki *et al*. 2009). Accordingly, phosphotyrosine proteins in sperm extracts could be dephosphorylated by the molecular form of acid phosphatase of boar SP (Wysocki & Strzezek 2003, 2006) and phosphotyrosine residues in sperm proteins could be completely dephosphorylated only by acid phosphatases isolated from the vesicular glands.

New approaches to improve the cryo-survival of boar sperm

Cryo-protectant agents

The biophysical changes brought about by the transition of water to ice during the relatively slow cooling most often used are the assumed main causes for sperm damage. If sperm are solely frozen in SP (neat semen) or extended with a buffer, such "unprotected freezing" is simply lethal, since ice is formed both extra- and intra-cellularly, damaging essential cell structures, particularly the membranes of organelles and the plasma membrane. Even when the extender contains a proper cryo-protectant agent (CPA), damage occurs, but many cells survive the process. Under these conditions, ice is formed in the aqueous extender medium surrounding the sperm and, as ice crystals grow in this extracellular milieu of free water, the amount of solvent decreases while the solute becomes more and more concentrated. Sperm lose intracellular water in order to compensate for this effective osmotic stress leading to a freeze-dehydration of the cells. Eventually, when temperatures pass ~ -80°C, the highly concentrated, viscous solution within and outside the sperm turns into a relatively stable glassy matrix, which is basically maintained when sperm are stored at -196°C (LN_2). The imaging of such concentrated medium, where sperm are embedded (the so-called veins) contrasts with the frozen free water (so-called lakes), when viewed using a cryo-scanning electron microscope (Cryo-SEM, Ekwall 2009). Interestingly, most sperm in the veins appear intact, i.e. they seem to survive the process of cooling. Even more interesting, intracellular ice is rarely formed, since the speed of cooling is usually low and the presence of the CPA increases viscosity, both of which add to the above process of cell dehydration (Bwanga *et al*. 1991a). Most cells are damaged during thawing, with membranes and axonemes deteriorating by the osmotic imbalance that has been created during cooling (Morris 2006; Morris *et al*. 2007).

As mentioned above, glycerol was the first recorded CPA added to a semen extender. This small, poly-hydroxylated solute is highly soluble in water interacting by hydrogen bonding and permeates across the plasma membrane at a low rate. Glycerol is, unfortunately, cell-toxic at body temperature and thus boar sperm are usually exposed to glycerol at ~5°C, which further slows permeation. Mixed with the other solutes of the extender in solution, it depresses the freezing point and ameliorates the rise in sodium chloride concentration during dehydration. Moreover, glycerol increases viscosity with lowering temperature to more than 100,000 cP at -55°C (Morris *et al*. 2006) retarding both ice crystal growth and dehydration speed on a kinetic basis. Glycerol also eliminates eutectic phase changes of the extender (Han & Bischof 2004),

thus becoming a very suitable CPA when added at 2-3%. It does not affect sperm cryo-survival in boars considered good freezers, and improves cryosurvival in those considered "moderate or bad freezers" (Hernández *et al*. 2007a).

A broad range of other solutes (mostly alcohols, sugars, diols and amides) has also been tested for CPA capacity (Fuller 2004). CPA capacity differed greatly in tests using boar sperm compared to the sperm of other species. Alcohols and diols can induce the formation of blebs, which are spherical cellular protrusions in the membrane. Sugars, which both increase viscosity and stabilize the membrane by interacting with phospholipids, have not led to a higher cryo-survival compared to glycerol (Hu *et al*. 2008). Replacing glycerol with amides (formamide; methyl- or dimethylformamide, MF- DMF; acetamide; methyl- or dimethylacetamide (MA- DMA) at ~5% concentration, has proven acceptable (Bianchi *et al*. 2008). Although cryo-survival was not dramatically enhanced in most boars, cryo-susceptible boars benefited somewhat from use of DMA, probably because the amide permeates the plasma membrane more effectively than glycerol, thus causing less osmotic damage during thawing. Additives other than CPAs increase cryo-survival too. At very low rates (<0.1%) N-acetyl-D-glucosamine has been able to enhance cryo-survival of boar sperm (Yi *et al*. 2002a) possibly interacting with the surfactant Orvus es Paste (OEP) (Yi *et al*. 2002b). The value of OEP or other surfactants has been confirmed (Karosas & Rodriguez-Martinez 1993, Pettitt & Buhr 1998) when used with egg yolk (Buranaamnuay *et al*. 2009). Use of low-density lipoproteins (LDL), most often isolated from egg-yolk from different species (Fraser & Strzezek 2007a; Jiang *et al*. 2007) has proven beneficial for sperm function post-thaw, particularly for DNA-integrity (see below).

Lipoproteins

Typically, boar sperm cryo-storage media contain up to 20% chicken yolk, whose active component (i.e. the low-density lipoprotein (LDL)-fraction), has been suggested to be largely responsible for protecting sperm against cold shock damage (Demianowicz & Strzezek 1996; Moussa *et al*. 2002; Jiang *et al*. 2007, Hu *et al*. 2008). Despite some success in species such as the horse (Clulow *et al*. 2007), yolks from duck and quail failed to improve the post-thaw quality of frozen boar sperm (Bathgate *et al*. 2006). In contrast lyophilized lipoprotein fractions isolated from ostrich egg yolk (LPFo) provided good protection of sperm cells by preventing physical damage and alteration of membrane fluidity during cooling at variable temperatures (i.e. from 5oC to 16°C or from 16°C to 5°C) and cryo-preservation (Strzezek *et al*. 2004; Strzezek *et al*. 2005a). Besides its protective action, LPFo has been shown to possess specific antioxidant properties (Strzezek *et al*. 2004). The overwhelming positive effects of LPFo on sperm quality characteristics have led to the development of a new semen preservation technology for liquid storage at different temperatures and cryo-preservation (Fraser & Strzezek 2005; Fraser & Strzezek 2007a,b). Moreover, AI of sows with liquid-stored or FT-semen supplemented with LPFo gave acceptable results (Fraser *et al*. 2007a). Investigations on the interaction between yolk lipoproteins and SP-proteins indicated that together they either enhance (Manjunath *et al*. 2002) or diminish (Vishwanath *et al*. 1992) sperm cryo-protection, suggesting that revisiting storage media without egg yolk could provide a more biosecure means of transporting and storing boar sperm by avoiding (contaminated) materials from other species.

Seminal plasma

The SP has a chequered history as a supplement for sperm storage media and current opinion is still divided over its benefits. The binding of SP-proteins to sperm stabilizes the plasma membrane components, masks the antigens exposed to the cell surface and prevents premature acrosomal

reaction (Muiño-Blanco *et al*. 2008). The boar SP contains a wide range of components that differ between species, males and ejaculates (Maxwell *et al*. 2007). This may explain the disparate results reported by different groups. It is now clear that mammalian SP contains components that can both inhibit and induce sperm capacitation, stimulate sperm motility and even provide protection against damage incurred during manipulation and storage, such as extension and cold-shock (Maxwell *et al*. 2007). The major proteins identified to date in boar SP are the non-heparin-binding proteins PSPI/PSPII heterodimer (PSPI/PSPII), the heparin binding spermadhesins AQN-1, AQN-3, AWN and the lipid-binding protein pB1. PSPI/PSPII, more specifically the PSPII subunit, inhibits zona penetration and increases sperm longevity, suggesting that this subunit can be used to improve the fertility achieved with stored sperm (Caballero *et al*. 2008). Incubation of FT-sperm with PSPI/PSPII prior to *in vitro* insemination resulted in an increased proportion of viable sperm but a reduced number of penetrated oocytes, compared with sperm incubated without PSPI/PSPII. The same treatment applied to fresh sperm had no effect on oocyte penetration (Caballero *et al*. 2004). Fertility was also improved after laparoscopic insemination of low doses of sex-sorted sperm pre-incubated in the presence of PSPI/PSPII (Garcia *et al*. 2007). The PSPI/PSPII heterodimer has also been shown to modulate the uterine immune response to the introduction of semen in both an inhibitory (Veselský *et al*. 1992) as well as a stimulatory manner (Rodriguez-Martinez *et al*. 2005), suggesting a direct effect on spermatozoa as well as a preparatory role for embryo attachment. Thus, addition of PSPI/PSPII to the sperm suspension medium prior to insemination may be influencing fertility by the modulation of both sperm function and the secretory and/or physical environment of the female reproductive tract.

AQN-1, a major heparin-binding protein prevalent in boar SP, may have a role in the congregation of sperm in the isthmic reservoir (Ekhlasi-Hundrieser *et al*. 2005) and both AQN-1 and -3 may regulate the initiation of capacitation (Dostalova *et al*. 1994). AWN appears to modulate sperm-oocyte interaction (Rodriguez-Martinez *et al*. 1998), suggesting that addition of these proteins to sperm prior to artificial insemination may improve fertility outcome. The lipid-binding protein pB1 has been implicated in the capacitation of epididymal boar sperm (Plucienniczak *et al*. 1999; Lusignan *et al*. 2007). Unlike homologous proteins found in the SP of other species, pB1 forms a complex with AQN-1 (Calvete *et al*. 1997). Manipulation of boar sperm storage media to remove this complex might prolong the shelf-life of sperm, or it could be exploited as a capacitation factor prior to IVF.

SP-vesicles (prostasomes) have been regarded as influencing fertility in humans (Kravets *et al*. 2000). Although probably of prostate origin, such has not yet been identified in the boar. Prostasomes consist of lipid and protein conglomerates that are concentrated in the sperm-rich fraction of the boar ejaculate but are of unknown function in this species (El-Hajj Ghaoui *et al*. 2007). Similar structures in other species have been identified as fusing with sperm membranes, possibly stabilizing the lipids and postponing the acrosome reaction (Frenette *et al*. 2002).

Recently, it has been shown that a 5-h period of dialysis of boar ejaculate in semi-permeable dialysis bags (12 to 14 kDa cut-off) prior to freezing had a significant effect on the polypeptide profiles of the SP (Strzezek *et al*. 2005b) as well as a significant improvement in post-thaw sperm quality characteristics, such as caffeine-stimulated sperm motility, plasma membrane integrity and mitochondrial status (Fraser *et al*. 2007b). Dialysis of ejaculates prior to freezing may be a useful technique to eliminate certain seminal plasma proteins with sperm toxic effects during cryo-preservation (Strzezek *et al*. 2005b).

Other additives

Boar sperm are susceptible to peroxidative damage induced by the cryo-preservation process (Hernández *et al*. 2007c) due to the high proportion of unsaturated fatty acids present in their

membranes (Cerolini *et al*. 2000). Hence, attempts have been made to add various antioxidants to different fractions of boar ejaculate to improve post-thaw sperm survival (Peña *et al*. 2003, Gadea *et al*. 2005), including butylated hydroxytoluene (BHT; Roca *et al*. 2004), catalase, superoxide dismutase (SOD; Roca *et al*. 2005), reduced glutathione (GSH; Woelders *et al*. 1996), and α-tocopherol (Breininger *et al*. 2005). The latter reduced cryo-induced oxidative damage to sperm membranes, protein tyrosine phosphorylation and the capacitation-like events (Satorre *et al*. 2007).

Additional additives that have shown some potential for improving quality of boar sperm post thaw are hyaluronan (Peña *et al*. 2004), platelet-activating factor (PAF; Kordan & Strzeżek 2002; Bathgate *et al*. 2007a) and platelet-activating factor: acetylhydrolase (Pafase; Bathgate *et al*. 2007a). PAF, a member of the family of the acetylated glycerophospholipids, is a component of plasmalemma lipids of boar sperm. PAF is involved in several sperm functions, including capacitation, the acrosome reaction and the regulation of motility of sperm, particularly during storage at 5° to 16°C (Kordan & Strzezek 2002). Supplementation of the cryo-preservation medium with PAF or the recombinant PAF acetylhydrolase appeared to have beneficial effects on the *in-vitro* quality of FT- sperm (Bathgate *et al*. 2007a).

Controlled freezing

Rates of cooling (and of thawing) can be controlled by use of programmed freezers, and "optimal" cooling rates are those that substantially diminished the period during which heat was released in the sample when water changed phases (i.e. ice was formed). Interestingly, experimentally determined optimal rates of the range 30-50°C/min (Thurston *et al*. 2003) have been theoretically predicted (Devireddy *et al*. 2004; Woelders & Chaveiro 2004) and confirmed by use of novel procedures, such as equilibrium freezing (Woelders *et al*. 2005). Boar sperm are still "best" (in terms of cryo-survival) cryo-preserved in standard lactose-egg yolk (or LDL)-based cooling and freezing media, the latter including a surfactant (often OEP) and glycerol (2-3% final concentration); cooled at 30 to 50°C/min and rapidly (1,000-1,800 °C/min) thawed. This protocol would serve most boars while for those with sub-optimal sperm freezability, the protocol must be modified, particularly regarding glycerol concentration and warming rates (Hernández *et al*. 2007b). The entire procedure takes most often 8-9 hours from collection to storage of the frozen doses in LN_2, is still tedious and inconvenient, and produces few AI-doses.

Packaging systems

The use of different packaging for extended sperm resulted in differences in cryo-survival. Boar sperm were processed in plastic straws of different volumes (0.25 to 5 ml) (Johnson *et al*. 2000), in flattened 5 ml straws (Weitze *et al*. 1987), in aluminium tubes (Fraser & Strzezek 2007b) or plastic bags of various types and constitution (Bwanga *et al*. 1991b; Karosas & Rodriguez-Martinez 1993; Mwanza & Rodriguez-Martinez 1993; Ortman & Rodriguez-Martinez 1994; Eriksson & Rodriguez-Martinez 2000a, b). The latter, denominated "FlatPack™", proved equally good or better than 0.25 ml straws in terms of sperm cryo-survival, despite the fact that they held 5 ml of semen, which could accommodate an entire dose for cervical AI with 5 billion sperm, thus pooling innumerable straws after thawing was not necessary. The use of FlatPack™ resulted in acceptable farrowing rates and litter sizes (Eriksson *et al*. 2002). FlatPack™ was considered as cryo-biologically convenient to dissipate heat during rapid cooling and warming as those smaller containers tested.

However, doses with such large sperm numbers are not the best use of the ejaculates. Since the introduction of intrauterine deposition of semen, small numbers of sperm are sufficient for successful insemination and the reduced volume of the dosage allowed the use of smaller containers. Recently, boar sperm have been frozen in highly concentrated (1-2 billion sperm/ml) small volumes (0.5-0.7 ml) in novel containers, the so-called "MiniFlatPack™" (Saravia *et al*. 2005). Interestingly, cryo-survival in MiniFlatPacks was equal or higher than survival in 0.5 ml plastic straws, suggesting the shape maintained the cryo-biological advantages of the FlatPack™ (Ekwall *et al*. 2007), including improvements in fertility (Wongtawan *et al*. 2006).

New simplified freezing of boar semen

Processing semen in the current manner is impractical and, therefore, unattractive for routine, commercial use. Sperm from boars that were semino-vesiculectomised sustained freezing and thawing equally well compared to sperm exposed to seminal vesicular proteins (Moore & Hibbitt 1977), indicating that the SP of the sperm-rich fraction (SRF) might not be necessary for cryo-survival or even fertility. Recently, it was determined that boar sperm contained in the first 10 ml of the SRF (also called Portion 1 or P1, containing about ¼ of all sperm in the SRF) were more resilient to handling (from extension to cooling) and cryo-preservation than the sperm contained in the rest of the ejaculate (Peña *et al*. 2003; Saravia *et al*. 2007; Rodriguez-Martinez *et al*. 2008). It appeared that it was actually the SP in this Portion 1 that was beneficial for sperm, either because of its higher contents of cauda epididymal fluid, its lower amounts of SP-spermadhesins or its lower bicarbonate levels. An attempt was very recently made to simplify the cryo-preservation protocol by freezing solely the P1-sperm, in concentrated form for eventual use with intrauterine AI. These sperm were packed into MiniFlatPacks™ for customary freezing using 50°C/min cooling rate. This "simplified" entire procedure, lasted 3.5 h compared to the "conventional freezing", which lasted 8 h. As controls, sperm from the SRF were compared to P1-sperm. Cryo-survival was equally good (above 60% of the processed cells, Saravia *et al*. 2009a).

There are several advantages to using this simplified, shorter protocol, namely the exclusion of primary extension and of recovering this conspicuously beneficial SP-aliquot by centrifugation; as well as waiving the need of an expensive refrigerated centrifuge. Moreover, inter-boar variation was minimized by use of P1-sperm, which, not only were the "best" sperm to be cryo-preserved, but also left the rest of the collected sperm for liquid semen processing. This simpler protocol ought to be an interesting alternative for AI-studs to freeze boar semen along with production of conventional semen doses for AI with liquid semen.

Advanced insemination strategies with FT- boar semen

Recently, farrowing rates ranging from 72 to 85% and 11-12 piglets born per litter have been achieved in FT-inseminated sows in Taiwan and Canada with semen cryo-preserved in Europe (Eriksson *et al*. 2002; Roca *et al*. unpublished observations). These results indicate that FT-semen has the potential for sufficient fertilization rates and can be used to improve international semen trade. However, the high sperm number currently required per AI-dose (5-6 x 10^9sperm) together with the sometimes inconsistent fertility, limit FT-semen usage to the introduction of genetics.

Therefore, it is of utmost importance to reduce the number of sperm per AI-dose without impairing fertility. At present, this objective can be achieved by means of the deep intrauterine insemination (DUI) procedure, which allows deposition of the sperm dose deep into a

uterine horn. Recently, the performance of DUI for swine AI and its suitability for an efficient application of FT-semen have been extensively evaluated (Bathgate *et al*. 2005; Roca *et al*. 2006c; Grossfeld *et al*. 2008; Vazquez *et al*. 2008). Overall, DUI appeared as a safe procedure leading to satisfactory fertility with as few as 1-2 x 10^9 FT-sperm per AI-dose. In this manner, in two more representative studies, farrowing rates above 70% with more than 9 piglets born per litter were achieved after insemination of 500 weaned sows under commercial conditions (Roca *et al*. 2003; Bolarin *et al*. 2005). Transcervical insemination could be another suitable AI-procedure for FT-semen, to allow deposition of sperm into the uterine body (Roca *et al*. 2006c). Unfortunately, it has been scarcely used with FT-semen, despite the promising fertility results achieved with liquid semen (Watson & Behan 2002; García *et al*. 2007). In the only report found using FT-semen, Abad *et al*. (2007) reported farrowing rates below 50% in weaned sows inseminated with 3 x10^9 live FT-sperm.

Fertility using commercial AI of FT-semen varies among trials, depending more on the interval between AI and ovulation than either post-thaw sperm quality or the sperm number inseminated. FT-sperm have a very short functional life span in the female genital tract, such that their fertilizing ability is dramatically impaired when inseminations are performed outside an interval 4-8 h before expected ovulation time (Wongtawan *et al*. 2006). Bolarin *et al*. (2005) achieved high farrowing rates above 80% in weaned sows using DUI with either 1-2 x 10^9 sperm per AI-dose, provided that the 4-8 h interval from AI to ovulation was respected. However, in contrast to these excellent results, the same authors and others (Wongtawan *et al*. 2006) achieved very low farrowing rates in sows using DUI outside of this "safe" pre-ovulatory interval, independent of how many living sperm were inseminated. Therefore, it is imperative that an accurate prediction of ovulation time is made to define an appropriate insemination timetable when FT-semen is used. As spontaneous ovulation takes place when two-thirds of the standing oestrus period has elapsed (Soede & Kemp 1997), appropriate insemination timetables can be established when the duration of standing oestrus is known. Finally, because seasonal differences in farrowing rates are greater for FT-semen than for liquid semen (Bolarin *et al*. 2008), the influence of season on the elapsed time between onset of oestrus and ovulation should also be considered.

Special freezing protocols for sex sorted boar sperm

A special application for FT-semen is related to sex sorted sperm. Currently, freshly collected boar sperm have been sexed successfully by flow cytometry and offspring were produced by surgical insemination (Johnson 1991), IVF (Rath *et al*. 1997, 1999; Abeydeera *et al*. 1998), ICSI (Probst & Rath 2003), and DUI (Rath *et al*. 2003; Grossfeld *et al*. 2005) under laboratory conditions. Due to the limited throughput of the sorting technology, a broader commercial application is not possible. Cryo-preservation might be a method of storage between sorting and insemination. Only a few studies investigated the freezing and thawing of sexed boar sperm. Sexed, frozen/thawed sperm have been subjected to IVF and DUI (Bathgate *et al*. 2007b, 2008). Whereas in-vitro fertilization with FT sperm was successful and pregnancies were initiated (Bathgate *et al*. 2007b), all sows returned to oestrus within 57 days. A combination of low sperm numbers and potentially compromised developmental capability of embryos derived from sex-sorted sperm may have resulted in this early stage loss of pregnancy based on fertilization with spermatozoa that are affected by non compensable defects (Bathgate *et al*. 2008).

Acknowledgements

Authors´ own studies have been funded by the Development Association for Biotechnology Research (FBF), Bonn, Germany; the Australian Pork Limited (APL); FORMAS and the Swedish Farmers' Foundation for Agricultural Research (SLF), Stockholm, Sweden; CICYT (AGL2005-00760), Madrid and GREM (04543/07) Murcia, Spain and the National Centre for Research and Development, Warsaw, Poland (NR12001404).

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Selection for boar fertility and semen quality - the way ahead

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Critical needs for the swine industry in terms of boar fertility evaluations are validation of semen quality estimates with *in vivo* reproductive data; estimation of the relative fertility of boars; and elimination of sub-fertile ejaculates. Single sire matings are the best way to validate semen quality estimates with reproductive performance. Sampling about 20% of the population provides an accurate estimation of the variability among boars and should be sufficient for this purpose. *In vitro* tests that measure univariate characteristics of ejaculates including motility and morphology appear to be just as accurate as those that measure multivariate traits such as *in vitro* fertilization in terms of predicting boar fertility. Reasons for this observation may be related to how properties of sperm cells are influenced by the sow reproductive tract. Several seminal plasma proteins show strong correlations with boar fertility and hold potential for being developed into tests that can rank the relative fertility of boars. Almost 90% of the variation in boar fertility was explained when the proportion of motile and acrosome-reacted spermatozoa was combined with relative amounts of 28 kDa, p*I* 6.0 and 55 kDa, p*I* 4.5 seminal plasma proteins. Consequently, combining different complementary tests improves estimations of boar fertility. Motility estimates routinely performed in most A.I. centres are a reasonable technique for identification and elimination of sub-fertile ejaculates. However, the accuracy with which they currently are conducted within the swine industry needs improvement.

Introduction

Estimation of fertility in boars presents some unique challenges for the swine industry. Unlike its beef and dairy counterparts, single-sire matings are rare within the commercial sector. This is because standard industry practices such as multiple matings; large numbers of sperm per insemination; high boar replacement rates; and the use of pooled semen hinder their use. Consequently, current approaches concentrate on quantification of physiological aspects of spermatozoa that are thought to be important for fertilization. Although progress is being made, identification of techniques for estimating boar and semen fertility via this strategy remains elusive. One of the main obstacles is that most semen tests evaluate the ability of spermatozoa to successfully complete only one of many steps in a complex process. Deficiencies in other areas can reduce fertilization even if the characteristic being measured is conducive for its occurrence.

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One way to address some of these challenges is to consider what types of information the swine industry needs to critically assess boar and semen fertility and then determine which fertility tests might best meet these needs. This premise is based on the assumption that there is not a single, *in vitro* test that can accurately predict boar fertility. However, perhaps a series of different tests administered at various times could. Hence, the objectives of this review are two-fold: first, to outline, perhaps speculatively, a set of requirements for the swine industry in terms of male fertility evaluations; and second, to examine our current understanding of relationships among selected tests for estimating semen quality and boar fertility. Special emphasis will be placed on how these techniques might be incorporated into strategies that are harmonious with the constraints of modern boar management systems. Hopefully, the end result will be a summary of relevant information that outlines what is possible now in terms of selecting for boar fertility and semen quality and what needs to be done subsequently to enhance the process.

Boar and semen fertility assessment needs

In order to be successful, strategies for fertility evaluations have to provide relevant information that can be obtained and used within the framework of normal management systems for boars. Consequently, a brief overview of the productive life of boars in commercial A.I. centres is shown (Fig. 1). Genetic evaluation of growth and physical characteristics including structural soundness typically are completed between 4 and 5 months of age. This coincides with the time when boars are moved into isolation facilities (Safranski 2008). The isolation period varies from 30 to 90 days based on the health status of the production system. The current trend within the industry is to isolate boars for 60 days or longer due to the problems associated with managing the P.R.R.S. virus (Amass & Baysinger 2006). During isolation, boars are trained for collection. When the isolation period is over, they enter production and are collected 2 to 5 times per week depending on sow breeding targets, season, genetic line, and other farm specific criteria (Knox *et al*. 2008). Finally, culling typically occurs when they are between 18 and 24 months old in order to maximize genetic improvement (Robinson & Buhr 2005).

An ideal situation for the swine industry would be to have an accurate estimate of relationships between the reproductive performance of boars and various semen quality tests when they enter production at 8 to 9 months of age or shortly thereafter. In order to accomplish this, quantitative data in the form of farrowing rates and litter sizes must be available at this time. This arguably is the most critical requirement. Without it, all subsequent assessments of boar and semen fertility are qualitative without a definitive reference point.

It is important to note that *in vivo* fertility data does not have to be obtained from every boar to establish these relationships as long as the sampling strategy accounts for the variability within the entire population. The 60 to 90 day isolation period provides an opportunity to obtain *in vivo* fertility data without influencing daily semen production destined for commercial farms. Semen collected from boars during this time typically is discarded so it would be available for "test" matings. In contrast, techniques that can accurately rank boars in terms of their relative fertility should be performed on several ejaculates from all boars during the isolation period with one occurring just prior to, or shortly after, their entry into the A.I. centre. Once *in vivo* fertility data become available from the subset of boars, the production of live pigs can be estimated for all boars based on their relative rank.

During the 10 to 16 month period that boars are producing semen destined for commercial farms, identification of sub-fertile ejaculates is important. This provides the main source of quality control and insures that ejaculates with low fertilizing potential are not used for inseminations.

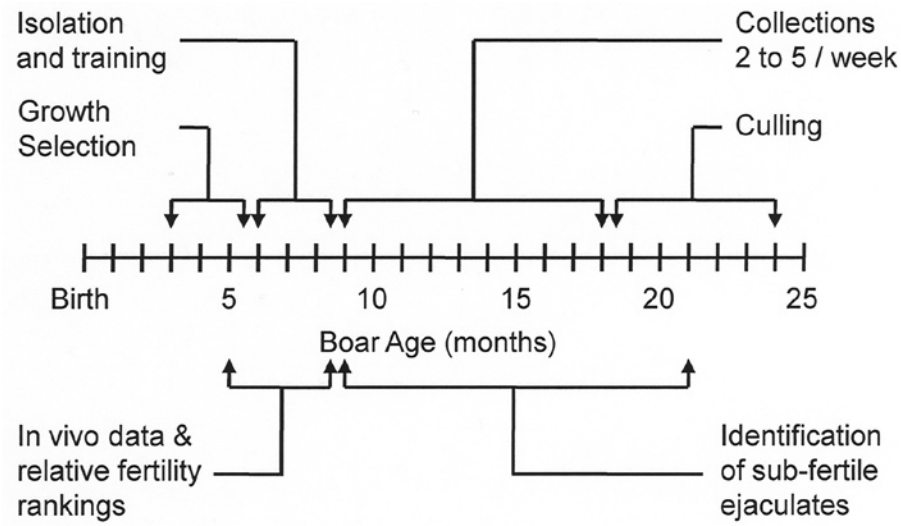


Fig. 1 Time line of productive life for modern boars housed in A.I. centres. Key management events are contained above the time line. Critical needs for evaluating boar fertility and semen quality are shown below the time line.

Screening for sub-fertility needs to be done on every ejaculate that is collected. These tests need to be performed accurately by A.I. centre personnel and their results have to be available immediately due to the short time interval between collection and production of insemination doses. It also may be necessary to reassess the relative fertility ranking of selected boars periodically during this phase. This would provide a way to monitor deviations from their fertility potential and make appropriate adjustments.

In summary, the three critical needs for boar fertility evaluations are to validate semen quality estimates with *in vivo* reproductive data; rank individuals in terms of their relative fertility; and identify and eliminate sub-fertile ejaculates. The first two should be met before or shortly after boars enter production, while the third one has to be addressed daily throughout their productive lives (Fig. 1). Consequently, determining which current techniques might be able to meet these critical needs is the next logical step in selecting for boar fertility and semen quality.

Obtaining *in vivo* fertility data for validation of semen quality estimates

Despite the reluctance of the swine industry to use of single-sire matings, the birth of live pigs is the best way to establish accurate reference points for male reproductive performance and validate *in vitro* fertility assessments. Results from a study conducted in a 200-head boar stud illustrate the importance of this process (Flowers 2002). In this particular study, each ejaculate with a motility score of 70% or greater was used to make insemination doses containing 1 to 9 billion total spermatozoa and used to breed between 75 and 100 sows from each insemination dose per boar. Changes in the number of pigs born alive in response to increasing the number of spermatozoa from 6 representative boars are shown (Fig. 2). The insemination dose at which litter size reached its peak and the actual number of pigs born alive at the optimal dose varied considerably among boars. These data demonstrate that there is a significant amount of variation in the fertility of boars currently used within the swine industry. Of particular interest was the identification of boars that demonstrated exceptional fertility at low insemination

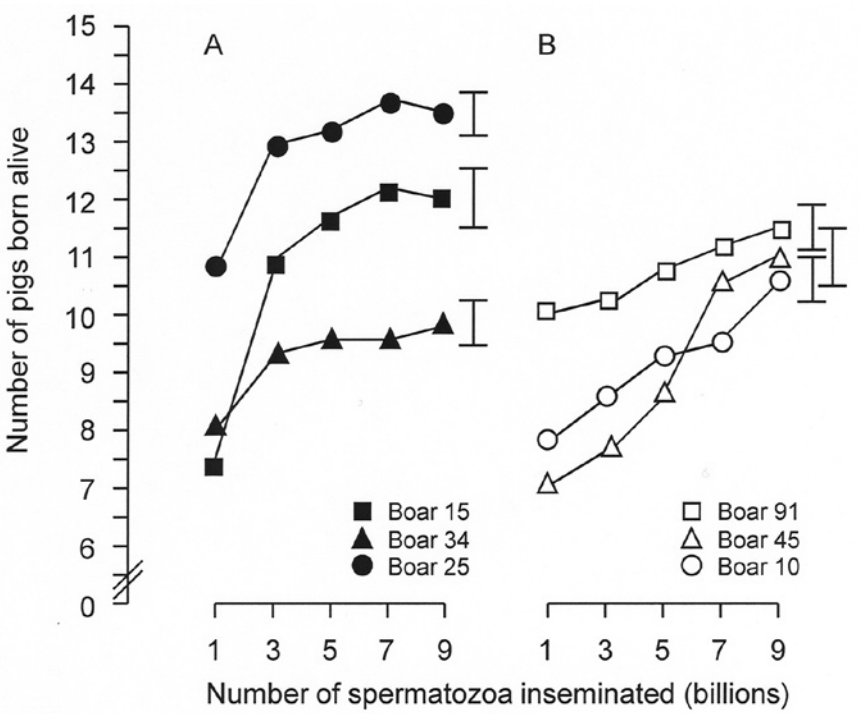


Fig. 2 Asymptotic (panel A) and linear (panel B) relationships between insemination doses and litter size (mean ± S.E.M.) in selected boars. (with permission from Flowers 2008).

doses generally thought to be suboptimal and their counterparts with poor results with doses commonly accepted as industry standards.

Ideally, it would be nice to have these data for all boars in an A.I. centre before they enter production. However, this is not practically or economically feasible. An alternative approach would be to evaluate a subset of the boars in the population. Fig. 3 contains frequency distributions for the farrowing index of boars used in the previous study. The farrowing index is an estimate of the live pigs a boar produces each time his semen is used to breed sows and is calculated by multiplying the farrowing rate by the average number of pigs born alive.

The goal of any sampling strategy is to obtain a subset that is representative of the entire population. In the context of boar fertility, a practical approach would be to consider boars as being sub-fertile; fertile; or exceptionally fertile. By industry standards, a reasonable estimation of sub-fertile would be a farrowing index of 8.0 or lower. This is because the decision boundaries for farrowing rate and number of pigs born alive are generally accepted as being 80% and 10 pigs (PigCHAMP 2008). Similarly, a farrowing index of 11 seems logical for the exceptionally fertile category. It corresponds to a 92% farrowing rate and 12 pigs born alive which often are used as reproductive targets in the industry (PigCHAMP 2008). If these groupings are applied to the farrowing index data (Fig. 3), then a random sample of 40 boars, or 20%, from this population should result in about 10, 23, and 6 boars from the sub-fertile, fertile, and exceptionally fertile groups, respectively. This should be sufficient to obtain reliable *in vivo* fertility data from boars in each classification and thereby an estimate for the entire population.

The insemination dose used to obtain *in vivo* fertility estimates deserves careful thought. In the above example, a dose of 3 billion total spermatozoa was inseminated because it is considered the industry standard (Knox *et al*. 2008). However, use of insemination doses lower than 3 billion

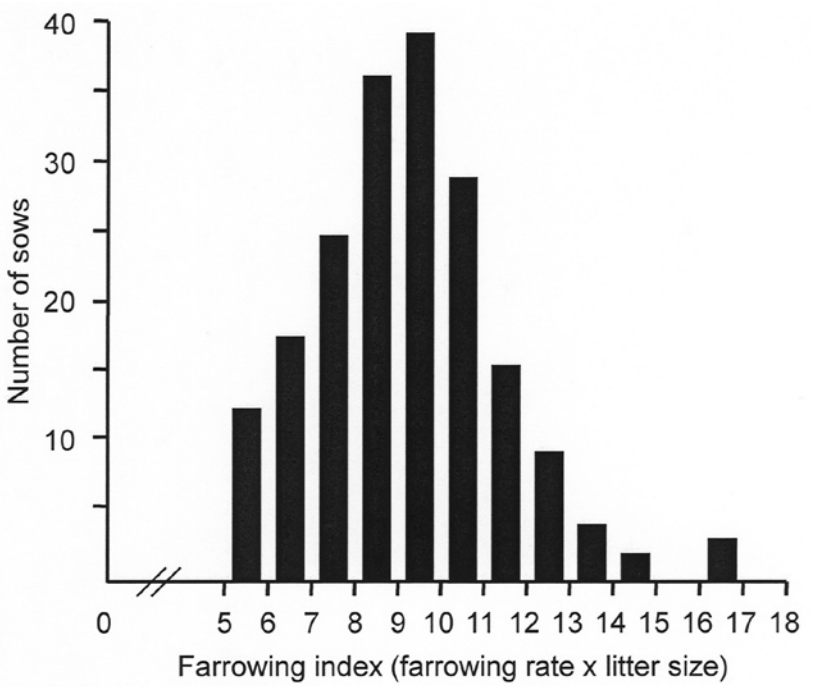


Fig. 3 Frequency distribution for farrowing index (farrowing rate x litter size) from a population of modern A.I. boars (n = 200).

spermatozoa enhances the identification of fertility differences among boars and increases the predictive value of many *in vitro* semen assessment techniques (Xu *et al*. 1998, Flowers 2002, Popwell & Flowers 2004, Ruiz-Shanchez *et al*. 2006). This decision, in part, depends on the relative importance of knowing the exact number of live pigs produced versus enhancing the ability of semen quality estimates to identify and rank fertile boars. Increasing the precision and accuracy of techniques for estimating boar fertility should also increase the number of live pigs produced.

Semen quality estimates for ranking relative boar fertility

A number of different techniques have been used to estimate the fertilizing ability of boar spermatozoa and, therefore, hold potential for identifying relative fertility differences. Excellent reviews describing the technical aspects associated with these tests and the physiology they mimic during fertilization are available (Rodriquez-Martinez 2003, Gadea 2005, Petrunkina *et al*. 2007, Foxcroft *et al*. 2008). What has not been addressed in as much detail is whether these tests can be integrated into a strategy to improve fertility estimates. Consequently, this possibility deserves further exploration.

Fertility estimates based on properties of spermatozoa

Selected techniques for estimating the fertility of boars by measuring various aspects of spermatozoa are summarized in Table 1. All are based on the premise that the proportion of sperm cells that possess a certain characteristic is either positively or negatively correlated with the ability of the ejaculate to fertilize ova. Those that measure motility, morphology, chromatin

structure, and surface proteins rely on estimation of a single trait. Others such as sperm binding, ooctye penetration, and in vitro fertilization estimate functional aspects of spermatozoa which, in essence, are the combination of many individual traits. Consequently, it is logical to assume that the ones that measure functional aspects would be better suited for ranking boar fertility than those that measure a single trait.

Table 1. Selected techniques for estimating semen quality and their relationship with *in vivo* fertility.

Technique	Correlation with *In Vivo* Fertility	References
Motility 7 to 10 days after storage	r = 0.36 to 0.46	Xu *et al*. 1998 Ruiz-Sanchez *et al*. 2006
Normal morphology at collection	r = 0.59	Xu *et al*. 1998
Spermatozoa with cytoplasmic droplets at collection	r = - 0.25	Ruiz-Sanchez *et al*. 2006
Spermatozoa with normal DNA structure	r = 0.27 to 0.91	Evensen *et al*. 1994
Sperm plasma membrane proteins	r = 0.38 to 0.53	Ash *et al*. 1994
Ubiquitin bound to spermatozoa	r = -0.31 to - 0.38	Lovercamp *et al*. 2007
Oocyte membrane binding assay	r = 0.80	Berger *et al*. 1996
In vitro fertilization – male pronuclear formation	r = 0.35 to 0.41	Ruiz-Sanchez *et al*. 2006

Surprisingly, the correlation coefficients between estimates of semen fertility and *in vivo* reproductive data do not support this assumption (Table 1). Those associated with a single physical aspect of spermatozoa were similar to those measuring their functional properties. Moreover, in the studies that used multiple regression techniques, motility and morphology estimates explained a large portion of the total variation observed in farrowing rates and number of pigs born alive in the population of boars being studied (Xu *et al*. 1998, Ruiz-Shanchez *et al*. 2006).

It is important to recognize that *in vivo* estimates of fertility were obtained via different methods for several of these studies. Work conducted by Xu *et al*. (1998) and Ruiz-Shanchez *et al*. (2006) used suboptimal insemination doses of 1.5 to 2 billion spermatozoa to obtain farrowing rates and litter sizes from their population of boars. In contrast, studies reporting very high correlations for normal DNA structure (Evensen *et al*. 1994) and oocyte binding (Berger *et al*. 1996) used heterospermic inseminations and paternity testing to estimate fertility in their boars. *In vivo* estimates of fertility varied from 2 to 98% of piglets sired with heterospermic inseminations, whereas farrowing rates and litter size normally were 70 to 90% and 9 to 12 pigs, respectively, with homospermic inseminations. Consequently, quantitative differences among correlations for some of these tests may be related to the manner in which *in vivo* estimates of boar fertility were obtained.

Nevertheless, the lack of consistent and significant advantages of multivariate techniques such as *in vitro* fertilization over those measuring univariate characteristics like motility has important implications for boar fertility evaluation. An underlying assumption for all semen tests is that characteristics of sperm cells measured *in vitro* reflect what happens to them *in vivo*. One fundamental difference between these two situations is the interaction of spermatozoa with the female reproductive tract, especially the oviduct. It is obvious that sperm-oviduct interactions are critical for successful fertilizations (Rodriquez-Martinez *et al*. 2005). What is not as clear is whether certain aspects of sperm function are influenced to a greater degree than others during their interaction with the female reproductive tract.

Results from a study originally designed to investigate the effect of the capacitation environment on *in vitro* fertilization efficiency may provide some insight into this question (Popwell 1999, Popwell & Flowers 2001). Briefly, ejaculates from boars were split and either processed for *in vitro* fertilization or used to inseminate sows. At selected time intervals after insemination, spermatozoa were recovered from the oviducts of sows or removed from the *in vitro* fertilization system and evaluated. Progressive forward motility decreased in both environments over time and tended to be

lower for spermatozoa recovered from the oviduct compared with those in the *in vitro* fertilization system (Fig. 4, top panel). In contrast, capacitation occurred very quickly *in vitro*, whereas a more protracted pattern was observed *in vivo* (Fig. 4, bottom panel).

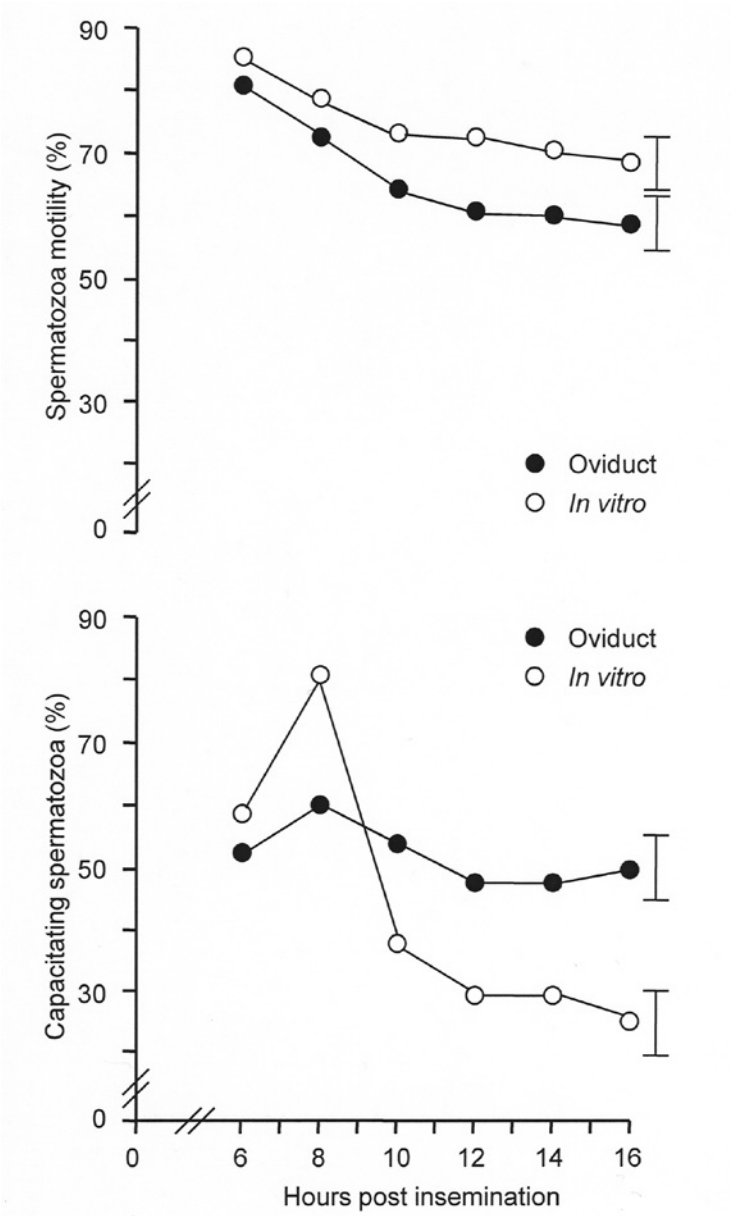


Fig. 4 Changes over time (mean ± S.E.M.) in the proportion of spermatozoa exhibiting progressive forward motility (top panel) and undergoing capacitation (bottom panel) after incubation *in vitro* and recovery from the oviducts (adapted from Popwell 1999 and Popwell & Flowers 2001).

These data help explain, in part, the lack of differences between univariate and multivariate tests in terms of their abilities to rank boar fertility. Estimates for progressive forward motility were equivalent between the *in vitro* and *in vivo* environments. Consequently, when the proportion of motile spermatozoa is estimated prior to insemination, this seems to be an

accurate reflection of the population present in the oviduct prior to fertilization. In contrast, the time course over which capacitation occurs *in vitro* does not appear to be representative of what occurs *in vivo*. Thus, there is variability in how the oviduct influences different functions of spermatozoa. Consequently, a component missing from functional tests that select for boar fertility is an estimation of how spermatozoa interact with the oviduct after insemination. Development of cell culture methodologies has potential to address this deficiency and preliminary investigations indicate that variations in the binding of sperm cells to oviductal explants *in vitro* are associated with boar fertility differences (Waberski *et al*. 2005). However, whether binding in vitro accurately reflects all the changes spermatozoa undergo while in the oviduct remains to be determined.

Fertility estimates based on seminal plasma proteins

Proteins in seminal plasma have been shown to influence many important processes associated with fertilization including regulating uterine function after mating (Rozeboom *et al*. 1998, Woelders & Matthijs 2001), ovulation (Waberski 1997), capacitation (Topfer-Petersen *et al*. 1998, Vadnais *et al*. 2005), oviductal binding (Petrunkina et al. 2001), and sperm-oocyte interactions (Caballero *et al*. 2004, Caballero *et al*., 2008). Moreover, when seminal plasma from boars of high fertility was used to replace that from boars of low fertility and vice versa, numbers of pigs born were increased and decreased, respectively (Flowers 1997). As a result, there has been increasing interest in using these as potential fertility markers for boars.

Selected seminal plasma proteins that have shown significant positive or negative correlations with farrowing rates and litter sizes are shown in Table 2. For the most part, correlation coefficients between relative amounts of these proteins and boar fertility are considerably stronger than those reported for various characteristics of spermatozoa (Table 1). This is particularly impressive because all the boars used in three of the studies had excellent motility and morphology estimates by industry standards (Flowers 1995, Ruiz-Sanchez 2006 as cited by Foxcroft *et al*. 2008, Turner & Flowers 2009).

Table 2. Relationships between relative amounts of selected seminal plasma proteins and *in vivo* fertility.

Seminal Plasma Proteins	Correlation with *In Vivo* Fertility	References
20 kDA, pI 6.0	r = - 0.76	Foxcroft *et al*. 2008
25-29 kDA, pI 5.9-6.2	r = 0.45 to 0.60	Flowers 1995 Foxcroft *et al*. 2008 Turner & Flowers 2009
55 kDA, pI 4.5-5.1	r = 0.56 to 0.62	Flowers 1995 Turner & Flowers 2009
60 kDA, pI 5.9	r = - 0.66	Foxcroft *et al*. 2008

It is clear that seminal plasma proteins modulate several important aspects of how spermatozoa interact with the oviduct after insemination. Consequently, it is tempting to speculate that variations in these proteins may reflect differences in the abilities that spermatozoa have to accomplish these tasks and, thus, prepare themselves for fertilization. Clearly, additional studies are required to validate this speculation. However, if it is correct, then quantification of specific seminal plasma proteins may provide a way to estimate how the oviduct and other parts of the female reproductive tract interact with spermatozoa after insemination, information that is clearly lacking from techniques currently used for male fertility evaluations.

Fertility estimates based on properties of spermatozoa and seminal plasma proteins

It is clear that techniques discussed previously have strengths and weaknesses in terms of their ability to rank the relative fertility of boars. In terms of those that estimate the properties of spermatozoa, motility and normal morphology appear to be just as good as those that measure more complicated aspects of sperm functions possibly because the latter do not account very well for the contributions of the oviduct. In contrast, some seminal plasma proteins show strong correlations with boar fertility and may be reflective of how effectively spermatozoa can undergo changes necessary for fertilization once they enter the sow. Thus, using combinations of these two types of assays should, in theory, enhance the ability to rank the relative fertility of boars.

A recent study investigated this possibility using heterospermic inseminations and subsequent paternity testing with 12 boars (Turner & Flowers 2009). Seminal plasma proteins and selected attributes of spermatozoa were measured in every ejaculate used for breeding. Relative concentrations of 26-28 kDa, p*I* 6.0 ($r^2 = 0.66$, $p < 0.001$) and 55-57 kDa, p*I* 5.6 ($r^2 = 0.05$, $p < 0.05$) proteins; the proportion of spermatozoa exhibiting an acrosome reaction ($r^2 = 0.15$, $p < 0.001$); and the proportion of motile spermatozoa ($r^2 = 0.03$, $p < 0.075$) explained 89% of the variation in the relative fertility in this population of boars. As mentioned previously, use of heterospermic inseminations and paternity testing has the advantage of increasing the variability in fertility compared to using farrowing rates and litter sizes. As a result, caution needs to be used when attempting to extrapolate these results to commercial situations using homospermic inseminations. However, the potential of combining several complementary techniques to rank the relative fertility of boars deserves a careful evaluation by the swine industry.

Semen quality estimates for identifying sub-fertile ejaculates

Acute and chronic stresses can temporarily reduce the fertility of boars (Flowers 1997). As a result, there is a need for procedures that can accurately screen for sub-fertile ejaculates. In theory any of the techniques discussed for ranking the relative fertility of boars could be used to accomplish this. However, the average time between collection and when the decision to keep or discard an ejaculate needs to be made is less than 1 hour in most commercial A.I. centres (Knox *et al*. 2008). This time limitation effectively eliminates all of the assays discussed previously (Tables 1 and 2) with the exception of motility and morphology evaluations.

Estimates of motility, albeit in various forms, are common in A.I. centres, while morphological evaluations are performed much less frequently and often in situations where a problem that isn't reflected by motility evaluations is expected (Knox *et al*. 2008). Therefore, it appears that the swine industry has procedures in place to provide data for meeting this critical need. Thus, the relevant question becomes how accurate are these routine evaluations for motility.

Results from a field study provide data to address this question (Flowers 1994). Video footage of ejaculates with different motilities were used either in training sessions or sent to personnel responsible for evaluating semen quality in A.I. centres in the southeastern U.S. Participants were asked to provide quantitative (actual percentages) and qualitative (keep or reject) estimates of motilities for each video segment. None of the participants were using CASA systems in their studs at the time the study began. The largest variances were associated with samples whose actual motilities were between 60 and 70% (Table 3). This was also the range in which the most mistakes were made in terms of keeping or rejecting ejaculates. Finally, there was a general tendency for technicians to overestimate motility.

Table 3. Accuracy and precision of boar sperm motility estimates by A.I. centre technicians in the southeastern U.S. (adapted from Flowers 1994).

Percentage of Motile Spermatozoa in the Ejaculate	Motility Estimate by Technician (%, mean + SEM)	Proportion of Technicians keeping Ejaculate	Proportion of Technicians rejecting Ejaculate
80	90 + 8	150 / 150	0 / 150
70	83 + 15	98 / 150	52 / 150
60	75 + 20	84 / 150	66 / 150
50	62 + 9	29 / 150	121 / 150
40	32 + 7	0 / 150	150 / 150

Previous studies have demonstrated that when insemination doses of 3 billion spermatozoa or higher are used, relationships between motility and reproductive performance in sows is asymptotic (Flowers 1997, Xu *et al*. 1998). The point at which fertility no longer increases at appreciable rates with increasing motilities is between 60 and 70%. Consequently, it appears that the accurate assessment of motility by A.I. centre personnel under field conditions is challenging, especially when ejaculates are close to physiologically relevant levels related to fertility. This compromises the ability of the swine industry, at least in the southeastern U.S., to meet the critical need of identifying sub-fertile ejaculates. Technologies associated with CASA have improved, while their costs have decreased over the past 10 years. These systems offer potential for improving the ability of A.I. centres to accurately estimate motilities and minimize the use of sub-fertile ejaculates.

Conclusion

An ideal situation for the swine industry would be able to have an accurate estimation of boar fertility by the time they enter A.I. centres around 9 months of age combined with the daily identification of sub-fertile ejaculates thereafter. Of these, the industry has the technology to identify sub-fertile ejaculates on a daily basis via routine assessment of motility. However, the accuracy at which this is currently being done within the industry is questionable and could be improved.

Development of tests that accurately rank the relative fertility of boars and procedures to validate these data with reproductive performance are necessary. The isolation period which typically lasts 60 to 90 days provides a reasonable time frame over which these data can be obtained without compromising semen used for breeding commercial sows. Single sire matings from about 20% of the boars being evaluated should be sufficient to obtain a good estimate of the variability in reproductive performance within the population. These data are viewed as critical in that they provide definitive reference points for all subsequent *in vitro* estimates of boar and semen fertility.

Ranking the relative fertility of all boars destined to enter production also needs to be done during the 60 to 90 day isolation period. This can be achieved with *in vitro* evaluations. Unfortunately, at the present time, there does not appear to be a single test that provides accurate estimates for relative fertility including those that measure the ability to fertilize ova *in vitro*. The proportion of motile spermatozoa after 7 to 10 days of storage appears to be as good as any other single test in terms of its predictive value. Moreover, it can be performed by most A.I. centres without additional investments in equipment or technical expertise. Most of the other techniques that can provide a relative ranking of boar fertility would likely have to be outsourced to external laboratories because they require specialized equipment and highly trained technicians.

Use of seminal plasma proteins as boar fertility markers is still a theoretical concept. However, it does show promise. It is an attractive technology from an industry perspective because if proteins with high correlations with male fertility are identified, then it should be possible to develop elisa-based tests that could be performed quickly and easily at A.I. centres. When seminal plasma protein data were combined with other estimates of semen quality the accuracy of predicting boar and semen fertility was improved significantly. Consequently, the way ahead for selecting for boar and semen fertility is through the application of multiple techniques performed at selected times during the productive life of boars.

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Changes in responsiveness to bicarbonate under capacitating conditions in liquid preserved boar spermatozoa in vitro

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Liquid-stored boar semen is commonly used for artificial insemination (AI) up to 72 h after dilution. Insemination with semen stored for longer periods generally results in reduced fertility. Standard semen parameters, i.e. motility and membrane integrity, usually give no indication of this reduction. Therefore, more sensitive methods are needed for detection of storage-induced changes in sperm quality. Capacitation has long been known to be an essential step in fertilization. In a number of studies bicarbonate has been shown to be the key capacitating agent in boar sperm in vitro (reviewed in Harrison & Gadella 2005). The ability of sperm to respond to bicarbonate in vitro by undergoing capacitatory changes can be measured as a sperm property crucial to fertilization (Petrunkina *et al*. 2005a; Silva & Gadella 2006). In this study we used calcium influx as a parameter to investigate the responsiveness of stored semen samples to bicarbonate. This parameter has been shown to be sensitive with respect to evaluating detrimental effect of cooling during liquid storage of boar sperm (Petrunkina *et al*. 2005b).

Three ejaculates from each of 14 boars of proven fertility were diluted in Beltsville Thawing Solution (BTS) extender to a concentration of 20 x 10^6 sperm / ml and stored at 17°C. After 12, 24, 72, 120 and 168 h of storage, motility was assessed in the diluted semen with a CASA-system, and membrane integrity was checked with propidium iodide (PI) and FITC-conjugated peanut agglutinin (FITC-PNA) using a flow cytometer. Samples were then washed through Percoll, loaded with the calcium probe Fluo-3-AM and PI, and incubated at 38°C in parallel in two variants of a Tyrode's medium. Medium A contained 15 mM bicarbonate as well as 2 mM Ca^{2+}, whereas bicarbonate was omitted from medium B; incubation in medium A was performed under 5% CO_2. Changes in Ca^{2+} influx were assessed on a flow cytometer at 3, 20, 40, 60, 90, 120, 150 and 180 min. The resulting kinetics of cell sub-populations were compared between media and storage time points, based on analyses of the non-agglutinated population.

During storage, motility declined only from 89.0 $\pm$ 3.2 to 74.4 $\pm$ 10.4% ($p = 0.001$) and membrane integrity from 83.1 $\pm$ 4.2 to 71.0 $\pm$ 20.9% ($p = 0.001$). However, bicarbonate induced marked changes in membrane permeability, as measured by increases in the population of Ca^{2+}-positive and PI-negative (live) cells as well as by increases in the population of PI-positive (dead) cells. After 12 and 24 h of storage, the population of Ca^{2+}-positive and PI-negative cells reached a maximum within 90 min of incubation in medium A, but as storage was prolonged the increase lessened although it reached its maximum more rapidly (after 40-60 min). A time point of 60 min was chosen for comparisons between storage periods and media. Values for the total % Ca^{2+}-positive / PI-negative cells in medium A declined significantly from 21.6 $\pm$ 6.4% at 12 h to 15.7 $\pm$ 2.78% at 72 h of storage ($p < 0.01$) after which they stayed at a constant level. At 3 min of incubation proportions of PI-positive (dead) cells in medium A varied between 9.8 $\pm$ 4.9% at 12 h of storage and 14.7 $\pm$ 3.3% at 168 h, whereas after 60 min of incubation their percentage had risen to around 46% regardless of storage period. In contrast, in medium B, 60 min values of both populations (i.e. Ca^{2+}-positive / PI-negative and PI-positive cells), though initially much lower than in Medium A, increased significantly through-

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out the whole storage period ($p < 0.01$) until they were comparable with the values in medium A. Response to capacitating conditions as measured by the change in % Ca^{2+}-negative / PI-negative cells between 3 min and 60 min of incubation (Δ_{60-3}) declined significantly in medium A during storage from 58.6 ± 8.2% after 12 h to 36.4 ± 8.6% after 168 h ($p < 0.001$), whereas in medium B it increased from 6.8 ± 4.4% to 24.0 ± 3.7% ($p = 0.001$). However, storage also resulted in a change in the sub-population distributions at the initial (3 min) incubation point. There were significantly higher ($p < 0.01$) percentages of Ca^{2+}-positive / PI-negative cells and PI-positive cells detected in both medium A and medium B: in medium A 10.1% Ca^{2+}-positive / PI-negative and 14.7% PI-positive after 168 h versus 3.3% and 9.8% respectively after 12 h; in medium B 9.1% Ca^{2+}-positive / PI-negative and 14.7% PI-positive after 168 h versus 2.1% and 7.2% respectively after 12 h. These data could be interpreted as indicating that storage has two effects. There was on the one hand a destabilization of the greater proportion of the population such that incubation even in the absence of bicarbonate caused membrane deterioration while incubation with bicarbonate caused such rapid membrane destabilization that the Ca^{2+}-positive / PI-negative state was increasingly short-lived before PI entered. These findings are in agreement with those of Petrunkina *et al*. (2005b), reporting that levels of Ca^{2+} uptake at the beginning of incubation under capacitating conditions are influenced by storage conditions. However, we also obtained evidence that the cohorts of cells that were less immediately responsive to bicarbonate became refractory, reducing the overall bicarbonate response in terms of appearance of Ca^{2+}-positive / PI-negative plus PI-positive cells.

As expected, motility and membrane integrity of the stored cells did not sufficiently reflect storage-dependent changes in sperm quality during prolonged storage. A recent review (Petrunkina *et al*. 2007) has proposed several requirements for the assessment of functional sperm parameters under capacitating conditions. In accordance with these proposals, we used a kinetic experimental approach with well-defined test and control media, considering equally both initial response and subsequent kinetics. Specific sperm response to bicarbonate as measured by intracellular increases in Ca^{2+} in live cells declined remarkably already after 72 h of liquid storage and dropped to almost zero in samples stored for 168 h. However, bicarbonate routinely caused large increases in dead cells within 20 min of incubation. In their original paper on fluo-3 detection of bicarbonate-mediated changes in boar sperm, Harrison *et al*. (1993) interpreted fluo-3-detectable Ca^{2+} entry as indicating the onset of a membrane destabilization eventually leading to cell death. Harrison (1996) proposed that if such destabilization were too rapid, fertilization would be compromised. Our findings suggest that boar semen contains a population of cells initially responsive to bicarbonate which becomes increasingly intrinsically unstable during storage and responds to bicarbonate too rapidly. In addition, more stable cohorts of cells exist that during storage become less responsive to bicarbonate. We suspect that the dual opposing effects of storage, namely destabilization and stabilization, may imply that after normal insemination life-time of most sperm in the female tract will be insufficient to ensure satisfactory fertilization levels, while the remainder may not respond to fertilizing conditions at all.

This work was supported by Development Association for Biotechnology Research (FBF e.V., Bonn)

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Intrauterine insemination of sows by using a two-chamber semen bag system

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Artificial insemination of sows is an effective method for intensive use of high breeding value boars. In sows, intracervical insemination (ICI) using 2 to 3 billion spermatozoa is an established method worldwide, resulting in a consistently high fertility (Watson *et al*. 2002). Using intrauterine insemination, the number of spermatozoa per insemination can be reduced even further. In intrauterine insemination (IUI), the tip of the catheter is placed in the corpus uteri depositing the semen even closer to the site of fertilization in the oviduct (Vazquez *et al*. 2008). If IUI can be performed as easily and efficiently as ICI, this will result in more semen doses per boar. This will allow a stronger selection among boars, and lower the costs per dose. This study investigated the effect on fertility, when reducing the amount of sperm per dose using IUI.

A total of 9272 multiparous Danish Landrace x Large White crossbred sows from seven Danish commercial herds were randomly distributed into three groups as shown in table 1. All sows were inseminated using heterospermic semen from Danish Duroc boars. Semen was collected using the gloved hand method collecting the whole ejaculate. Semen quality was evaluated using subjective microscopic motility score. Semen concentration was measured using NucleoCounter SP100. Semen was extended using EDTA boar semen extender. The same batch of semen comprising semen from 6 to 10 boars was used in all three groups in each herd. One dose from each batch was analysed for content of sperm per dose using SP100.

Table 1. Method of insemination, no. sperm per dose and volume in the three groups.

Group	Method of AI	No. Sperm per dose	Volume (L)
ICI	Traditional AI with normal bag	2 x 109	80
IUI-750	IUI with two-chambered bag	750 x 106	30* + 50
IUI-500	IUI with two-chambered bag	500 x 106	20* + 60

* volume of chamber containing extended semen

Estrus detection was performed for all sows daily from day 4 after weaning using the "five-point-plan" as described by Madsen *et al*. (2002). Prior to insemination, sows were stimulated according to the "five-point-plan". A boar was present in front of the sows during insemination. Sows were inseminated when estrus was detected and inseminated at 24-hour intervals, until standing reflex was no longer present. All inseminations (ICI and IUI) were carried out by farm workers. Before the IUI, the farm workers had been thoroughly instructed by a veterinarian. For intracervical insemination, a standard semen bag was used. To avoid a negative dilution effect when using the low number of sperm for the IUI, a Porcivet two-chamber bag (Joergen Kruuse A/S, Denmark) was used. One chamber was filled with extended semen, the other chamber contained EDTA-extender. Prior to insemination, the semen and extender were mixed in the double-chambered bag. A traditional flexible foam tip catheter was used for intracervical insemination. The catheter used for intrauterine insemination was the Porcivet Cervi-slip catheter (Jørgen Kruuse A/S, Denmark). The tip of the IUI catheter was fixed in the cervical canal after which the inner catheter was led gently through the cervix into the uterus.

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Analyses of semen doses showed a mean content of 2093 x 10^6, 764 x 10^6 and 510 x 10^6 sperm per dose for ICI, IUI750 and IUI500, respectively. The deviation was 249, 70 and 43 respectively. This is as expected during production of semen doses and is therefore estimated not to have an influence on the results of this trial.

The average parity in all three groups was 4.1. Farrowing rate and total number of piglets born are shown in table 2.

Table 2. Number of sows and results achieved in the three groups.

Group	Total number of sows	Total number of litters	Farrowing rate	Total born piglets per litter
ICI	3,099	2,793	90.2	16.5*
IUI-750	3,077	2,807	91.3	16.3
IUI-500	3,021	2,684	88.9	16.2*

* Significant different ($p < 0.05$)

A significant difference of 0.3 total piglet per litter ($P = 0.0139$) was seen between the control group and IUI500. There was no significant difference in litter size between the control group and IUI750. There was no significant difference between the control group and the two IUI-groups in farrowing rate.

Intrauterine insemination could not be performed in 1.2 percent of the sows as the inner catheter could not pass through the cervix into the uterus. Whenever IUI was not possible, the sows were inseminated using ICI. Sows inseminated using IUI did not show signs of pain or discomfort. At the beginning of the trial, attending farmers were mainly positive to IUI. However, thorough training in performing the technique is necessary. Also, they felt that IUI required more time than ICI, since an extra catheter needed to be inserted.

Intrauterine insemination can lead to a significant reduction in the number of sperm per insemination under farm conditions - without affecting fertility negatively. The insemination dose containing heterospermic semen can be lowered from 2 x 10^9 to 750 x 10^6 sperm per dose with this technique. These results demonstrate that the total number of sperm needed for successful AI is far lower than the standard number of sperm per dose.

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Binding of porcine spermatozoa to uterine epithelial cells modulates the female immune response and might indicate the formation of a pre-oviductal sperm reservoir

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Inseminations in pigs are characterized by the tremendous amount of spermatozoa needed for successful fertilisation. If, in contrast, spermatozoa are deposited at the tip of the uterine horn a fraction of the usual porcine insemination dose suffices (Johnson 1991, Vazquez *et al*. 2005). It thus seems to be the uterine passage where the need for such high sperm numbers arises. In the past the provision of sufficient sperm numbers to reach acceptable fertility rates did not pose a problem due to the abundance of spermatozoa in one single boar ejaculate. However, modern biotechnological procedures, such as sex sorting of spermatozoa, require insemination of sperm portions containing no more then 50 x 10^6 spermatozoa. To facilitate insemination with such small sperm doses also for conventional AI-techniques, more knowledge has to be gathered about fundamental sperm transport and selection mechanisms within the uterus. Previous studies on the pig uterus (Lovell & Getty 1968), the utero-tubal junction (Rodriguez-Martinez *et al*. 1990) and the oviduct (Wagner *et al*. 2002) suggested that spermatozoa are indeed subject to close interaction and even binding with the epithelial structures of the female genital tract. The present study aimed to further our understanding of such interactions specifically in the porcine uterus.

For this purpose an ex vivo model was developed using uterine segments of 10 cm derived from 50 freshly slaughtered peri-ovulatory German Landrace gilts. In each segment 100 x 10^6 spermatozoa were incubated for 60 min at 38°C. The sperm cells originated from ejaculates provided by 4 boars of the same breed and of proven fertility. Previous to incubation spermatozoa were either washed and diluted in the semen extender Androhep™ or diluted with autologous seminal plasma without further washing. The spermatozoa were subsequently flushed out of the segments, counted and their viability parameters were established flow cytometrically using the stains PI and JC1 for membrane integrity and mitochondrial membrane potential respectively.

The results indicated a retention of viable spermatozoa within the uterine cavity since only $55 \pm 7\%$ of the intact spermatozoa (PI-/JC1+) were rediscovered in the flushing, while the damaged sperm population (PI+/JC1-) was flushed out almost in its entity ($93 \pm 12\%$; $p < 0.03$). The effect was more emphasised in the sperm population, which had been washed and diluted in Androhep™ ($p < 0.05$). The location of the uterine segments in relation to the cervix had no effect on the numbers of recovered sperm cells. Neither were differences observed between segments from the right and left uterine horn.

In order to determine the physiological reasons behind the observed uterine sperm retention an in vivo experiment was performed looking for changes in uterine gene expression in response to insemination by measuring endometrial mRNA concentration. Because many processes concerning reproduction such as ovulation and implantation involve the immune system, the

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mRNAs chosen to be examined encoded for 6 cytokines suggested to have impact on reproductive outcome (GM-CSF, IL-10, CXCL8, TNF-α, TGF-β & IL-6) and 2 products of the arachidonic acid metabolism (COX-2, ALOX-5). For the experiment 68 periovulatory gilts received a 90 ml uterine infusion consisting of either Androhep™ (AH) or seminal plasma (SP) with or without spermatozoa. Endometrial tissue samples were taken 3h after treatment. The mRNA copy numbers were determined by qRT-PCR and compared with baseline expression obtained from 12 otherwise identically treated non-inseminated sows. Results showed that all chosen genes displayed baseline expression. However, insemination without spermatozoa led to a general up-regulation of all examined genes, which was significant in case of IL-10 (SP: 1.5-fold), CXCL8 (AH: 7.1-fold), TNF-α (AH: 1.9-fold) and COX-2 (AH: 7-fold). Interestingly, despite their majorly different composition Androhep™ and seminal plasma elicited a significantly different response only concerning the arachidonic acid metabolite COX-2. Most surprisingly however was the result that the presence of spermatozoa led to a significant down-regulation back to baseline levels for every mediator tested.

In conclusion, based on the described results we propose the hypothesis that the immense amounts of spermatozoa needed for insemination in pigs is at least partially due to sperm binding sites along the endometrium, which need to be saturated before unbound sperm can proceed to the oviduct. Interestingly, the majority of bound spermatozoa are membrane intact and possess functional mitochondria, i.e. are potential candidates for fertilisation. The exact binding mechanism and the biological consequences remain, however, elusive. The sperm binding sites might serve several purposes. For once, the bound spermatozoa might serve as a secondary reservoir to feed the oviductal reservoir, in case of a delayed ovulation. The fact that bound sperm are viable support this presumption. On the other hand, they could also function as part of a negative selection mechanism, which prevents boars with oligozoospermia to mate successfully, a fact that would have to be overcome for low dose insemination for instance by changing the site of application close to the tip of the uterine horn as described by Vazquez *et al*. (2005). Furthermore, the observed sperm-associated modulation of uterine gene expression strongly suggests that sperm binding to endometrial cells has a considerable influence on the female immune response. The down-regulation of inflammation-relevant cytokines and arachidonic acid metabolites might serve to tightly control the issuing post-mating inflammatory reaction described in pigs (Matthijs *et al*. 2003). As a long-term perspective it could also be part of the tolerance induction process against paternal antigens or otherwise set off the preparation of the uterus for the reception of the conceptus.

We gratefully acknowledge the financial support of the Hans Wilhelm Schaumann Foundation and the German Research Foundation.

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Sperm survival following colloid centrifugation varies according to the part of the sperm-rich fraction used

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Spermatozoa present in the first 10 ml (portion 1, P1) of the sperm-rich fraction (SRF) of boar ejaculates show increased resilience to cooling and cryopreservation compared to those in the rest of the ejaculate (Saravia *et al*. 2008) presumably because of differences in exposure to seminal plasma. A new technique for selecting the most robust animal spermatozoa, Single Layer Centrifugation (SLC), has recently been developed at SLU (Morrell & Rodriguez-Martinez 2009). It was shown that SLC of SRF using Androcoll-P™ followed by storage at room temperature (22°C) in extender without antibiotics resulted in sperm preparations of high motility which survive longer than unselected sperm samples (Wallgren *et al*. 2008). However, it is not known how SLC using P1 instead of SRF would affect boar sperm survival. Furthermore, the technique requires scaling-up to enable larger volumes of ejaculate to be processed. Scaling up has been shown to be possible for stallion spermatozoa (Morrell *et al*. unpublished data).

In experiment 1, P1 from 12 ejaculates (4 boars, 3 ejaculates per boar) and 12 SRF from the same boars (again 3 ejaculates from each boar), collected on different occasions, were extended in Beltsville Thawing Solution (BTS) without antibiotics to achieve a sperm concentration of $100x10^6$ mL. Aliquots (1.5 ml) of these sperm samples were prepared by SLC on Androcoll-P™ by centrifugation at 300xg for 20 min. The resulting sperm pellets were washed in 5 ml BTS + bSA (5 mg/ml) by centrifugation at 500xg for 10 min. before resuspending in 1.5 ml of the same extender. The sperm suspensions were stored in a styrofoam box at room temperature (22°C) for up to 6 days. Sperm motility was assessed subjectively on a daily basis using phase contrast microscopy, after incubating the sperm suspensions at 38°C for 30 min. Differences between means were tested for statistical significance by analysis of variance, where $P < 0.05$ was considered significant.

In the second experiment ("scale-up"), larger volumes of extended semen were used, e.g. 3 ml or 4.5 ml were pipetted on top of 4 ml Androcoll-P™ and 10 ml, 12.5 ml or 15 ml extended semen were pipetted on top of 15 ml Androcoll-P Large, an optimized formulation for larger tubes. After centrifugation, the sperm pellet was resuspended in 3 ml or 10 ml BTS with bSA (1.25 mg/ml) respectively. Sperm motility was analysed by computer assisted sperm analysis (CASA) on 5μl aliquots of sperm samples placed in a pre-warmed Makler chamber (Sefi Medical Instruments, Haifa, Israel), depth 10 μm, using a Mika Cell Motion Analyzer (MTM Medical Technologies Montreux, Switzerland) and a microscope equipped with a warm stage and phase contrast optics (20x objective, Optiphot-2, Nikon, Japan). Two hundred spermatozoa per sample were examined.

In experiment 1, the mean differences in % sperm motility between control (non-SLC-selected) and SLC-selected samples were as follows: time 0 h, P1 +13.3%, SRF +16.6%; at 24 h, P1 +23.7%, SRF +23.4%; at 48 h, P1 +10%, SRF +24.6%; at 72 h, P1 +9.6%, SRF +39.2%; at 96 h, P1 +9.4%, SRF +39.2%; at 120 h, P1 +6.7%, SRF +25.8%. The non-SLC-selected samples showed bacterial growth after the first 24 h, which may have contributed

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to the subsequent deterioration in sperm motility. SLC-selected sperm samples from SRF had a higher motility than non-selected sperm samples and retained their motility for longer ($P < 0.001$), even at this relatively high storage temperature (22°C) without antibiotics. SLC-selected spermatozoa from SRF had significantly better motility than those from P1 at 24, 48, 72 and 96 h ($P < 0.01$), although not at 0 or 120 h ($P > 0.05$), and retained their motility longer. SLC-selected spermatozoa from P1 were significantly better than controls only at 0 and 24h ($P < 0.01$).

For experiment 2, there were no differences in yield or motility when 3 ml or 4.5 ml extended ejaculate was used on 4 mL Androcoll-P™ nor between 10 ml, 12.5 ml or 15 ml on 15 ml Androcoll-P™-"Large". In a further experiment where 4.5 ml were compared with 15 ml on the "small" and "large" colloids respectively, there was no difference between the "small" and "large" preparations (sperm motility: "small" = 81.7 ± 7.7, "large" = $84.9 \pm 6.1\%$; yield: "small" = $43.7 \pm 10.1\%$, "large" = $42.4 \pm 23\%$). The motility of the unselected spermatozoa in these experiments was $77.4 \pm 19\%$. It was interesting to note that the yield of spermatozoa was lower than in the previous experiment (performed 15 months earlier) which may be due to deteriorating quality in the semen of boars as they age.

The findings indicate that SLC selected the most motile spermatozoa from both P1 and SRF. Moreover, SLC-selection removed bacterial contamination occurring during semen collection, enabling the sperm suspensions to be kept without antibiotics in the semen extender or requiring a reduced temperature. SLC-selection of SRF resulted in better sperm motility than SLC of P1 suggesting a potentially critical relationship between initial sperm numbers and accessory gland secretions. These observations are of interest to the swine AI-industry for the following reasons: (i) reducing the use of antibiotics in semen extenders; (ii) simplifying sperm storage requirements; (iii) providing additional information about the effect of seminal plasma on boar sperm survival. Further studies on all these issues are warranted. Finally, the results of the second experiment showed that it was possible to scale-up the volumes of semen used from the 1.5 ml used in the first experiment to 15 ml. This experiment is continuing, to scale-up to even larger volumes and to assess other parameters of sperm quality.

Funded by the Swedish Farmers´ Foundation for Agricultural Research and FORMAS, Sweden.

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The role of gene discovery, QTL analyses and gene expression in reproductive traits in the pig

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The reproductive performance of the sow is one of the key factors affecting production profitability of the pig industry. Reproductive traits are in general, lowly heritable, and with reliable markers, they can be used to enhance current selection procedures for improvement of these traits. To find potential markers, large scale quantitative trait loci (QTL) and candidate gene studies have been conducted for reproductive traits. The present review discusses QTL and candidate gene discovery, large scale SNP association studies, gene expression profiling and discovery of miRNA regulation of pig reproductive tissues. Many QTL have been found for reproduction traits and a limited number of useful genes (e.g.: ESR1, PRLR, FSHB, EPOR and RBP4) have been found to have significant associations with reproductive traits. Expression studies with reproductive tissues have revealed differential expression within a few gene networks which need further mapping and association analyses to select prospective gene markers. The near completion of the pig genome sequence and the development of high density SNP chips will allow for large scale SNP association studies for pig reproductive traits in the future. Collection of appropriate phenotypes in large numbers and in broad populations representative of the swine industry are required if such genomic studies will ultimately be successful.

Introduction

Reproductive efficiency in pig breeding herds can best be measured as pigs per sow per year among all breeding females. Pork producers are also increasingly concerned with the length of sow productive life in a herd. Productive sows represent those animals which can farrow a litter of pigs, lactate for ~ 21 days, return to estrus, successfully conceive, complete gestation, and finally farrow again and again over many parities. The recent advances in pig genomics including whole genome sequencing have provided the identification of useful candidate genes and QTL (quantitative trait loci), expressed sequence tags (ESTs), single nucleotide polymorphisms (SNPs) and microRNAs; which all may affect reproductive phenotypes. This review focuses on the studies previously conducted and the roles they may play in improved reproductive performance.

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Quantitative approaches to improved reproduction

Reproductive traits in the male and female differ considerably. In males, reproductive traits or performance may be measured by testis size, semen volume, sperm concentration of the ejaculate, sperm quality and libido or breeding aggressiveness. Reproductive traits in females include age at puberty, estrous cycles and expression, litter size, weaning to estrus interval and farrowing interval. The component traits of litter size are ovulation rate, fertilization rate, embryo survival and uterine capacity. Fertilization rate is contributed in part by the boar. Embryo survival and uterine capacity have also been viewed in part as under the genetic control of the embryo/fetus. Hormone levels and control of hormone receptors are also important traits under consideration. Genetic differences have been observed both among breeds and lines. Those differences can be most effectively exploited through the use of crossbreeding. Within breed or line, heritability estimates are measures of the additive genetic variation that can be manipulated via selection of superior animals. Estimates of genetic parameters, heritabilities and genetic correlations vary for several reasons, including the breed(s) studied, method of analysis and sampling variation. Estimates of heritabilities for several traits are summarized (Lamberson 1990, McLaren & Bovey 1992). Estimates for most male traits are moderate (e.g. 0.4, testis wt.) and would be expected to respond to selection while most of the heritabilities for the female traits (e.g. 0.07, number born alive) are low and progress utilizing selection is expected to be more limited. Hence, while progress can be made using conventional selection, marker assisted selection (MAS) using useful genes and markers offer an opportunity to improve selection programmes for reproductive traits and reduces generation interval and enhances the accuracy of selection (Spotter & Distl 2006).

Candidate genes and QTL

To identify genetic markers the first approach has been to use genome scans using microsatellites to find quantitative trait loci (QTL) (Rathje *et al*. 1997, Rohrer *et al*. 1999, Wilkie *et al*. 1999) and the second approach has been to use candidate genes thought to play a role in controlling phenotypes (Rothschild *et al*. 1996, Drogemuller *et al*. 2001, Jiang *et al*. 2001). QTL analysis is the identification of genomic regions that are responsible for genotypic differences in a desired trait. Most QTL analyses have used at least three generations hence it is time-consuming to produce such pig populations for reproductive traits. Several QTL were found for both male and female reproductive traits (Table 1 and 2). However, initial studies revealed that chromosomes 8 and X harbored many QTL for female and male reproductive traits, respectively. Generally, QTL regions cover 10-20cM regions which are difficult to use in selection programmes. Thus fine mapping of QTL is necessary to develop markers to use in marker assisted selection programmes (Distl 2007). Several QTL have been found for reproduction traits but further research is required to find the causative genetic variation in the gene influencing the trait. Studies on the association of positional candidate genes are progressing and these studies require useful commercial populations for validation. The requirement for the candidate gene approach is to test the gene variants in different populations (Rothschild *et al*. 2000). Because lowly heritable reproductive traits are influenced greatly by management and other environmental influences, it is important to test the association of candidate genes with phenotypic traits in different populations under different farm conditions. There may be inconsistencies in associations of candidate genes with phenotypes in different studies but this does not mean that the gene marker does not work. Failure to be predictive of a trait may be the result of the small sample size used to test the association. In addition, association studies are usually affected by differences in the frequency of alleles and genotypes responsible for the candidate gene effects, different linkage phases between the marker and causal mutation in different populations and epistatic effects (Distl 2007).

Table 1. QTL for female reproductive traits in pigs.

Trait	SSC	Population*	Reference**
Age at puberty	1, 10	WC x M	Rohrer *et al*. 1999
	7, 8, 12	LW x Lr	Cassady *et al*. 2001
	7, 8, 12, 15	LW x Lr	Holl *et al*. 2004
Ovulation rate or Number of corpora lutea	4, 8, 13, 15	LW x Lr	Rathje *et al*. 1997
	8	Y x M	Wilkie *et al*. 1999
	8, 3, 10	WC x M	Rohrer *et al*. 1999
	8	Y x M	Braunschweig *et al*. 2001
	9	LW x Lr	Cassady *et al*. 2001
	9	LW x Lr	Holl *et al*. 2004
	3	M x D	Sato *et al*. 2006
Uterine capacity	8	WC x M	Rohrer *et al*. 1999
Gestation Length	9	Y x M	Wilkie *et al*. 1999
Litter size	6	GMP x M	Yasue *et al*. 1999
	7, 12, 14, 17	LW/Lr x M	De Koning *et al*. 2001
Total number born	8	LW x M	King *et al*. 2003
Number born alive	11	LW x Lr	Holl *et al*. 2004
	1	(LW x Lr) x Lc	Buske *et al*. 2006a
	7, 16, 18	LW x F Lr	Tribout *et al*. 2008
Number of still born	4	Y x M	Wilkie *et al*. 1999
	5, 13	LW x Lr	Cassady *et al*. 2001
	5	LW x Lr	Holl *et al*. 2004
	6, 11, 14	LW x F Lr	Tribout *et al*. 2008
Teat number	1, 3, 10	WC x M	Rohrer 2000
	1, 7	GMP x M	Wada *et al*. 2000
	1, 8, 6, 7, 11	LW x Lr	Cassady *et al*. 2001
	2, 10, 12	M x DP	Hirooka *et al*. 2001
	8	LW x M	King *et al*. 2003
	1, 8	M, P, WB crosses	Beeckmann *et al*. 2003
	5	M, P, WB crosses	Lee *et al*. 2003
	10	M, P, WB crosses	Dragos-Wendrich *et al*. 2003
	12	M, P, WB crosses	Yue *et al*. 2003
	X	M, P, WB crosses	Cepia *et al*. 2003
	5, 10, 12	Ib x M	Rodriguez *et al*. 2005
	3, 7, 8, 16, 17	LW x M	Bidanel *et al*. 2008

* F Lr: French Landrace; LW: Large white; Lr: Landrace; Y: Yorkshire; M: Meishan; WC: White composite; D: Duroc; GMP: Gottingen miniature pig; Lc: Leicoma, P: Pietrain; WB: Wild boar; DP: Dutch piglines; Ib: Iberian
** Most of the data were obtained from http://www.animalgenome.org/QTLdb/pig.html.

A list of some associations of candidate genes with female reproductive traits in different populations is presented in Table 3. The first discovered and perhaps most important being *ESR1*, which is a steroid hormone receptor mediating the actions of estrogens. The association of *ESR1* with litter size was first reported by Rothschild *et al*. (1996) who found a PvuII polymorphism in intron 9 of *ESR1* in Meishans, Meishan Synthetic lines and Large White populations. Among the PvuII genotypes (AA, AB and BB), the BB sows farrowed 2.3 and 1.5 piglets more than the AA sows for both the total number of piglets born (TNB) and the number born alive (NBA) traits respectively in Meishan synthetics and larger differences among purebred Meishan. Similar results of *ESR1* associations were found in Large White and Yorkshire populations by subsequent studies (Table 3) though the effects were smaller but still quite significant. Short

Table 2. QTL for male reproductive traits*

Trait	SSC	Population	Reference
Testicular weight			
300 d	X, 1, 7, 5	White Duroc x Erhualin	Ren *et al*. 2008
220 d	X	Meishan x White composite	Rohrer *et al*. 2001
180 d	X	Meishan x Large White	Bidanel *et al*. 2001
90 d	X, 1	White Duroc x Erhualin	Ren *et al*. 2008
60 d	X, 3	Meishan x Duroc	Sato *et al*. 2003
Epididymal weight			
300 d	7, 3	White Duroc x Erhualin	Ren *et al*. 2008
180 d	4, 10, 13, 15 and X	Meishan x Large White	Bidanel *et al*. 2001
90 d	2	White Duroc x Erhualin	Ren *et al*. 2008
Seminiferous tubular diameter			
300 d	16	White Duroc x Erhualin	Ren *et al*. 2008
90 d	X, 14, 13, 5	White Duroc x Erhualin	Ren *et al*. 2008
Serum testosterone concentration			
300 d	7, 13	White Duroc x Erhualin	Ren *et al*. 2008
Plasma FSH levels	3, 10, X	Meishan x White composite	Rohrer *et al*. 2001

* The data were obtained from http://www.animalgenome.org/QTLdb/pig.html.

et al. (1997) showed an additive effect of the B allele with average effect of 0.8 piglets in first parity and approximately 0.7 piglets in later parities using four Large White-based commercial pig lines with more than 4200 first parity records and over 4700 later parity records. This large population is a good example of the population sizes needed for reproductive trait studies. A favorable meta-analysis reported an association of the B allele with TNB and NBA using 15 studies in more than 9000 sows (Alfonso 2005). On the contrary, some much smaller studies reported an association of superior litter size with the A allele rather than the B allele (e.g. Van Rens *et al*. 2002). In addition, no significant association of *ESR1* with litter size was mentioned in different swine populations (Depuydt *et al*. 1999, Drogemuller *et al*. 2001, Isler *et al*. 1999). Many of these were small studies and environmental effects may have prevented seeing the significant effect of *ESR1*. Recently, Muñoz *et al*. (2007) reported five silent mutations in the coding region of *ESR1* and found an association of a SNP C1227T with litter size. Because of unaltered amino acid sequence by the reported polymorphisms of *ESR1,* the associated *ESR1* polymorphisms may not be causal mutations, and instead might be linked with the causative SNP. In addition, the genetic background (associations found only in Large White and Yorkshire populations) may be important to consider when using *ESR1* as a possible marker for marker assisted selection programmes (Rothschild *et al*. 1996). Similarly *ESR1* (*Ava*I) and *ESR2* (PvuII) polymorphisms showed significant differences in semen volume and live sperm concentration (Terman *et al*. 2006). *ESR1* is used by many breeders and breeding companies worldwide.

An essential process to establish pregnancy in pigs is a shift in endometrial prostaglandin (PG) F secretion from an endocrine (toward the myometrium and uterine vasculature) to an exocrine (toward the uterine lumen) orientation (Bazer & Thatcher 1977). This is mediated by interactive effects of estrogens and prolactin (Gross *et al*. 1990). Therefore, another important candidate gene associated with reproductive traits is the prolactin receptor (*PRLR*). In pigs, this gene was found to be associated with age at puberty, ovulation rate, uterine length and litter size (Table 3). Initial results for litter size were first presented by Vincent *et al*. (1998) and later confirmed by Van Rens & Van der Lende (2002) and Van Rens *et al*. (2003). These genotypes

Table 3. Candidate genes associated with female reproductive traits in pigs.

Trait	Associated genes†	SSC	Polymorphism	Polymorphism location	Population*	Reference
Age at puberty	*PRLR*	16	Alu site	-	LW x M	Van Rens & Vander Lende 2002
	AKR1C2	10	Ile16 Phe	Nt179 in coding region	¼ M	Nonneman *et al*. 2006
	PAX5	1	C/T	Intron 9	D x BT and Lr x BT	Kuehn *et al*. 2008
Ovulation rate	*PRLR*	16	Alu site	-	Lr x M	Van Rens *et al*. 2003
	*GNRHR***	8	-	3'UTR	M x LW	Jiang *et al*. 2001
	NCOA1	3	T/T	Exon 11	M x LW	Melvile *et al*. 2002
	MAN2B2	8	A/G	Nt1574 mRNA	M x WC	Campbell *et al*. 2008
Uterine length	*PRLR*	16	Alu site	-	Lr x M	Van Rens *et al*. 2003
	FSHB	2	FSHBMS microsatellite	5'flanking region	LW x M	Li *et al*. 2008
Uterine capacity	*EPOR*	2	C/T	Intron 4	Yx Lr x CW x LW	Vallet *et al*. 2005a
	sFBP	-	Ser-Arg	Exon 1	M x W	Vallet *et al*. 2005b
Litter size	*ESR1***	1	PvuII site	Intron	M x SL and LW	Rothschild *et al*. 1996
					LW	Short *et al*. 1997
					Lr	Chen *et al*. 2000
					M x LW	Van Rens *et al*. 2002
					LW	Matousek *et al*. 2003
					Czech LW	Goliasova & Wolf 2004
			C/T	Exon 5	M x LW	Muñoz *et al*. 2007
	PRLR	16	-	-	LW	Vincent *et al*. 1998
					SL	Droggemuller *et al*. 2001
					M x LW	Van Rens & Vander lende 2002
	*FSHB***	2	-	Intron	Y x EL	Li *et al*. 1998
					Lr and Y	Zhao *et al*. 1999
			Alu site	Promoter	LP, DP and Lr	Du *et al*. 2002
			FSHBMS microsatellite	5'flanking region	LW x M	Li *et al*. 2008
	RBP4	14	-	-	LW	Olliver *et al*. 1997
				Intron	SL	Rothschild *et al*. 2000
				Intron	GW	Spotter *et al*. 2009
	BF	7	-	Intron	(LW x Lr) x Lc	Buske *et al*. 2005
	LIF	8	-	Exon 3	GL	Spotter *et al*. 2009
			C/T	Intron 1	LW	Lin *et al*. 2009
	FUT1		Hha1	-	(LW x Lr) x Li	Buske *et al*. 2006b
	RNF4	6	C/T	Intron 5	CQ	Niu *et al*. 2009

† *AKR1C2*: Aldo keto reductase 1C2; *BF*: Properdin; *EPOR*: Erythropoietin receptor; ESR1: Estrogen receptor 1; FSHB: Follicle stimulating hormone beta; *FUT1*: fucosyl transferase 1; *GNRHR*: Gonadotropin releasing hormone receptor; *LIF*: Leukemia inhibitory factor; *MAN2B2*: Mannosidase 2B2; NCOA1: Nuclear receptor coactivator 1; *PAX5*: Paired box 5; *PRLR*: Prolactin receptor; RBP4: Retinol binding protein 4; *RNF4*: ring finger protein 4 gene; *sFBP*: Secreted folate binding protein.

* BT: Yorkshire x maternal Landrace composite; CQ: Chinese Qingping; CW: Chester White; D: Duroc; DP: Duli pigs; EL: Erhualian line; GL: German landrace; GW: German large white; LP: Laiwu pigs; Lr: Landrace; Lc: Leicoma; LW: Large white; M: Meishan; SL: Synthestic lines; Y: Yorkshire; W: White European breed cross; WC: White composite

**Some of the mentioned gene information can be seen in http://www.animalgenome.org/QTLdb/pig.html

were associated with significant ($P < 0.01$) differences in male reproductive traits such as ejaculate volume and spermatozoa concentration in ejaculate (Kmiec & Terman 2004).

During pregnancy, the pig uterus secretes a large amount of proteins in response to progesterone. These proteins are required to nurture the litter (Roberts & Bazer 1980). Among them, retinol binding protein (RBP) is secreted by endometrial epithelial cells to deliver retinol to the uterine lumen (Roberts *et al*. 1993). High levels of RBP secretion on day 12 of pregnancy verify its importance during that period of time (Trout *et al*. 1991). The *RBP4* gene was investigated as a candidate gene and initial results, based on a limited number of sows, indicated an additive gene effect for the favorable allele of 0.52 ± 0.30 pigs per litter in a Large White Hyperprolific line and 0.45 ± 0.43 in the control (Ollivier *et al*. 1997). It was again verified as a useful candidate gene for litter size (Rothschild *et al*. 2000) using an *MSP1* PCR-RFLP assay and the favorable A allele had an approximate additive effect of 0.23 pigs per litter ($P < 0.05$) for TNB and 0.15 pigs per litter for NBA in commercial Landrace lines. However, other small studies were unable to identify a significant association of *RBP4* with litter size in selected lines (Blowe *et al*. 2006) and synthetic lines (Drogemuller *et al*. 2001). This gene has been suggested for use only in Landrace populations.

The association of follicle stimulating hormone (*FSH*) with reproductive traits has been well studied. The association of a retroposon element in intron 1 of the follicle stimulating hormone-β (*FSHB)* gene with litter size was studied and it was found that the non-retroposon homozygous allele (BB) females produced on average 2.53 piglets more than the retroposon homozygous allele (AA*)* sows for total number born (TNB) and 2.12 for number born alive in the first parity in Landrace and Yorkshire pigs (Zhao *et al*. 1999). An Alu element with a difference in poly A length in the regulatory element of the *FSHB* gene was also found to be associated with litter size. These results indicated that *FSHB* was associated with pig litter size or it is linked with other genes (Du *et al*. 2002). Li *et al*. (2008) conducted a study on the association of a microsatellite 4Kb upstream of the *FSHB* gene in a Large white x Meishan F2 population and found a significant association with higher number of piglets at weaning and greater litter weight at weaning ($P < 0.05$).

In addition, the associations of polymorphisms in aldo keto reductase 1C2 (*AKR1C2*), erythropoietin receptor (*EPOR*), fucosyl transferase 1 (*FUT1*), gonadotropin releasing hormone receptor (*GNRHR*), leukemia inhibitory factor (*LIF*), mannosidase 2B2 (*MAN2B2*), nuclear receptor coactivator 1 (inhibin beta A), paired box 5 (*PAX5*), properdin (*BF*), ring finger protein 4 gene (RNF4) and secreted folate binding protein (*sFBP*) with different female reproduction traits have been reported. Using 119 SNPs from 95 genes, Fan *et al*. (2009) carried out association analyses for six reproductive traits (total number born, TNB; number born alive, NBA; still born number, SBN; mummy number, MN; gestation length, GL and non productive days, NPD) recorded in 2,066 animals for six parities. It was found that 23 genes were significantly ($P < 0.05$) associated with at least three reproductive traits. In parity 1, *COL9A1, NST, ADAM12, WARS2, DKFZ* and *LRP5* were significantly ($P < 0.05$) associated with both TNB and NBA while *COL1A2, CALCR* and *IGFBP5* were significantly ($P \leq 0.01$) associated with SBN; and *IL6* and *ESR2* were significantly ($P \leq 0.01$) associated with MN and NPD, respectively. During later parities, *CASR, ESR2, WARS2, NST, IFNγ* and *BMP8* had significant association ($P < 0.05$) with TNB and NBA. The genes *MC4R, FBN1, IGFBP2* and *SFRP4* were significantly ($P < 0.05$) associated with GL in several parities. It should be noted that ESR1 was not tested in this initial study. For male reproduction traits Lin *et al*. (2006) mentioned the association of *GNRHR* with motility, plasma droplet ratio and abnormal sperm rate; inhibin beta A (*INHBA)* with plasma droplet ratio and abnormal sperm rate and inhibin beta B (*INHBB*) with sperm concentration.

Though many candidate genes examined so far have direct physiological roles in different stages of reproduction, this is not mandatory. Instead, a QTL could be the result of genetic

variation in regulatory protein or initiation factor genes that then affect the expression of genes involved in pig reproduction. This concept of "polygenic paradox" was illustrated by Pomp *et al*. (2001) using the example of putative regulation of FSHB gene.

ESTs and gene expression in pig reproductive tissues

Gene expression analysis is also a useful approach to understanding the biological basis of reproduction and large numbers of expressed sequence tags (EST) to study gene expression for these traits are essential. The Midwest consortium (Tuggle *et al*. 2003) isolated ESTs from female reproductive organs and deposited 21,499 ESTs representing 10,574 genes in public databases. Out of these ESTs, 3,183 sequences were from the anterior pituitary; 3,900 were from term placenta; 4,505 were from the peri-implantation uterus; 4,165 were from embryo/fetus tissue; 1,544 were from hypothalamus and 1,643 were from ovary. Gorodkin *et al*. (2007) presented an expression study based on 35 tissues representing 98 cDNA libraries and 1,021,891 sequences assembled by the Distiller assembly program, and concluded that gene diversity is greater in the brain and testis, which are major components of reproduction. The expression profiles of the hypothalamus-pituitary-gonadal axis between animals with different reproductive performance are extremely useful in exploring variation among reproductive traits in a given situation. Using differential display PCR, Bertani *et al*. (2004) reported 125 EST sequences were differentially expressed in the anterior pituitary gland between control line and a line selected for ovulation rate and embryo survival. The differential expressions of the genes G-beta like protein, ferritin heavy chain and follicle stimulating hormone beta subunit, were confirmed by northern blotting of anterior pituitary RNA between the above lines. The expression levels of ferritin heavy chain and G beta like protein were less in a selected line for enhanced reproduction compared to the control line, whereas the FSH beta gene was increased in the selected line.

To date only a limited number of detailed transcriptome analyses on pig folliculogenesis have been conducted. Two of them (Caetano *et al*. 2004, Gladney *et al*. 2004) used whole follicles and were performed on pigs from lines selected for reproductive traits. It was found that follicle sizes were bigger in the selected line than control line. By using differential display PCR, Gladney *et al*. (2004) found that 152 genes were up regulated and 20 genes were down regulated in follicles of the selected line. Three differentially expressed genes were confirmed by northern hybridization. These genes were calpain 1 light subunit (*CAPN4*), cytochrome P450 aromatase and cytochrome P450 side chain cleavage enzymes. Similarly, microarray analysis of pooled follicles with two versions of human cDNA arrays (UniGem V1.0 and V2.0) resulted in 33 and 21 differentially expressed probes between selected and control line. Northern hybridization of differentially expressed mRNA resulting from microarray analysis confirmed the reduced expression of follistatin (*FST1*) and increased expression of nuclear receptor subfamily 4, group A, member 1 (*NR4A1*) in the line selected for ovulation rate and embryo survival. It was hypothesized that less expression of *CAPN4* is favorable to decreased follicular degradation and apoptosis, which promotes the recruitment of a larger pool of follicles for ovulation (Gladney *et al*. 2004). Similarly, low follistatin increases the bioavailability of activins, which are required for follicular growth and differentiation (Hasegawa *et al*. 1994). Higher expression of *NR4R1* increases the sensitivity of follicles to steroid hormones (Gladney *et al*. 2004). To know the expression profile of the ovary and follicle in the above mentioned selected and control lines, a microarray with 4608 probes was prepared at the Nebraska Medical Center Core Facility using the GMS417 Arrayer (Genetic Microsystems). Mixed model analysis of these microarray data (Caetano *et al*. 2004) found evidence for differential expression of 71 and 59 genes in

the whole ovary and ovarian follicles during the follicular phase of the estrous cycle between animals from the selected and control lines. The genes involved in steroid biosynthesis (e.g., cytochrome P450 side chain cleavage enzyme, steroidogenic acute regulatory protein and others), tissue remodeling (plasminogen activator inhibitor II) and apoptosis (calpain light chain I) were all differentially expressed between the lines. It was suggested that the differential expression of ovarian genes between the select and control lines was due to the effect of selection for increased reproduction on the frequency of allelic variation within the genes themselves or from allelic variation in genes that control the genes found to differ between lines (Caetano *et al*. 2004).

An ovarian transcriptome analysis was conducted on the effect of luteinization on preovulatory follicles (Agca *et al*. 2006). This microarray analysis detected the decreased regulation of 107 genes and increased regulation of 43 genes during the transition from preovulatory estrogenic to luteinized follicles. The decreased regulated genes belonged to cytoskeletal proteins, regulators of cytoskeleton, nuclear proteins, nucleic acid binding proteins, metabolic enzymes, mitochondrial transporters, proteins involved in the oxidative stress response, ligands, receptors and receptor pathways (predominantly cAMP response system), and cell proliferation/differentiation functional groups. The increased expression of certain genes during luteinization were for proteins that are involved in cell adhesion and migration, cell growth inhibition or angiogenesis. Another ovarian transcriptome study (Bonnet *et al*. 2008) found 79 differentially expressed transcripts associated with terminal follicular growth. These genes were involved in functional networks required for cellular growth, cell cycle, proliferation, cancer, reproductive system development and function, molecular transport, protein synthesis and lipid metabolism. Genes in glutathione metabolism (e.g. *GSTA1*, glutathione S-transferase alpha 1; and *MGST1*, microsomal glutathione S-transferase 1) and lipid metabolism (e.g. *CYP19A*, P450 aromatase A and many others) were up regulated, and the ribosomal protein genes (e.g. *CALU*, calumenin; *SLC40A1*, solute carrier family 40 and others) and cell shape genes (e.g. *TUBA1B*, tubulin, *CAPNS1*, calpain, small subunit 1 and others) were down regulated in terminal antral follicular development especially in large follicles compared to small and medium ovarian follicles. A quantitative RT-PCR study found significant ($P < 0.05$) correlations between oocyte number and the expression levels of *ESR1* and *IGFR1* in ovaries of Duroc x Meishan and PIC lines (Hu *et al*. 2006). Whitworth *et al*. (2005) compared expression profiles of germinal vesicle stage oocytes to that of 4-cell stage and blastocyst stage embryos produced from *in vitro* fertilization and culture and *in vivo* derived embryos. *In vivo* blastocyst stage embryos had higher expression of gene networks pertaining to ribosomal function and ion transport than that of *in vitro* blastocyst stage embryos. Both *in vitro* embryo stages had lower expression of plasma membrane-protein-tyrosine-phosphatase activity, and increases in expression of the nucleolus, small nucleolar ribonucleoprotein complex, and RNA binding and processing gene networks than *in vivo* embryo stages. The complete list of differentially expressed genes in these stages of embryos can be obtained from http://genome.rnet.missouri.edu/Swine/Publications. The utilization of these transcript profiles allows the identification of differentially expressed genes associated with embryogenesis as well as developmental competency associated with *in vitro* fertilization systems.

Studies of the endometrium transcriptome were conducted to determine the differential expression of genes in the uterus during the estrous cycle and pregnancy (Green *et al*. 2006). A total of 4,827 genes were significantly differentially expressed at some time during the estrous cycle. Clustering of these genes identified six main patterns across the estrous cycle. These patterns are related to the functions of the endometrium such as sperm maturation, blastocyst growth and position, and conceptus development and attachment. These data may be useful for transgenic and cell transfer approaches to improve reproduction efficiency. Ross *et al*. (2007) identified numerous endometrial genes aberrantly expressed following exogenous estrogen exposure prior to implantation that is associated with total pregnancy loss. In addition to different periods

of estrous cycle and pregnancy, the transcriptome profile of uterine epithelium was studied in comparison to other tissues using a 20,400 70 mer second generation pig oligonucleotide array (Pigoligoarray; www.pigoligoarray.org), Steibel *et al*. (personal communication). This study found that 286 transcripts were differentially expressed in uterine epithelium relative to liver, muscle and brain stem.

Because fertilization is an efficient process in pigs, mortality can be assessed throughout gestation by comparing conceptus/fetus numbers to the number of corpora lutea. Pigs suffer from a high incidence of prenatal mortality, ranging from 20 to 46% at term (Pope 1994). The occurrence of embryonic mortality can be broadly broken into two phases; the peri-implantation stage of development, Days 10-18 of gestation; and post-implantation development between Day 18 and 114 of gestation. In gilts where ovulation rate is not sufficient to exceed uterine capacity, the majority of prenatal mortality is thought to occur during the peri-implantation period of development (Anderson 1978). Transcript discovery and/or profiling during conceptus transition from a 9-10 mm sphere on Day 11-12 to a transient tubular shape (15-20 mm) and into a long filamentous thread (greater than 150 mm) over the course of a few hours has been achieved through quantitative RT-PCR (Green *et al*. 1995, Kowalski *et al*. 2002, Yelich *et al*. 1997a,Yelich *et al*. 1997b), differential display RT-PCR (Wilson *et al*. 2000), suppression subtractive hybridization (SSH) (Ross *et al*. 2003a), expressed sequence tag (EST) library construction and analysis (Smith *et al*. 2001), utilization of embryonic based cDNA array (Lee *et al*. 2005), serial analysis of gene expression (SAGE) (Blomberg *et al*. 2005) and Affymetrix GeneChip microarray (Ross *et al*. 2009). Several transcripts have been consistently identified as differentially expressed during transition through these critical developmental stages such as interleukin 1β (Ross *et al*. 2003b, Lee *et al*. 2005), steroidogenic acute regulatory protein (STAR) (Blomberg *et al*. 2005, Lee *et al*. 2005) and cyclooxygenase-2 (Wilson *et al*. 2000, Ross *et al*. 2009). The utilization of the Affymetrix GeneChip identified 192 transcripts with the putative ability to serve as molecular markers due to transient up or down regulation during early stages of trophoblastic elongation (Ross *et al*. 2009). In addition 482 transcripts were differentially expressed in filamentous day 12 conceptuses compared to large spherical conceptuses representing biological processes associated with cell motility, ATP utilization, cell growth, metabolism and intracellular transport (Ross *et al*. 2009). Expression of numerous genes and gene products characterize this developmental process, which is associated with the production of steroids (primarily estrogen), prostaglandins, cytokines and morphogenic factors having a tremendous influence on maternal recognition of pregnancy, immunological tolerance and growth of the conceptus.

Following the peri-implantation period, rapid fetal growth ensues and during the period from Days 21 to 45, uterine capacity becomes limiting. Uterine capacity is defined as the maximum number of piglets carried to term when potentially viable conceptuses are not limiting (Christenson *et al*. 1987). Uterine crowding significantly affects fetal weight, placental weight and protein secretion on both Days 25 and 35 of gestation (Vallet & Christenson, 1993), suggesting the expression and regulation of transcripts in placental and fetal tissue during this stage of gestation is responsive to environmental stresses. Wesolowski *et al*. (2004) conducted a fetal transcriptome study using pig fetuses at 21, 35 and 45 days of gestation and identified 17 differentially expressed genes which play a major role in musculoskeletal growth, immune system function, and cellular regulation. Comparison between Meishan and European Large White composite gilts identified numerous single nucleotide polymorphisms within the transcriptome of day 25 placental tissues (Bischoff *et al*. 2008).

Using human microarrays, differential expression of genes in testis between boars of high and low steroidogenesis was studied and results indicated that seven genes were over expressed in boars with high steroidogenesis. Among them three (*CYB5, CYP19A1,* and *CYP11A1*) are involved in steroidogenesis (Stewart *et al*. 2005).

MicroRNAs

The numerous transcriptional profiling experiments conducted in pig reproductive tissues assess transcript diversity and genomic regulation of gene expression, but do not assess post transcriptional regulation of mRNAs and how it influences cellular phenotypes in these tissues. MicroRNAs (miRNA) are single-stranded RNA molecules of about 18–24 nucleotides in length, and through their ability to confer post transcriptional gene silencing (PTGS) (Bartel 2004), may have a significant regulatory role in reproductive tissues. MicroRNAs are organized throughout the genome in multiple ways that allow the expression of an individual miRNA alone (Mendell 2005), clustered with other miRNAs (Lee *et al*. 2002) or, expressed within introns of transcribed mRNA (Kim 2005). RNASEN, DGCR8, exportin 5 and DICER are all critical enzymes for production of mature miRNA capable of conferring PTGS (Hutvagner & Simard 2008). Conditional knockout of DICER, the enzyme responsible for final processing of a mature miRNA, during oogenesis causes infertility through major disruption of the miRNA repertoire in the oocyte (Tang *et al*. 2007). In addition to the importance of miRNA in regulating oogenesis, reproductive tract development is also dependent on miRNA function (Hong *et al*. 2008). During implantation in mice, miR-101a and miR199a post-transcriptionally regulate cyclooxygenase 2 (COX2) (Chakrabarty *et al*. 2007), an enzyme whose transcript is also differentially expressed during the opening of the implantation window in the pig (Ashworth *et al*. 2006).

Because miRNA:mRNA complementation sites are relatively short and often imperfectly paired, slight changes in sequence, due either to RNA editing or the presence of a SNP in either the recognition site of the target gene or the miRNA could significantly alter PTGS in reproductive tissues. RNA editing can also produce variations in miRNA function, particularly, adenosine-to-inosine editing (Pfeffer *et al*. 2005, Athanasiadis *et al*. 2004). For example, the miRNA, miR-376 undergoes *tissue specific* RNA editing that converts an adenosine to an inosine. In addition to RNA editing, SNPs also affect miRNA function. Recent screening of miRNAs has revealed the occurrence of 323 SNPs in 227 known human miRNA genes (Duan *et al*. 2007). This study further demonstrated the ability of a SNP in miR-125a to prevent DROSHA recognition, in so doing blocking its ability to be processed into a pre-miRNA, thereby reducing its effectiveness in conferring PTGS (Duan *et al*. 2007).

In essence, miRNA influence, which can be impacted by appropriate enzyme expression and function, RNA editing and SNPs in both target genes and miRNAs; have a distinct ability to dramatically alter the phenotype of cells by influencing the mRNA and protein repertoire through PTGS, potentially altering the efficiency of function. The importance being that very few miRNAs have the ability to post-transcriptionally influence hundreds of mRNAs (Rajewsky 2006). The reports on miRNAs in different pig reproductive tissues are very limited. MicroRNAs are expressed in porcine reproductive tissues such as ovary (Kim *et al*. 2006, Pratt *et al*. 2008), oocytes (Ross and Prather, unpublished data), and day 33 fetus and extra-embryonic membranes (Huang *et al*. 2008). Further research is required to examine miRNA expression profiles in other reproductive tissues of the pig that require significant transcript turnover and proteome reorganization to function efficiently.

Whole genome sequencing, SNP discovery and SNP chips

The development of advanced methods to improve genotyping in large scale projects and the reduction of genotyping costs have allowed for large scale SNP discovery. These studies will allow genotyping of hundreds of thousands of SNPs and genes. Such large scale SNP association studies pertaining to pig reproduction traits are still in infancy. Following efforts to sequence the

human, cow and chicken genomes, sequencing for the pig genome began. Initial sequencing was done by a Sino-Danish team (Wernersson *et al*. 2005) and resulted in a .66X coverage of the swine genome. Since then an international effort led by individuals at several U.S. universities and centers around the world have focused on sequencing the pig genome at the Sanger Institute in the UK. Now in its third year, the International Swine Genome Sequencing committee has raised nearly 20 million dollars and nearly 75% of the pig genome is sequenced (http://www.sanger.ac.uk/Projects/S_scrofa/) to 6X coverage. It is expected that the sequencing will be completed by the end of 2009. This sequence information can be further mined for SNPs as was the case when nearly 100,000 SNPs were identified from the existing 1.2 Gb of porcine sequence (Kerstens *et al*. 2009). Initial work by the Danish-Sino team led to the first 7K SNP chip (Vingborg *et al*. 2008). Furthermore, the next generation sequencing technology revolutionized the ability to identify many more SNPs With the help of such technology, the International Swine SNP Consortium designed a 60K Illumina Infinium iSelect™ SNP Bead Chip for pigs and produced over 1 million SNPs. The SNPs used for this chip were a small group of previously validated SNPs in the public domain and the SNPs identified de novo by second generation sequencing on the Illumina Genome Analyzer (Solexa) and Roche 454 FLX sequencer (Groenen *et al*. 2009). These were then chosen on the basis of minor allele frequency and spacing when known from existing sequence information. Initial work suggests the chip have well over 50,000 useful SNPs with excellent minor allele frequency and is already being employed. This chip has the potential to modernize association trials and lead to effective genomic selection (Solberg *et al*. 2008).

Phenomics

The development of gene expression arrays, sequence information, SNP chips and other genomic tools is relatively well advanced. But if we are to better use high density SNP chips for association trials or investigate other gene action such as imprinting and epigenetics then clearly more useful and varied phenotypes must be collected. This includes the usual traits like number born and number born alive but also ovulation rate, embryo survival, hormone levels and other traits of interest. This area of collecting new and interesting phenotypes is called phenomics (Freimer & Sabatti, 2003). These traits must be measured not on tens of animals or hundreds of animals but on thousands. Furthermore, utilization of the available tools and resources to impact specific reproductive traits in swine requires cognizance that selection and improvement of single traits, such as number born alive, can have deleterious effects on other traits such as post-natal performance (Foxcroft *et al*. 2007). An effort is needed to determine the relevance of various alternative measurable traits to improvements in swine reproduction. Reproductive physiologists and animal geneticists need to participate more collaboratively to accomplish such data sets.

Acknowledgements

Efforts and information supplied by colleagues around the world is appreciated. Work discussed here has been supported in part by PIC USA, Monsanto Choice Genetics, Newsham Choice Genetics, the National Pork Board and by Hatch and State of Iowa Funds and funding provided by the College of Agriculture and Life Sciences at Iowa State University.

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Proteomic analysis of mammalian gametes and sperm-oocyte interactions

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Proteomic analysis occupies an increasingly important place in gamete and embryo biology as an independent tool of discovery and as a means of follow-up to transcriptional profiling. Proteomics have been and will be increasingly helpful in many areas of reproductive biology, including applied science and technology development. Areas likely to be impacted most rapidly by proteomic knowledge include fertility evaluation in male farm animals, male infertility diagnostics in humans, assessment and optimization of oocyte and embryo culture protocols, selection of fittest oocytes for assisted fertilization and selection of most competent embryos for embryo transfer. Oocyte proteomics will help us understand the process of oogenesis and oocyte maturation, and to discover non-invasive markers of oocyte quality. Sperm proteomics correlate with normal sperm structure and function and can be applied to discover novel biomarkers of farm animal fertility and diagnostic markers of human male infertility. Putative receptors participating in fertilization, as well as proteins acquired onto sperm surface from epididymal fluid and seminal plasma, have been discovered by proteomic analysis. An added level of information is provided by advanced proteomic approaches, capable of identifying posttranslational modifications such as phosphorylation, glycosylation and ubiquitination which play important functions in gametogenesis, fertilization and embryo development. By no means exhaustive, the present paper reviews some of the most interesting proteomic studies of mammalian gametes and embryos published in the last decade.

Introduction

Genomic revolution brought us transcriptional profiling which allowed for the identification of dozens of genes involved in mammalian gametogenesis, fertilization and preimplantation embryo development. The next major challenge facing reproductive biology is to apply meaningful approaches to analyze these gene products at the protein level. Proteomic analyses could therefore be used as a follow up to transcriptional profiling, or as an independent discovery tool. Proteomic follow-up of transcriptional profiling is important because of a lack of consistency and repeatability in microarray data wherein multiple levels of verification are necessary to confidently confirm differential expression. Also, some of the identified transcripts could be products of untranslated pseudogenes, or their transcription levels may differ from their translation levels. Other transcripts could represent non-coding RNAs with

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distinct biological functions that are not to be translated. Finally, protein half life varies greatly and can be a cause of discrepancies between mRNA and the protein level of a given gene product in a given cell type.

Cataloging of the oocyte proteome will be instrumental in studies of the process of oogenesis and oocyte maturation. In addition, proteomic approach can be used to identify non-invasive markers of oocyte quality in the oocyte proteome and oocyte secretome. Quality control of oocyte maturation is of importance for farm animal embryo transfer technology and, in particular, for human assisted fertilization. If sensitive biomarkers are identified in the oocyte secretome, human oocytes or fertilized eggs could be cultured individually in media drops and the oocyte-exposed media could be collected and evaluated for the presence or abundance of protein biomarkers associated either with normal or deviant oocyte quality. Based on such a test, oocytes or zygotes with highest developmental potential could be selected for fertilization or embryo transfer, respectively.

Sperm proteomics correlate with normal sperm structure and function and can also be used for comparison of normal and subfertile/infertile sperm samples. Furthermore, the carryover of proteins translated during the haploid phase of spermatogenesis, the spermiogenesis, can be informative of the mechanisms involved in spermatid differentiation into a spermatozoon.

During fertilization, sperm structures such as the acrosome are lost but their remains (e.g. the acrosomal shroud or ghost) can be collected from the egg coats of the fertilized oocytes. By tagging spermatozoa with biotin before fertilization, sperm proteins that interacted with the egg coat can be purified and identified by protemics. This can lead to the identification of sperm surface receptors involved in sperm-zona pellucida interactions during fertilization. Of equal importance are the proteomics of seminal plasma and epididymal fluid, since it is in the epididymis where many sperm surface proteins are acquired that convey sperm fertilization-potential. Advanced proteomic approaches, capable of identifying posttranslational modifications such as phosphorylation, glycosylation and ubiquitination, pick up where conventional proteomics leave off, adding a new layer of information to straight protein identification in gamete and embryo biology.

While not all inclusive, the present paper reviews a select group of recent reports on proteomes of mammalian gametes, with particular emphasis on reports relevant to sperm fertilizing ability and oocyte developmental potential. Readers are referred to other recent review articles to complement the information reviewed here (Sirard *et al*. 2003, Aitken & Baker 2008; Katz-Jaffe & Gardner 2008; Oliva *et al*. 2008). Some proteins related to the ubiquitin-proteasome pathway are discussed in more detail, as this pathway is the main focus of research in our laboratory.

Oocyte secretome

The importance of assessing oocyte secretome, i.e. the protein composition of culture media enriched by oocyte-secreted or oocyte-leaked proteins, lies in its potential for identifying molecular biomarkers of good or poor oocyte competence for fertilization and embryo development. Thus far, ubiquitin was reported as the only informative marker of mouse and human embryo quality (Katz-Jaffe *et al*. 2006). Ubiquitin is the central protein of the substrate-specific, proteolytic ubiquitin-proteasome pathway (Glickman & Ciechanover 2002). Ubiquitin can occur as a free protein (as seems to be the case of secretome in good quality oocytes and embryos), or covalently linked to other proteins in form of a monomer (monoubiquitination), dimer (diubiquitination), tetramer (tetraubiquitination) or polymer (polyubiquitination). Tetra and polyubiqutiantion serve as signals for protein degradation by the 26S proteasome, an event

central to a number of physiological cellular mechanisms (e.g. cell cycle regulation, antigen presentation by immune system cells) and pathologies (e.g. Alzheimer's, AIDS, liver cirrhosis). The housekeeping function of ubiquitin-proteasome pathway, i.e. the recycling of outlived and damaged proteins is certain to play an important role in oocyte and embryo metabolism.

Oocyte proteome

Mass spectrometry of protein spots excised from two dimensional PAGE gels of porcine oocytes before and after meiotic maturation revealed 35 abundant oocyte proteins, including zona pellucida proteins, cytoskeletal proteins, redox proteins and ubiquitin-system proteins (Ellederova *et al*. 2004). Relative abundance of these proteins was compared to that of actin, revealing that spermine synthase, peroxiredoxin and ubiquitin C-terminal hydrolase L1 (UCHL1) were extremely abundant in the porcine oocyte proteome, even more so than actin (Ellederova *et al*. 2004). Among these proteins, UCHL1 amount doubled between the germinal vesicle (GV) and metaphase II (MII) stage of oocyte maturation (Ellederova *et al*. 2004).

The role of UCHL1, and related UCHs and ubiquitin-specific proteases (USP) is to remove ubiquitin from substrate proteins (deubiquitination) and regenerate the available monoubiquitin pool by disassembling the multi-ubiquitin chains. A detailed study of porcine fertilization revealed that UCHL1 is concentrated in the oocyte cortex, where it may regulate the events of sperm oolemma-fusion and sperm incorporation (Yi *et al*. 2007a). Based on these studies, a role of UCHL1 and a related enzyme UCHL3 was proposed in the regulation of anti-polyspermy defense. Remarkably, electrofusion of a donor cell fibroblast during somatic cell nuclear transfer (SCNT) produced an UCHL1 free cortex area on the oocyte pole to which the donor cell was fused (Yi *et al*. 2007a). The cortical accumulation of UCHL1 is also observed in bovine (PS, unpublished) and murine (Sekiguchi *et al*. 2006; Yi *et al*. 2007a) oocytes. The gracile axonal dystrophy *(gad)* mutant mice expressing a truncated form of UCHL1 (Saigoh *et al*. 1999) were subfertile in mating studies and appeared to produce many polyspermic zygotes by *in vitro* fertilization (Sekiguchi *et al*. 2006). It is not clear whether the *in vivo* produced *gad* mutant zygotes are also polyspermic.

A follow up study confirmed the high abundance of UCHL1 in porcine oocytes and provided evidence that this enzyme participates in the regulation of oocyte meiosis-I, particularly at metaphase-anaphase transition (Susor *et al*. 2007). Such a role is consistent with the requirement of ubiquitin-dependent proteolysis for metaphase-anaphase transition in somatic cells, wherein the ubiquitin conjugating and deubiquitinating enzymes are constituents of the anaphase promoting complex (APC)(Pesin & Orr-Weaver 2008). In summary, UCHL1 is likely important for protein turnover in the oocyte and embryo, but it also has more specific functions in the cell cycle control during meiosis, and in the regulation of oocyte cortex and anti-polyspermy defense during fertilization. Remarkably, UCHL1 was also identified as one of ten most abundant proteins in the bovine oocyte proteome (Massicotte *et al*. 2006). Besides UCHL1, ubiquitin conjugating enzyme E2D3, chaperone proteins HSP70, HSC71, cyclophilin A (CYPA), and CCTε, glutathione-S-transferase GSTM5 (anti-oxidant), 2,3-bisphoglycerate mutase (2,3-BPMG; glycolytic pathway enzyme), epidermal fatty acid binding protein E-FABP (lipid transport protein), and two actin isoforms were identified (Massicotte *et al*. 2006).

The major vault protein (MVP; lung resistance-related protein/LRP) was also revealed to be an abundant protein in the pig oocyte proteome (Ellederova *et al*. 2004). A more recent study demonstrated that MVP, which is the major ribonucleoprotein component of the cytoplasmic vault particle (Kickhoefer & Rome 1994), accumulated during oocyte maturation in porcine ova and showed aberrant accumulation patterns in morphologically abnormal human

oocytes (Sutovsky *et al*. 2005). High MVP levels would appear to be desirable in oocytes and preimplantation embryos as this drug resistance-related protein is thought to have a cell protecting activity (Scheffer *et al*. 2000). High content of MVP protein could have positive influence on oocyte maturation and preimplantation development; the MVP containing vault particles protect cells from stress and conveys resistance to chemotherapy drug treatment in cancer cells (Steiner *et al*. 2006). The turnover of MVP in porcine oocytes and zygotes is regulated by ubiquitin-dependent proteasomal proteolysis (Sutovsky *et al*. 2005). Translation and turnover of MVP appears to be misregulated in cloned porcine embryos and could contribute to their reduced developmental competence (Antelman *et al*. 2008).

Proteomes of immature and mature bovine oocytes were compared using saturation labeling (2-D DIGE)(Berendt *et al*. 2009). Ten proteins were identified as significantly different in their amount between immature and mature oocytes. These included the Ca(2+)-binding protein/translationally controlled tumor protein, enzymes of the Krebs and pentose phosphate cycles, clusterin, 14-3-3 epsilon signaling protein, elongation factor-1 gamma, and redox enzymes including GST Mu 5 and peroxiredoxin-3 (Berendt *et al*. 2009).

Large scale proteomic profiling of mouse metaphase II oocytes identified 380 proteins, including 53 putative phosphoproteins (Ma *et al*. 2008). According to gene ontology annotation, most abundant categories included protein metabolism, protein binding, and hydrolase activity.

Sperm proteomics

Comprehensive analysis of the human sperm proteome was undertaken as part of a contraceptive target identification project at Wyeth Research, an effort that also generated a valuable depository of testicular and epididymal transcriptomes and sperm protein sequences (Johnston *et al*. 2005). A total of 1,760 proteins were identified, of which 1,350 proteins were associated with the soluble sperm fraction, 719 with the insoluble fraction, and 309 identified in both fractions. The high content of insoluble fraction-associated proteins in the sperm proteomes is likely a result of a high level of disulfide bond cross-linking and detergent resistance observed in the sperm accessory structures. Dominant ontologies by protein function included, in decreasing order of identified protein number, protein folding & degradation, cytoskeletal function, cell growth/maintenance and metabolism in the soluble fraction and cytoskeleton, protein folding/degradation, and cell growth/maintenance in the insoluble fraction (Johnston *et al*. 2005). Twenty seven different subunits of the 26S proteasome, a multi-subunit protease implicated in fertilization process (Sutovsky *et al*. 2004), were identified.

Baker *et al*. (2008a) identified 829 rat sperm proteins by using prefractionation of sperm extracts with narrow range immobilized pH gradient (IPG) gel strips. This technique, described by (Essader *et al*. 2005), can increase the chance of identifying low abundance proteins. Identified sperm proteins were clustered according to subcellular localization, molecular function, and biological processes in which they were known to participate. By protein annotation/localization, 82% of identified proteins were intracellular, 29% were mitochondrial proteins, and only 14% were annotated as cytosolic proteins, which is understandable due to low abundance of cytoplasm in spermatozoa. Molecular function was attributed to 600 proteins, of which 69% possessed a binding domain and 66.5% contained a catalytic domain. Among proteins of interest to gamete and fertilization biologists were superglobulin family protein IZUMO and ADAM family disintegrin-proteins implicated in sperm-oolemma fusion during fertilization, ion transporters, 26S proteasome subunits implicated in both spermatogenesis and fertilization, proteins related to spermatid elongation/differentiation and proteins related to sperm maturation.

The same group also analyzed 858 proteins in the mouse sperm proteome and found some of the same proteins as described above for rat sperm proteome. As an example of this conserved proteome composition, they reported that 26 of the 42 identified proteases were subunits of the 26S proteasome (Baker *et al*. 2008c). Other proteins of interest included previously characterized sperm proteins IZUMO, zonadhesin, CatSper4, and ODF1 & 2.

Proteomic analysis can be used to compare protein composition of fertile and infertile/defective spermatozoa, potentially identifying novel biomarkers of male fertility. In such comparisons of high and low fertility bull sperm proteomes, 125 differentially expressed proteins have been identified, including proteins involved in sperm-oocyte interactions such as IZUMO, CRISP and ADAM-family proteins including fertilin alpha (Peddinti *et al*. 2008). Proteins involved in metabolism, cell signaling, spermatogenesis and cell motility were upregulated in the high fertility group. In particular, proteins involved in EGF signaling, PDGF signaling, oxidative phosphorylation, and pyruvate metabolism pathways were upregulated in the high fertility group (Peddinti *et al*. 2008).

For human infertility research, proteomes of normozoospermic men and asthenozoospermic patients were compared, and 17 differentially expressed proteins were identified (Oliva *et al*. 2008). They were actin-B, annexin-A5, cytochrome C oxidase-6B, histone H2A, prolactin-inducible protein and precursor, calcium binding protein-S100A9 (2 spots), clusterin precursor, dihydrolipoamide dehydrogenase precursor, fumarate hydratase precursor, heat shock protein-HSPA2, inositol-1 monophosphatase, 3-mercapto-pyruvate sulfurtransferase/dienoyl-CoA isomerase precursor, proteasome subunit-PSMB3 (20S proteasomal core subunit beta 3), semenogelin 1 precursor and testis expressed sequence 12.

In a separate study from the same group, the relative abundance of 58 identified proteins correlated significantly with the expression of other proteins in sperm samples from 47 infertile patients and 10 fertile donors (de Mateo *et al*. 2007). Several proteasomal subunits were identified, with inter-correlated expression patterns. The 26S proteasome has been increasingly implicated in several steps of mammalian fertilization (Yi *et al*. 2007b). Eight proteins, including proteasomal subunit PSMA6 and mitochondrial membrane protein prohibitin correlated positively with sperm DNA fragmentation revealed by TUNEL assay (de Mateo *et al*. 2007). Prohibitin also showed increased levels in sperm samples with abnormally low ratios of protamine 1 to protamine 2, an indicator of sperm chromatin structure. Of importance, prohibitin ubiquitination in the bull sperm mitochondria has been implicated in the mechanism of targeted degradation of paternal mitochondria after fertilization (Thompson *et al*. 2003).

Proteomics have been applied to identify sperm antigens that could induce autoimmune infertility in humans (Bohring & Krause 2003). Human antisperm antibodies were found to recognize 18 major antigens in the preparations of human sperm plasma membrane and acrosomal membrane proteins, including heat shock proteins HSP70 and HSP70-2, disulfide-isomerase-ER60, caspase-3 and two different proteasomal subunits (Bohring & Krause 2003). Major proteins most frequently identified by human antisperm antibodies in the proteomic screen of mouse sperm extracts included apo lactate dehydrogenase (LDHC4), voltage-dependent anion channel (VDAC2), outer dense fiber protein ODF2 and glutathione S-transferase mu5 (Paradowska *et al*. 2006).

Epididymal fluid and sex accessory gland

Studying epididymal fluid (EF) proteome, which represents the secretome of epididymal epithelial cells and proteins released from epididymal spermatozoa, is of importance to gamete and fertilization biology. It is during epididymal maturation and storage when spermatozoa

Table 1. Listing and proposed functions of most abundant and reproductively essential proteins identified in mammalian gametes and reproductive tissues or fluids by proteomic analysis.

	Protein designation	HUGO nomenclature	General function and/or proposed function in reproductive process
Oocyte	Spermine synthase	SMS	Conversion of spermidine to spermine, a polyamine involved in cell growth and maintenance.
	peroxiredoxins	PDX1, PDX2, PDX3	Maintenance of redox potential; support oocyte metabolism, energy balance.
	Ubiquitin C-terminal hydrolase L1	UBL1	Protein deubiquitination, disassembly of multiubiquitin chains, maintenance of stable pool of unconjugated ubiquitin. Proposed to have a function in oocyte meiosis, fertilization and anti-polyspermy defense. Female mutant mouse (*gad* mutant) is subfertile.
	Ubiquitin	UBB	Protein turnover/recycling, cell cycle control; also present in oocyte secretome. Mutations are lethal.
	Ubiquitin-conjugating enzyme E2D3	UBE2D3	Protein ubiquitination; protein turnover/recycling.
	Major vault protein	MVP	Cell protection, drug resistance, nucleo-cytoplasmic transport. Supports embryo development, may protect oocyte and embryo during in vitro culture.
	Zona pellucida glycoproteins	ZPA, ZPB, ZPC	Structural proteins of the egg coat, zona pellucida; oocyte protection, sperm receptor activity during sperm-zona binding, induction of sperm acrosomal exocytosis during fertilization. KO mice are infertile.
	Hath shock chaperone proteins	HSP70, HSC71, CYPA, CCT-ε	Stress defense, protein folding, prevention of protein aggregation; protein targeting for recycling, protein transport.
	Glutathione-S-transferase	GSTM5	Cell-protectant; anti-oxidant; maintains redox balance.
	Epidermal fatty acid binding protein	E-FABP	Lipid transport.
	Actin	ACTB	Cytoskeletal component.
	Clusterin	CLU	Sperm maturation, complement regulation, lipid transport, cell-cell adhesion.
Spermatozoon	IZUMO		Sperm-oolemma adhesion during fertilization; KO mouse is infertile.
	ADAM family disintegrins/ fertilins	ADAM2, ADAM3	Sperm-oolemma adhesion during fertilization.
	Ion channels/transporters	CATSPER1	Mutant mouse is infertile due to lack of hyperactivated sperm motility.
	26S proteasome subunits	PSMA1-7 PSMB1-10 PSMC1-6 PSMD1-14	Degradation of ubiquitinted proteins; role in acrosomal exocytosis and sperm-egg coat penetration. Mutations of several subunits are lethal.
	Protamines	PRM1, PRM2	Sperm DNA hypercondensation.
	Zonadhesin	ZAN	Sperm-zona binding.
	Cysteine rich secretory proteins	CRISP1 & 2	Sperm-zona binding; sperm-oolemma adhesion.

Table 1. Listing and proposed functions of most abundant and reproductively essential proteins identified in mammalian gametes and reproductive tissues or fluids by proteomic analysis.

	Protein designation	HUGO nomenclature	General function and/or proposed function in reproductive process
	Outer dense fiber proteins	ODF1, ODF2	Formation of sperm flagellar structures.
	Protein kinase A-anchoring proteins	AKAP3, AKAP4	Sperm motility, PKA sequestration in the sperm tail principal piece.
	Sperm adhesion molecule 1	SPAM1	Hyaluronidase activity, sperm maturation, storage, sperm penetration through cumulus oophorus; abundant in sperm membrane lipid rafts.
	Tubulins	TUBA, TUBB	Cytoskeleton; major component of sperm flagellar axoneme.
Epididymal fluid	Clusterin	CLU	Sperm maturation, sperm protection from stress, microbes, immune attack; complement regulation, lipid transport, cell-cell adhesion.
	Alpha-mannosidase	MAN2	Protein deglycosylation, branched sugar trimming, sperm maturation.
	Lactoferrin	LTF	Iron-binding glycoprotein; regulates iron homeostasis, protects cells from bacteria and viruses, has anti-inflammatory effect.
	Osteopontin/secreted phosphoprotein 1	SPP1	Cytokine; regulation of fertilization and preimplantation embryo development.
	Outer dense fiber proteins	ODF1-4	Formation of sperm flagellar structures.
	Alpha-L-fucosidase 2	FUCA2	Protein deglycosylation.
	Cathepsin D	CTSD	Proteolysis; lysosomal component.
Seminal plasma	Bovine seminal plasma proteins	BSP1, BSP3, BSP5	Sperm binding to and detachment from oviductal sperm reservoir, sperm capacitation, maintenance of sperm motility during storage.
	Clusterin	CLU	Sperm maturation, Sperm protection from stress, microbes, immune attack, complement regulation, lipid transport, cell-cell adhesion.
	Spermadhesin	SPADH2	Sperm binding to oviductal reservoir; sperm-egg binding.
	Semenogelin I & II	SEMG1, SEMG2	Add viscosity to seminal plasma; form vaginal plug in rodents; proteolytic cleavage of semenogelins occurs during semen liquefaction.
	Osteopontin/secreted phosphoprotein 1	SPP1	Sperm-egg binding, regulation of polyspermy in vitro, recombinant SPP1improved embryo development in vitro.
	Serum albumin	ALB	Blood carrier protein; cholesterol acceptor during sperm capacitation.
	Phospholipase A2	PLA2	Lipid regulation, fatty acid hydrolysis, cell signalig.
	Fibronectin	FN1-3	Cell adhesion, extracellular matrix formation.
	Lactoferrin	LTF	Iron-binding glycoprotein; regulates iron homeostasis, protects cells from bacteria and viruses, has anti-inflammatory effect.
	Laminin	LAM	Cell adhesion, extracellular matrix formation.

acquire their fertilizing potential through posttranslational modifications of testicular origin-sperm proteins and addition of new epididymis-secreted proteins onto sperm surface. The acquisition of fertilizing capacity is first observed between the distal caput and proximal corpus epididymis, suggesting that secretory proteins of these epididymal regions are of particular importance for sperm function (Moore 1981). In recent years, the understanding of epididymal function expanded well beyond sperm storage function (Cornwall 2009). Most abundant epididymal proteins are those governing sperm maturation, epididymal protein turnover and sperm storage. Early biochemical and proteomic studies established that clusterin alone represents ~20-40 % of total epididymal fluid protein in various mammalian species (Dacheux *et al*. 2003). Clusterin is a glycoprotein found in a number of tissues and biological fluids. Besides sperm maturation, clusterin has been implicated in complement regulation, lipid transport, cell-cell interactions and various diseases (Rosenberg & Silkensen 1995). Other abundant EF proteins include cytosine rich secretory protein CRISP implicated in sperm-oocyte interactions, lactoferrin, mannosidase and hexosoaminase (Syntin *et al*. 1996).

Proteins of the EF are secreted by epididymal epithelial cells via specialized membrane vesicles, apical blebs and epididymosomes, allowing for apocrine secretion of cytosolic proteins that in other cell types do not enter secretory pathways (Hermo & Jacks 2002, Baska *et al*. 2008, Cornwall 2009). Proteomics of human epididymosomes revealed 146 proteins, including enzymes (27%), adhesion molecules (14%), transporters and protein trafficking molecules (13%) and signal transducers (12%) (Thimon *et al*. 2008). Analysis of bovine caput and cauda epididymis epididymosomes by liquid chromatography quadrupole time-of-flight tandem mass spectrometry (LC-QToF-MS/MS) identified 10 proteins, including aforementioned clusterin and also aldose reductase, an enzyme involved in steroid hormone metabolism (Frenette *et al*. 2006). Cauda epididymal fluid proteins have also been analyzed with regard to fertility of dairy bulls. A total of 118 differential spots were identified in 2D gels of the EF fluid from high versus low fertility bulls. Amounts of alpha-L-fucosidase 2 and cathepsin D were more than two fold greater in high-fertility bulls, while prostaglandin D-synthase was upregulated in low fertility bulls (Moura *et al*. 2006). In another study, the same group used *in vitro* fertilization to assay the effect of seminal plasma from high and low fertility sires on sperm ability to penetrate the oocytes. Proteins identified as beneficial to fertilization included seminal plasma glycoproteins BSP A1/A2, BSP A3 and BSP 30 kDa, and also clusterin, albumin, phospholipase A(2) and osteopontin (Moura *et al*. 2007a). Osteopontin has been used as an additive to porcine *in vitro* fertilization medium, to improve embryo development and reduce the rates of polyspermic fertilization (Hao *et al*. 2006; Hao *et al*. 2008).

Proteomic data on boar seminal plasma have been reviewed previously (Strzezek *et al*. 2005). Most abundant proteins include spermadhesins, cell protectants with antioxidant properties, immune suppressors, sperm decapacitating factors and proteins implicated in the regulation of sperm motility via protein phosphorylation such as phosphotyrosine protein acid phosphatase.

Bovine seminal plasma proteins BSP-A1/A2, BSP-A3 and BSP-30 kDa represent 40-57 % of bovine seminal plasma proteins (Nauc & Manjunath 2000). Spermadhesins, clusterin and osteopontin are also highly abundant (Moura *et al*. 2007b). The BSP proteins coat the sperm head and have been implicated in various aspects of sperm function, including sperm capacitation, maintenance of sperm motility within female reproductive system and sperm binding to and release from the oviductal sperm reservoir (Gwathmey *et al*. 2006). Detailed analysis of bull seminal plasma by 2D SDS-PAGE and MS/MS revealed 99 proteins, including BSP proteins, clusterin, spermadhesin 2 and osteopontin, but also 49 minor proteins that were not previously reported in seminal plasma of any species (Kelly *et al*. 2006). Seminal plasma

is one of eleven human body fluids covered by a recently established proteomic database (Li *et al*. 2009). Human seminal plasma was found to contain 923 proteins, with most abundant proteins being semenogelins, fibronectin, lactoferrin, laminin and serum albumin (Pilch & Mann 2006). Seminal plasma proteins display a wide range of posttranslational variants (Fung *et al*. 2004). The many roles of the above seminal plasma proteins include adding viscosity to seminal plasma, protecting spermatozoa from stress, microbes and immune cells, providing energy substrates, preventing premature sperm capacitation and mediating the interactions between spermatozoa and oviductal epithelial cells (Calvete & Sanz 2007, Muino-Blanco *et al*. 2008).

Sperm-oocyte interaction proteomics

During fertilization, sperm structures undergo irreversible changes, and some of them are lost or partially retained on the egg vestments. Proteomic studies profited from these irreversible and relatively stable binding reactions for identification of sperm proteins involved in interactions with oocyte surface.

van Gestel *et al*. (2007) isolated, purified and solubilized plasma membranes from boar spermatozoa and incubated such preparations with porcine zona pellucida fragments. They resolved the complex sperm and ZP proteins on 2D gels and identified the potential ZP-binding sperm proteins by using Q-Tof Nanospray MS/MS. They identified four dominant proteins in these ZP-bound sperm membranes, including spermadhesin AQN-3, lactcadherin/p47, fertilin beta and peroxiredoxin 5. Except for peroxiredoxin 5, all three remaining proteins have been implicated previously in sperm-oocyte interactions. Other sperm membrane-associated proteins included eight ADAM family disintegrins, ion channels and transporters, enzymes, secretory pathway associated proteins, acrosomal surface proteins and various plasma membrane receptors.

Sperm head tail-separation and subcellular fractionation of the sperm head components were applied to identify mouse sperm head proteins potentially involved in different steps of fertilization (Stein *et al*. 2006). To distinguish the sperm surface proteins from the rest, spermatozoa were surface biotinylated and membrane surface, membrane vesicle and acrosomal matrix fractions were recovered. Of the identified proteins, one third were products of genes for which the mutations have been shown previously to cause subfertility or infertility. Identified acrosomal proteins included glycolytic and proteolytic enzymes and enzyme inhibitors, receptor proteins for zona pellucida, secretory pathway proteins and proteins involved in protein folding (Stein *et al*. 2006). Identified proteins with a proposed function in fertilization included those implicated in sperm zona interactions (e.g. SP38/IAM38/ZPBP2), sperm-oolemma binding (ADAM-family proteins) and various acrosomal enzymes.

Biotinylated oolemmas were harvested to identify mouse oocyte surface proteins potentially involved in fertilization and zygotic development. The putative surface-labeled proteins identified by biotinylation included heat shock proteins HSP70 and HSP90a, chaperone proteins GRP94 and GRP78, oxygen regulated protein ORP150, calreticulin, calnexin and protein disulfide isomerase (Calvert *et al*. 2003).

Some proteins involved in sperm capacitation and acrosome reaction assemble within designated membrane microdomains, the lipid rafts (Boerke *et al*. 2008). Some of these proteins were identified in the mouse detergent-resistant membrane fractions (Miranda *et al*. 2009). Among the most abundant proteins were those previously implicated in fertilization process, including IZUMO and SPAM1. Other proteins of interest were membrane raft-associated proteins caveolin 2 and flotilin 2, TEX101 and hexokinase 1.

Posttranslational modification proteomics in gamete research

Phosphoproteomics are particularly important for sperm studies due to the importance of sperm protein phoshorylation for the process of sperm capacitation (Bailey *et al.* 2005). In spite of intensive research, relatively few capacitation-related phosphoproteins have been identified. A predominant one is the tyrosine phosphorylated p32 antigen/group of antigens migrating at 32 kDa in boar sperm extracts. These are acrosomal proteins including acrosin-binding protein sp32 and two triosephosphate isomerase isoforms (Bailey *et al.* 2005).

Proteins phosphorylated on Tyrosine residues were detected by 2-D Western blotting with anti-phosphotyrosine antibodies in non-capacitated versus capacitated mouse spermatozoa and identified by MS/MS. Among proteins identified were 20S proteasomal core subunit alpha 6 (PSMA1), VDAC, tubulin, PDHE1 beta chain, glutathione S-transferase, NADH dehydrogenase (ubiquinone) Fe-S protein 6, acrosin binding protein sp32-precursor and cytochrome b-c1 complex (Arcelay *et al.* 2008). Findings from an earlier study of capacitated human sperm phosphoproteome by the same group zeroed in on phosphoproteins in the sperm tail principal piece that are likely to convey signals for hyperactivated sperm motility observed in capacitated spermatozoa, including the A-kinase anchoring proteins (AKAP3 and AKAP4). Also identified was the valosin-containing protein (VCP) involved in substrate presentation to 26S proteasome (Ficarro *et al.* 2003). This study used Fe3 + immobilized metal affinity chromatography (IMAC) to enrich phosphopeptides generated by enzymatic digestion of sperm phosphoproteins prior to MS/MS. Preliminary data from a large scale proteomic study of sperm phosphoproteome have been presented recently, but not yet published as a peer-reviewed article (Baker *et al.* 2008b).

Oocytes have also been subjected to phosphoproteomics. Phosphoprotein profiling of bovine oocyte maturation identified 40 proteins belonging to various protein families such as protein kinases, cell cycle regulators, chaperone proteins, and cytoskeletal proteins (Bhojwani *et al.* 2006). Identified proteins included some found in other oocyte proteome studies discussed above, such as MVP, heat shock proteins and peroxiredoxin 2. Proteins differentially phoshorylated during oocyte maturation included beta-tubulin, beta-actin, cyclin E2, aldose reductase and UMP-synthase, protein disulfide isomerase 2 and peroxiredoxin 2.

We have been particularly interested in posttranslational modification of gamete proteins by ubiquitination. Ubiquitinated proteins can be purified by using the agarose-immobilized recombinant UBA domain of ubiquitin binding-protein p62 (Vadlamudi *et al.* 1996). Such proteins can be resolved on PAGE and excised for proteomic identification. Using this approach, we have identified three abundant, ubiquitinated proteins in porcine oocytes: MVP (Sutovsky *et al.* 2005), UCHL1 (Yi *et al.* 2007a), and TFAM (Antelman *et al.* 2008). Ubiquitin binds covalently to an internal Lys-residue of its substrate proteins through its C-terminal residue Gly-76, backed by Gly-75 and Arg-74. Consequently, trypsin digestion of ubiquitinated proteins around the ubiquitinated substrate Lys-residue produces a unique fingerprint-peptide with Gly-Gly adjunct and a mass increased by 114 Da (Peng *et al.* 2003). This "Gly-Gly" modification can be accounted for in proteomic database searches and used to identify the position of the ubiquitinated Lys-residue on the substrate protein. Studies are in progress to identify such ubiquitin-modified sites in several sperm and oocyte proteins that participate in fertilization.

Protein S-nitrosylation is a covalent posttranslational modification that results in the formation of an S-NO bond on a thiol group of a cysteine residue of the modified protein. Nitrosylation was examined in human spermatozoa by Lefievre *et al.* (2007). Sperm proteins in which nitrosylation was identified included proteins that were shown to be nitrosylated in other cell types (e.g. tubulin, GST and several heat shock proteins), as well as sperm/seminal plasma proteins such as the A-kinase anchoring proteins AKAP3 and AKAP4, and semenogelin 1 and

2. Similar to other sperm proteomes discussed above, subunits of the 20S proteasomal core were represented prominently.

Conclusions

Table 1 summarizes the proposed functions of the most prominent proteins found in mammalian gametes and secretions of the male sex accessory glands. Altogether, the present review illustrates positive impact of proteomics on our understanding of mammalian gametogenesis, fertilization and preimplantation embryo development. An outstanding challenge in basic gamete and embryo research is the optimization of strategies for a high throughput proteomic validation of transcriptional profiling data. Antibody arrays are being developed by several companies. Recent examples of such arrays applied to the study of mammalian oocyte and embryo is the screening of 400+ signal transduction proteins in porcine maturing oocytes by using Kinex antibody microarray (Pelech *et al*. 2008). The problem with antibody arrays is that at the present time only a small portion of changes observed in protein expression and posttranslational modification seem to be repeatable and validated by immunoblotting (Pelech *et al*. 2008). The large number of oocytes necessary for proteomic analysis is another limitation, particularly in studying rodent and human oocytes. This issue will likely be mitigated by an increased use of large animal models in basic gamete research and by steadily increasing sensitivity and decreasing total protein requirement of novel proteomic protocols. Differential isobaric tagging of a protein sample, akin to the differential probe labeling employed by microarray technology, will allow for the accurate comparison of protein composition between samples.

Acknowledgements

I wish to thank Ms. Kathy Craighead for editorial assistance. Some of the work discussed in this paper was supported by the Food for the 21st Century Program of the University of Missouri, and by the National Research Initiative Competitive Grants # 2007-01319 and 2005-3502-15549 from the USDA Cooperative State Research, Education and Extension Service.

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Mapping quantitative trait loci for reproduction in pigs

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Reproductive performance is a critical component of sustainable animal production systems. The low heritability of reproductive performance traits such as litter size, ovulation rate, and prenatal survival and their expression only in females limits improvement of these traits through traditional selective breeding programs. However, there is abundant evidence of genetic variation in these traits between pig breeds, which could be exploited to improve reproductive performance through selective breeding. The Chinese Meishan breed is one of the most prolific pig breeds known, displaying greater litter size than commercial Western breeds, such as Large White, through higher levels of prenatal survival for a given ovulation rate. But Meishan pigs have poor growth rates and high carcass fat content. However, increasing the number of viable and productive offspring per reproductive female reduces financial and environmental costs and improves the sustainability of the system. Thus, the superior Meishan alleles for reproduction traits are potentially commercially valuable. As only a fraction of the genes / loci that underpin the Meishan's superior reproductive performance have been identified to date, it is evident that the genetics of reproductive performance merits further investigation. In an earlier study we mapped a QTL (quantitative trait loci) with effects on embryo survival and litter size to the distal end of pig chromosome 8 (King *et al*. 2003). The objective of this study is to identify QTL affecting ovulation rate, teat number, litter size, number born alive and embryo survival, and characterize candidate gene(s) underlying such QTL.

Our strategy to identify genetic markers for reproduction traits combines identifying QTL (regions of the genome linked to the phenotypes) through genome scans using interval mapping and testing genes recognized as candidates on both positional and physiological grounds. The QTL analyses involve testing for associations between variation in the trait(s) of interest and the inheritance of chromosomal segments from the parental animals. The inheritance of chromosomal segments through the QTL mapping population is tracked by genotyping the population for polymorphic genetic markers – microsatellites and single nucleotide polymorphisms (SNPs). The three-generation Roslin Institute Meishan x Large White F2 QTL mapping population was genotyped for ten additional markers across the QTL found previously on chromosome 8 and for 127 markers evenly spaced across the rest of the genome. The marker genotypes and trait data were lodged in the resSpecies database (www.resSpecies.org). Linkage maps were constructed using Multimap and Crimap (Green *et al*. 1990) and the resulting maps checked for anomalous double recombinants with the chrompic function. Anomalous genotypes were checked and corrected or omitted from the analysis. The marker orders in the linkage map exhibited good agreement with international reference linkage maps. QTL analyses were performed using the "fixed QTL allele" model on the GridQTL portal (www.gridqtl.org.uk/) (Seaton *et al*. 2002).

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A genome-wide scan was conducted for QTL with effects on the following traits: embryo survival, total ovulation rate, total teat number, litter size, and number born alive. This scan revealed QTLs for litter size (total number born) on SSC6 (102 cM), SSC8 (114 cM), SSC13 (135 cM) and SSC18 (37 cM); QTL for number born alive on SSC8 (114 cM); QTL for embryo survival on SSC8 (135 cM) and SSC10 (111 cM) and QTL for total ovulation rate on SSC7 (56 cM), SSC13 (38 cM), SSC15 (8 cM), and SSC18 (37 cM), and for total teat number on SSC5 (57 cM), SSC6 (20 cM) and SSC18 (0 cM). These results were used as background effects in further analyses to confirm the presence or absence of these QTL and to potential reveal additional QTL. This new scan revealed QTL for litter size (total number born) on SSC8 (114cM) and SSC13 (35 cM), confirming two of the previous ones; QTL for number born alive on SSC13 (41 cM); QTL for embryo survival on SSC8 (135 cM), and QTL for total ovulation rate on SSC7 (9-13 cM), SSC13 (39 cM), and SSC14 (21 cM), and for total teat number on SSC5 (56 cM), SSC11 (0 cM) and SSC12 (3 cM). These QTL require further studies to confirm the effect on the different traits. As noted above, QTL with effects on litter size and embryo survival had been discovered co-located at the distal end of chromosome 8 in an earlier study (King *et al*. 2003). The fine mapping of these QTL enabled by genotyping the population for additional markers revealed two QTL locations - a QTL for embryo survival on chromosome 8 (SSC8) at 134 cM, a QTL for litter size at position 114 cM, together with a QTL with effects on the number born alive. The emerging pig genome sequence and the homologous regions of the human and mouse genome sequences will be inspected for potential positional and comparative positional candidate genes. The SPP1 (Secreted phosphoprotein 1) gene, which is known for its role in communication between the developing embryo and the sow, with a key role in conceptus implantation and maintenance of pregnancy, is a strong candidate gene as it is located under the peak of the SSC8 QTL with effects on embryo survival.

The resolution for the QTL found previously has been improved by the addition of a further ten markers across the region of interest. The genome scan revealed twelve QTLs of which only two are annotated in the pigQTLdb (www.animalgenome.org/QTLdb/pig.html). Future studies will involve fine mapping the QTL revealed by the genome scan and prioritisation of positional candidate genes underlying the QTL for functional analyses. For example, candidate genes for embryo survival QTL will be examined for their expression at the RNA and protein levels in relevant tissue samples (feto-placental tissue) from animals of different breeds.

We thank the British Pig Executive (SCH) and BBSRC (ALA, CJA, CSH) for financial support and the Birrel-Gray Travelling Scholarship (SCH) and the Society for Reproduction and Fertility Travel Grant (SCH) for the awards to attend the conference.

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Global protein profiling of porcine cumulus cells in response to native oocyte secreted factors in vitro

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Until recently, the traditional view was that the oocyte played a passive role in folliculogenesis, relying on paracrine signalling from surrounding somatic cells to acquire developmental competence. However, recent evidence suggests that the oocyte plays an active role in the process of folliculogenesis by secreting soluble factors that act on the neighboring somatic cells. Studies using rodent and ruminant animal models have shown the importance of oocyte-secreted factors for cell proliferation and differentiation, steroidogenesis, metabolism, and, in certain species, in determining ovulation rate. However, little information is available in the pig species. Therefore, the objective of the current study was to determine the effects of native oocyte-secreted factors on cumulus cell protein expression in the pig follicle.

Three groups of four sows were euthanized on day 19 $\pm$ 1 after the 1st post-weaning oestrus at a time point when the pre-ovulatory follicle population was established and the oocytes should have acquired full developmental competence. Follicle size and follicular fluid oestradiol concentration were used to confirm that oocytes were derived from highly oestrogenic follicles that had not been exposed to the preovulatory-LH surge. Cumulus-oocyte complexes (n = 19 $\pm$ 2 per sow) were aspirated from these large pre-ovulatory follicles, washed three times in PVA TL-HEPES, and denuded (DO) from their cumulus cells. Concomitantly, gilt ovaries were obtained from a local slaughterhouse and oocytectomized cumulus complexes (OOX) were prepared by microsurgically removing the oocytes from their surrounding cumulus cells. Groups of 16 OOX were then cultured for 22h with or without the DOs obtained from individual sows (n = 4 groups of OOX per treatment per replicate) in 36 μl droplets of modified M199 medium under mineral oil at 38.5ºC in a humidified atmosphere of 5% CO_2. For each of the three replicate cultures, 3 groups of 16 OOX incubated with or without DOs were pooled and subjected to 2-dimensional gel electrophoresis on 7cm IPGstrips pH 3-10 in the first dimension and 10% SDS-PAGE slab gels in the second dimension. The SYPRO Ruby (BioRad, CA) stained gels were imaged on a Typhoon Trio (GE Healthcare). Due to incomplete isoelectric focusing for one replicate, only the gel images from the remaining two replicates were finally analysed using Progenesis SameSpots software (Nonlinear Dynamics, UK). Individual spot intensities were normalized with the total spot volume from the gel of origin. A preparative gel containing the protein from the cumulus cells of 500 cumulus-oocyte complexes was also run under the same conditions but using an 18 cm IPGstrip pH 3-10. The preparative gel was matched with the analytical gels and protein spots of interest were manually excised and sent to a mass spectrometry facility for identification by LC MS/MS (Centre Genomique du Quebec, Sainte-Foy, Canada). To confirm proper matching between the analytical and preparative gel, 6 abundant protein spots of interest from the analytical gel were also sent for identification.

More than 600 spots were matched across the 4 gels of which 14 proteins were found to be differentially expressed when the OOX were incubated alone or with DOs. Interestingly, 9 of

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the 14 differentially expressed proteins were up-regulated (1.2- to 2.7-fold change) in the absence of oocytes. Conversely, 5 of the 14 differentially expressed proteins were down-regulated in the OOX incubated without DOs; however, the magnitude of the down-regulation was lower, with only a 1.1- to 1.2-fold change, representing a 10-20% decrease in protein abundance.

These results suggest that removing the oocyte from the cumulus-oocyte complex leads to specific, and predominantly stimulatory, effects on cumulus cell protein expression. Amongst the 13 proteins that were identified, 5 up-regulated proteins and 1 protein that was down-regulated in OOX incubated without DOs appear to be of particular interest (Table 1). First, 14-3-3η is a member of the 14-3-3 protein family that modulates the interactions between proteins. The 14-3-3 proteins are involved in signal transduction and interact with various receptors including the glucocorticoid receptor, androgen receptor, insulin and IGF-1 receptor, as well as FSHR. In addition, they are also involved in the regulation of cell cycle progression, apoptosis and metabolism. NPM1 is a nuclear chaperone capable of acting as a transcription coactivator and modulates cell proliferation and apoptosis. P4HB is a key enzyme in collagen biosynthesis that appears to be essential in the peri-ovulatory period after the LH surge. Collagen synthesis is considered to be essential for repairing the ruptured follicle after ovulation but may also be necessary for the follicle-luteal transition that occurs at ovulation. TCTP is involved in modulating apoptosis but also alters cell morphology and positively regulates cell proliferation. The Rho GDP-dissociation inhibitor 1 prevents the activity of Rho GTPase which is involved in the organization of the actin cytoskeleton and in cell proliferation. Finally, PGAM1 is a glycolytic enzyme that catalyzes the interconversion of 2- and 3-phosphoglycerate. Changes in the activity of this enzyme modify the quantity of glucose 6-phosphate available for the pentose-phosphate pathway which ultimately modulates the amount of NADPH available for downstream biosynthetic pathways such as steroid biosynthesis. Altogether, these observations suggest that the porcine oocyte potentially modulates cumulus cells with regards to metabolism, apoptosis, proliferation and steroidogenesis. These findings are, therefore, consistent with the concept that oocytes secrete soluble factors that act on the cumulus cells to modulate their functions and phenotype.

Table 1: Identity of 6 proteins whose expression was differentially regulated when oocytectomized cumulus complexes (OOX) were cultured with or without the denuded oocytes (DOs) harvested from large, oestrogenic porcine follicles that had not been exposed to the pre-ovulatory LH surge in vivo.

Spot #	↓↑ in OOX w/o DOs (fold change)	Protein name (symbol)	Accession #	Theoretical MW (kDa)/pI	# of unique peptides	Protein coverage (%)
2	↑ (2.7)	14-3-3 protein eta (YWHAH)	UPI0000EB0531	28 / 4.8	21	60
3	↑ (2.1)	Nucleophosmin (NPM1)	P06748	33 / 4.6	10	39
4	↑ (1.8)	Prolyl 4-hydroxylase, beta subunit (P4HB)	P05307	57 / 4.7	15	32
5	↑ (1.7)	Translationally-controlled tumor protein (TCTP)	P13693	20 / 4.8	6	31
20	↑ (1.2)	Rho GDP-dissociation inhibitor 1 (ARHGDIA)	P19803	23 /5.0	8	43
23	↓ (1.15)	Phosphoglycerate mutase 1 (PGAM1)	P18669	29 / 6.7	20	78

Nutritional and lactational effects on follicular development in the pig

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In sows, follicular development is inhibited during lactation, and weaning the piglets allows recruitment and selection of follicles that will undergo preovulatory maturation and ovulate. Lactation inhibits GnRH secretion, and in turn LH secretion, through neuroendocrine stimuli induced by suckling. Pituitary response to GnRH and the sensitivity of the hypothalamo-pituitary unit to oestradiol positive feedback are also reduced. The impact of lactation on the reproductive axis is further complicated by the physiological and metabolic adaptations that are developed for milk production and that depend on nutrient intake, nutrient needs and body reserves. A strongly catabolic state during lactation amplifies the inhibition of LH secretion, thereby inducing a delay of oestrus and ovulation after weaning. Nevertheless, post-weaning ovulation is less delayed nowadays than in the 1970's or 80's. Nutritional deficiency has also deleterious effects on embryo survival, which are likely related to alterations in follicular growth and maturation. The physiological mechanisms by which information on the metabolic changes is transmitted to the hypothalamus-pituitary-ovary axis are not fully understood in the sow. Glucose, insulin and leptin are the most likely signals informing the hypothalamus of the metabolic state, yet their roles have not been definitely established. At the ovarian level, folliculogenesis is likely to be altered by the reduction in insulin and IGF-I concentrations induced by nutritional deficiency. More knowledge is needed at the intrafollicular level to better understand nutritional effects on follicular development, and also on occyte quality and embryo development.

Introduction

In sows, as in numerous mammalian species, parturition is followed by a period of anovulation. Suckling by piglets is the main factor that inhibits the activity of the hypothalamo-pituitary-ovarian axis and the nutritional deficiency related to milk production can amplify the inhibition of the reproductive axis. Lactational and nutritional effects on fertility in sows have been extensively reviewed (Britt *et al*. 1985, Aherne & Kirkwood 1985, Foxcroft 1992, Einarsson & Rojkittikhun 1993, Quesnel & Prunier 1995). However, nutritional effects on reproductive performance after weaning appear to have changed over the past 20 years towards less delayed ovulation after weaning and deleterious effects are frequently reported on embryo survival (Table 1). Moreover, extensive research has been conducted to better understand the physiological and metabolic mechanisms underlying the nutritional effects on reproduction. The purpose of

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this review is first to update knowledge on the impacts of lactation and nutrition on follicular development, and second to examine potential mechanisms mediating these effects.

Table 1. Influence of feed or protein supply (High or Low) during lactation on the weaning-to-oestrus interval (WOI), ovulation rate (OR) and embryo survival (ES) in sows from first or second parity.

References		WOI (days)		OR		ES (%)	
	Parity	High	Low	High	Low	High	Low
Feed supply							
Mullan *et al*.1991	1	8.7	19.2*	-	-	-	-
Zak *et al*. 1997a	1	3.7	5.4*	19.9	15.4*	87	64*
Zak *et al*. 1998	1	4.2	6.3*	14.4	15.6	83	72
Van den Brand *et al*. 2000	1	5.1	5.4	18.2	16.9†		
Vinsky *et al*. 2006	1	5.3	5.4	18.3	18.2	79	68*
Kirkwood *et al*. 1987	2	4.3	5.8*	18.2	18.7	83	68*
Kirkwood *et al*. 1990	2	6.0	8.9*	17.6	17.7	83	72*
Baidoo *et al*. 1992	2	5.9	7.3*	16.4	17.2	81	67*
Protein supply							
King & Martin 1989	1	7.5	16.0*				
Jones & Stahly 1999	1	7.7	11.7*				
Mejia-Guadarrama *et al*. 2002	1	5.4	5.3	23.4	20.0*	72	73

* $P < 0.05$; † $P < 0.1$.

Effect of nursing and milk production on follicular development

Neuroendocrine and metabolic consequences of nursing and milk production

In sows, the effects of nursing and milk production cannot be dissociated since lactation is not maintained without suckling by the young. During lactation, stimulation of the teats by piglets and piglet proximity elicit neuroendocrine reflexes that induce the release of neurotransmitters and neuropeptides in the central nervous system of the sow. Neuropeptides include the endogenous opioid peptides (EOP) that have a morphine-like biological activity and include endorphins, enkephalins and dynorphins. These factors, in turn, stimulate the secretion of the pituitary hormones, prolactin, growth hormone (GH), adrenocorticotropin (ACTH), thyroid-stimulating hormone (TSH) and oxytocin (Fig. 1, reviewed by Kraeling & Barb 1990, Estienne & Barb 2005). Thereby, secretion of insulin-like growth factor I (IGF-I), cortisol and thyroid hormones is also stimulated.

These hormones are involved in milk production through different pathways including the regulation of udder development, nutrient uptake and body reserves mobilization (for review, see Père *et al*. 2008). Oxytocin plays an essential role in milk ejection during each nursing, but could also facilitate the mobilization of body reserves (Valros *et al*. 2004). Prolactin is essential for the initiation and the maintenance of milk production, and milk protein synthesis (Farmer *et al*. 2008). Elevated concentrations of GH are thought to favour the preferential drive of glucose and lipids to the mammary gland through the anti-lipogenic action of this hormone. Together with elevated concentrations of IGF-I, they could also minimize mobilization of endogenous proteins and thus spare lean tissue. Cortisol is known to enhance mobilization of energetic substrates from body reserves and thyroid hormones stimulate protein synthesis by the mammary gland, amongst many metabolic actions.

Because of the great use of glucose by the mammary gland, mean blood concentrations of glucose are lower during lactation than during pregnancy. In contrast, concentrations of non-

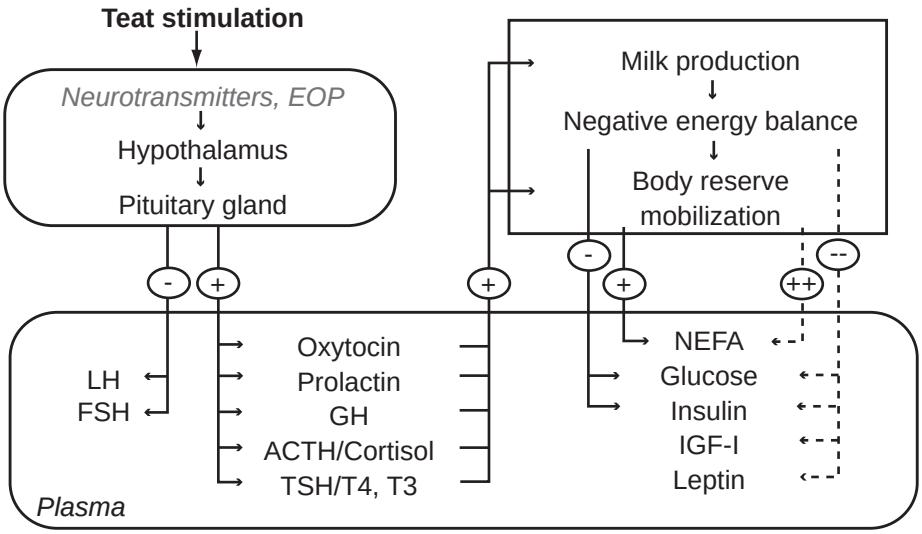


Fig. 1 Schematic representation of neuroendocrine and metabolic consequences of suckling and milk production.

Energy balance of sows during lactation may vary from moderately (plain line) to highly negative (interrupted lines). Plasma concentrations of metabolites and metabolic hormones are summarized from various studies (e.g. Rojkittikhun *et al*. 1992, Schams *et al*. 1994, Kraetzl *et al*. 1998, Quesnel *et al*. 1998a, Govoni *et al*. 2007).

EOP: endogenous opioid peptides, NEFA: non-esterified fatty acids, ACTH: adrenocorticotropin, TSH: thyroid-stimulating hormone, LH: luteinizing hormone, FSH: follicle-stimulating hormone.

esterified fatty acids (NEFA) are elevated during lactation, which indicates lipid mobilization from adipose tissue. Fat catabolism is induced by the elevated concentrations of GH and also by energy deficiency when sows are not allowed to consume feed *ad libitum* in early lactation. Elevated concentrations of NEFA are believed to induce the peripheral insulin resistance observed during lactation (Père & Etienne 2007), which is a major physiological adaptation to enhance glucose availability for the mammary gland. Preprandial concentrations of glucose and insulin generally decrease as lactation progresses and milk production increases.

In situations of severe nutritional deficiency, further physiological and metabolic adaptations are developed to maintain a high level of milk production at the expense of maternal body reserves (Fig. 1). Such adaptations are observed in high-yielding multiparous sows and also in most primiparous sows because they have a lower feed intake than multiparous sows but a relatively high milk production. When sows are in a strongly catabolic state induced by feed or protein restriction, IGF-I concentrations are reduced presumably because of the uncoupling of the link between GH and IGF-I secretion (Quesnel *et al*.1998a, 2005a, Mejia-Guadarrama *et al*. 2002). A low level of IGF-I facilitates the mobilization of endogenous protein, which provides amino acids that may be used for gluconeogenesis and milk protein synthesis. Insulin secretion is reduced which allows further mobilization of lipids from adipose tissue. Feed restriction also affects post-prandial concentrations of leptin (Mao *et al*. 1999), but not pre-prandial concentrations (Prunier *et al*. 2001, Estienne *et al*. 2003). Pre-prandial concentrations appear to be highly influenced by sow adiposity (Estienne *et al*. 2000, 2003, de Rensis *et al*. 2005).

Effects of suckling on the reproductive axis

During the last month of pregnancy, follicular development and LH secretion are inhibited by progesterone and oestrogens secreted by corpora lutea and the foeto-placental units respectively (reviewed by Quesnel & Prunier 1995). At parturition, circulating concentrations of progesterone and oestrogens fall and LH secretion immediately increases. Two or three days after parturition, LH secretion is inhibited again in suckled sows (de Rensis *et al*. 1993a). This suckling-related suppression of LH episodic secretion appears to be due to the inhibition of the GnRH pulse generator (Kraeling & Barb 1990). The inhibitory effect is mainly due to EOP during established lactation, whereas its development in early lactation is opioid-independent (de Rensis *et al*. 1993b, 1998).

In addition to the inhibition of GnRH secretion, the pituitary responsiveness to GnRH is also altered (Quesnel & Prunier 1995). It is low after parturition and increases as lactation progresses. The reduced responsiveness of the pituitary gland can be due the direct action of EOP on the adenohypophysis (Estienne & Barb 2005). It may also be related to LH stores in the pituitary gland, which are depleted just after farrowing and are progressively restored during lactation.

Mean concentrations of plasma LH and number of LH pulses are low during the first two weeks of lactation, and then progressively increase (*e.g.* Shaw & Foxcroft 1985, Jones & Stahly 1999). This partial resumption of LH secretion can be permitted by the increase in pituitary LH-response to GnRH and (or) by a decrease in suckling intensity in the course of lactation. Indeed, the total time spent nursing was shown to decrease from the 13th day of lactation onwards (Valros *et al*. 2002). Variations in follicle-stimulating hormone (FSH) are less marked than variations in LH, probably because FSH release is influenced by ovarian secretions during lactation (Quesnel & Prunier 1995). Steroid secretion remains low during lactation and therefore does not influence LH secretion. In contrast, FSH secretion is inhibited by inhibin secreted by growing follicles (Wheaton *et al*. 1998).

In the ovaries, follicular growth up to 2 mm in diameter does not require gonadotrophic support; growth from 2 to 4 mm requires FSH support and is stimulated by LH, while growth and maturation from 4 mm to the ovulatory size (6-10 mm) requires a high frequency of LH pulses. Consistently, large follicles (≥ 5 mm) can be observed on the ovaries after parturition, and then only small and medium-sized follicles (no larger than 3 mm) are present during the second week of lactation. Afterwards, follicular growth resumes as a consequence of the progressive increase in the frequency of LH pulses. However, follicles generally do not develop beyond 5 mm in diameter during lactation. Beside this general pattern of follicular growth, great variability between sows has been reported in follicular populations before weaning. Using transrectal ultrasonography, Lucy *et al*. (2001) described sows with relatively inactive ovaries with no follicles larger than 2 mm in diameter and other sows with large follicles (6 mm) present. These authors also suggested the existence of non-ovulatory follicular waves during the week before weaning, consisting of a cohort of follicles that grow to 4-6 mm and then regress. This needs to be further investigated.

During lactation, the positive feedback action of oestradiol on LH release is also impaired. After parturition, the hypothalamo-pituitary unit does not respond to oestrogen positive feedback and its responsiveness is partially recovered in the third and fourth weeks of lactation (for review, see Quesnel & Prunier 1995). However, the amplitude of the LH surge after the injection of a massive dose of oestradiol benzoate remained lower than that observed in similarly treated cyclic or prepubertal gilts. The impaired responsiveness of the sows to oestradiol benzoate could be due to a low pool of LH stores or to the inhibitory effects of EOP on GnRH secretion and pituitary response to GnRH. The dysfunction in the oestradiol-induced positive feedback

could also be a long-term effect of pregnancy and parturition (Quesnel & Prunier 1995). This hypothesis was based on studies in sows weaned just after parturition. Weaning piglets at birth, instead of 3 to 5 weeks after birth, induces a high incidence of anoestrus and cystic ovaries and delayed ovulation. Anoestrus and cystic ovaries probably result from a lack of an ovulatory LH surge due to reduced hypothalamic response to oestradiol positive feedback. Additionally, the duration of lactation influences the hypothalamic responsiveness to oestradiol in weaned sows; the amplitude of the LH surge induced by exogenous oestradiol is higher after 35 than after 21 of lactation (Edwards & Foxcroft 1983).

Effects of weaning on the reproductive axis

Weaning the piglets removes the inhibition originating from the suckling stimuli, which induces an immediate increase in LH secretion and, although less clear, in FSH secretion. Some follicles are recruited and selected from a pool of medium-sized follicles (2-3 mm) to undergo preovulatory maturation and to ovulate, whereas the other medium-sized follicles become atretic. The preovulatory follicular growth and associated oestradiol production lead to oestrous behaviour and ovulation within 4 to 6 days after weaning, on average.

Variation in the weaning-to-oestrus interval (WOI) is related to the degree of inhibition of LH secretion during lactation (Shaw & Foxcroft 1985). The degree of inhibition of LH secretion during lactation influences follicular development before weaning and the resumption of follicular growth after weaning (Quesnel *et al*. 1998b). Bracken *et al*. (2006) reported that the average diameter of follicles at weaning was one factor controlling the WOI duration. The degree of inhibition of LH secretion before weaning also influences LH secretion after weaning (Shaw & Foxcroft 1985, van den Brand *et al*. 2000).

Does the intensity of suckling modulate the activity of the reproductive axis?

Assessing to what extent the intensity of suckling stimuli can modulate the inhibition of LH secretion is not easy. Most strategies that increase suckling intensity (e.g. increasing litter size, litter weight, age of nursed piglets or nursing frequency) also increase milk yield and can affect sow metabolic state. In multiparous sows, a reduction in LH secretion was reported in association with a longer average nursing duration and not in association with a more catabolic state (Hultén *et al*. 2002a, b). In an attempt to dissociate suckling and metabolic effects, we compared sows that nursed 13 or 14 piglets and were fed *ad libitum* to sows that nursed only 7 piglets and were subjected to feed restriction (Quesnel *et al*. 2007). The sows that nursed a large litter had smaller follicles at weaning than sows with a small litter. Yet, despite a similarly negative energy balance in sows from the two groups, plasma IGF-I concentrations were lower in sows that nursed a large litter. Therefore, we were not fully successful in maintaining an equivalent metabolic state across groups.

Although most sows remain anoestrus throughout lactation, ovulation can occur before weaning. Lactational ovulation is followed by a regular oestrous cycle of 21 days, thereby inducing a delayed oestrus after weaning. Because oestrous behaviour is not usually recorded during lactation, data are scarce on the incidence of lactational ovulation in commercial farms. In a field study based on 7 farms and 492 sows, 3% of sows on average were reported with lactational ovulation, as detected by high concentrations of progesterone at weaning (Auvigne *et al*. 2006). One may wonder whether strategies implemented at farm level to alleviate side

effects of high prolificacy, such as partial weaning of the heaviest piglets, increase the incidence of lactational ovulation. Moreover, welfare concerns promote the development of housing systems that allow the animals to express their natural behaviour, such as group-housing. Hultén *et al*. (1995) reported that 28% of the sows housed in groups during weeks 4 and 5 of lactation ovulated before weaning, whereas none of the singly housed sows ovulated during the corresponding period.

Nutritional effects on follicular development in the sow

Impact of nutritional deficiency on performance of reproduction

A severe feed restriction associated with body weight loss during lactation delays oestrus after weaning in primiparous sows and to a lesser extent, in second-parity sows (Table 1). In the most recent experiments, the weaning-to-oestrus interval was little influenced by nutritional restriction during lactation (Table 1). This change is probably the consequence of indirect selection on WOI along with genetic selection on prolificacy. Together with the moderately extended WOI, deleterious effects of nutritional restriction are occasionally observed on ovulation rate and consistently on embryo survival (Table 1). Reduction in both ovulation rate and embryonic survival can partly explain the so-called "second litter syndrome" or "second parity dip", i.e. the smaller second litter as compared to the first one. Ultimately, a severe negative impact on embryo survival could explain the reduction in farrowing rate observed after the first weaning. In a field study including nearly 1700 sows, a longer weaning-to-service interval was reported when bodyweight loss during lactation increased above 5% for primiparous sows and above 10% for older sows (Thaker & Bilkei 2005). Yet, all sows returned to oestrus within 7 days after weaning when bodyweight loss did not exceed 15%. Subsequent farrowing rate and litter size were depressed when bodyweight loss exceeded 10%.

Nutritional effects at the hypothalamo-pituitary level

Feed restriction associated with a strongly catabolic state inhibits the episodic secretion of LH during lactation (Mullan *et al*. 1991, Zak *et al*. 1997a, 1998, Quesnel *et al*. 1998b). Since the frequency of LH pulses is tightly controlled by the GnRH pulse generator, the nutritional anoestrus is likely due to the inhibition of GnRH secretion. It is worth noting that a severe restriction in crude protein or digestible energy also delays the post-weaning oestrus through impairment of GnRH and LH secretion during lactation (King & Martin 1989, Koketsu *et al*. 1996, Jones & Stahly 1999, Yang *et al*. 2000a). Such restrictions do not occur naturally in herds, but may help understand the mechanisms mediating nutritional effects.

Almond & Dial (1990a, b) suggested that failure to return to oestrus after weaning might also be due, in part, to an increased sensitivity of the hypothalamo-pituitary unit to the negative feedback of oestrogens. In their experiments, however, anoestrus was thought to be due to the seasonal influence on reproduction. In ewes, seasonal anoestrus involves an increased sensitivity of the hypothalamus to oestradiol negative feedback and high feed intake can modify the sensitivity of the hypothalamus to oestradiol (Forcada & Abecia 2006). To our knowledge, a nutritional influence on the sensitivity of the hypothalamo-pituitary unit to oestradiol has not been investigated in the sow.

Metabolic mechanisms mediating nutritional effects at the hypothalamo-pituitary level

The relationships between nutrition and reproduction are most often viewed through energy balance. The influence of energy deficiency on GnRH secretion has been extensively reviewed (e.g. Barb *et al*. 2001a, Wade & Jones 2004) and general pathways have been proposed. Feed intake modulates the availability of oxidizable metabolic fuels (glucose and fatty acids), which in turn modulate hormonal secretion (insulin, insulin-like growth factors, leptin, etc) by various organs and tissues. In the model proposed by Wade & Jones (2004), available substrates and hormones can modulate the activity of the neurons of GnRH by two ways (Fig. 2). First, the availability in substrates could be detected by the area postrema, located in the hindbrain. This area has a permeable blood-brain barrier and can monitor substrate concentrations in the blood and cerebrospinal fluid. Second, metabolic hormones could modulate substrate availability and neuronal activity directly in the part of the forebrain which contains neurons of GnRH. The first pathway has been inferred from experiments in rodents and sheep where oxidization of substrates was inhibited by chemical inhibitors. In female rats and guinea pig, glucose deprivation induced by an inhibitor of glycolysis interrupts ovulatory cycles, but this effect disappears when the area postrema has previously been destroyed. However, lesions of the area postrema do not block the inhibitory effects of feed deprivation. Therefore, this pathway alone cannot explain the nutritional inhibition of GnRH secretion (Wade & Jones 2004).

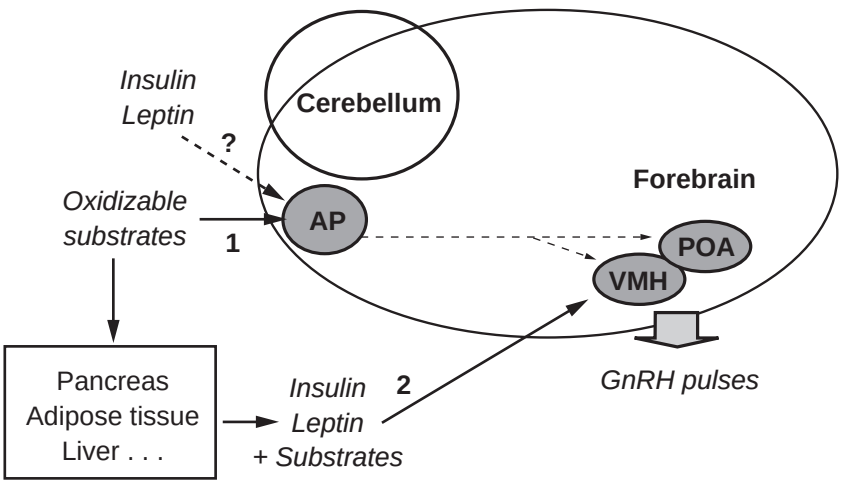


Fig. 2 Schematic representation of potential pathways by which nutrient availability could influence GnRH secretion, based on studies in rodents and small ruminants (adapted from Wade & Jones 2004).

Pathway 1. The availability of oxidizable substrates (glucose and fatty acids) is detected by the area postrema (AP) and information is transmitted to the area which contains neurons of GnRH and neurons sensitive to sexual steroids (ventromedial hypothalamus – VMH- and preoptic area – POA). Pathway 2. Metabolic substrates modulate hormonal secretion by various organs and tissues, which in turn can modulate the activity of the neurons of GnRH.

In the sow, the metabolic mechanisms underlying the nutritional effect on the reproductive axis are not yet fully understood. In swine as in all monogastric animals, metabolic energy is mainly provided by glucose. In gilts, experimentally-induced glucose deprivation reduced LH pulse frequency (Barb *et al*. 2001b). Glucose administration to feed-restricted gilts, and the associated rise in circulating concentrations of insulin, induces a rapid increase in LH pulse frequency similar to that observed in response to refeeding (Booth 1990). Conversely, administration of insulin can stimulate LH secretion, as does increased energy supply (Cox *et al*. 1987). Based on experiments with diabetic pigs, it was suggested that insulin enhances the sensitivity of the hypothalamo-pituitary unit to ovarian positive feedback signal and pituitary responsiveness to GnRH (Cox *et al*. 1994, Angell *et al*. 1996). In lactating primiparous sows, increasing mean plasma concentrations of insulin and glucose by feeding a starch-rich diet compared to a fat-rich diet stimulates LH pulses in early lactation (van den Brand *et al*. 2000). Collectively, these findings support the hypothesis of a positive effect of glucose and insulin on LH secretion in the pig.

Leptin and IGF-I have been identified as putative signals linking metabolic state and neuroendocrine regulation of reproduction, thereby playing a role in puberty attainment in gilts (Barb *et al*. 2001b, 2008). In the post-partum sow, however, there is no evidence for a causal relationship between IGF-I and LH concentrations. Regarding leptin, Barb *et al*. (2008) described, in a very comprehensive review, how it is involved in the control of appetite, energy homeostasis and LH secretion. These authors suggested that leptin can link metabolic state and fertility in the gilt and also in the post-partum sow. However, the importance of the role of leptin in the prolonged lactational anoestrus has not been established. De Rensis *et al*. (2005) reported that fatter sows at farrowing had greater concentrations of leptin, lost more backfat during lactation and had extended WOI and reduced farrowing rate than thinner sows.

In contrast, it appears from literature on the influence of protein supply (Jones & Stahly 1999, Yang *et al*. 2000a, Quesnel *et al*. 2005a) that restriction in protein alone during lactation impairs LH secretion without reducing fat tissue reserves and leptin concentrations. It induces an intense mobilization of endogenous protein and alters amino acids profiles. The reduced concentrations of insulin in protein-restricted sows could play a major role in mediating the inhibitory impact of protein restriction. Besides, as some amino acids are necessary for the synthesis of neurotransmitters involved in the secretion of GnRH, we suggested that a reduction in their availability could participate in the inhibition of GnRH secretion during lactation (Quesnel *et al*. 2005a, b). This hypothesis needs to be further investigated.

Physiological and metabolic mechanisms underlying nutritional effects at the ovarian level

Compared with sows fed *ad libitum,* primiparous sows fed half the *ad libitum* feed supply had inhibited secretion of LH, fewer follicles larger than 4 mm at weaning and two days later, and reduced follicular concentrations of IGF-I (Quesnel *et al*. 1998b). Clowes *et al*. (2003a, b) reported that protein restriction and the associated loss in maternal body protein impair folliculogenesis after weaning, as shown by reduced number and diameter of follicles and reduced follicular content of IGF-I and oestradiol at weaning. Additionally, follicular fluid recovered from sows subjected to feed or protein restriction showed a poor ability to support oocyte maturation *in vitro* (Zak *et al*. 1997b, Yang *et al*. 2000b, Clowes *et al*. 2003b). Nutritional deficiency during lactation can therefore alter both follicular growth and maturation after weaning.

Follicular development is controlled by interactions between gonadotrophins, metabolic hormones (such as insulin and IGF-I) and intra-ovarian growth factors (including IGFs; for review, see Webb *et al*. 2007). Notably, it is well established that insulin and IGF-I increase ovarian

response to gonadotrophins and stimulate steroidogenesis (Adashi *et al*. 1992). Therefore, it is likely that reduced concentrations of insulin and IGF-I in feed-restricted sows impair ovarian responsiveness to the gonadotrophic stimulation at weaning and alter subsequent follicular development and ovulation rate. To our knowledge, however, there is no data on the nutritional effects on intra-follicular IGF binding proteins, which modulate IGF-I bioavailability. Whether elevated concentrations of GH during lactation may inhibit follicular development remains unknown, since both stimulatory and inhibitory actions of GH were reported on follicular steroidogenesis *in vitro* and *in vivo*.

The potential role of leptin at the ovarian level is not clear. Leptin has been shown to attenuate *in vitro* oestradiol secretion by follicular cells stimulated by insulin, IGF-I or gonadotrophins in several species, including the pig. Given that negative energy balance is associated with both low leptin and impaired folliculogenesis, it seems unlikely that direct effects of leptin at the ovarian level are involved in the impairment of folliculogenesis. Nevertheless, Gregoraszczuk *et al*. (2007) reported recently an *in vitro* synergistic action of leptin with IGF-I on oestradiol secretion by pig follicles. Another candidate, adiponectin, has been recently proposed as a mediator of the nutritional effects at the ovarian level. Adiponectin is an adipocyte-derived hormone that plays an important role in lipid metabolism and glucose homeostasis. It was shown to induce preovulatory changes in porcine granulosa cells *in vitro* (Ledoux *et al*. 2006) and to enhance the stimulatory action of IGF-I on steroidogenesis in the rat (Chabrolle *et al*. 2007). Low plasma concentrations of adiponectin have been associated with reproductive disorders related to obesity, including the polycystic ovarian syndrome (Campos *et al*. 2008). Whether adiponectin secretion is influenced by excessive fat loss associated with severe energy deficiency is not known.

Feed or protein restriction has also been shown to impair oocyte quality, i.e. its ability to be fertilized and develop into an embryo (Zak *et al*. 1997b, Yang *et al*. 2000b). Like follicular development, oocyte quality is influenced by a complex hormonal background, including gonadotrophins, IGF-I and steroids (Webb *et al*. 2007, Hunter & Paradis, 2009). Extensive research has been conducted on nutritional effects on oocytes and embryos, mainly from cyclic gilts. Findings support the concept that alteration of these hormones may underlie the impact of nutritional inadequacy on oocyte quality. In turn, follicular and oocyte quality influences embryonic development and survival. To our knowledge, however, little is known on nutritional effects on intrafollicular characteristics (IGF system, glucose utilization, insulin and leptin receptors...). The metabolic cues supporting the impact of the metabolic state on oocyte maturation and embryo survival are beyond the scope of the present review.

Interestingly, detrimental consequences on ovulation rate or embryo survival were mostly reported together with a relatively short WOI duration. Sow energy balance becomes positive as soon as piglets are weaned. However, metabolic state cannot be determined only as a function of energy balance, as previously shown (Quesnel *et al*. 2007, Zak *et al*. 2008). Consistently, IGF-I concentration requires several days after weaning before it is restored to normal (van den Brand *et al*. 2001, Mejia-Guadarrama *et al*. 2002). Presumably, these sows that ovulate soon after weaning have not fully recovered from their lactational catabolic state. Evidence for this hypothesis was provided by experiments where post-weaning insemination was delayed. Extending artificially the weaning-to-oestrus interval by treatment with altrenogest, an analogue of progesterone, or inseminating sows at the second oestrus after weaning ("skip-a-heat") resulted in increased ovulation rate and/or higher embryo survival (Martinat-Botté *et al*. 1994, Clowes *et al*. 1994, Wellen *et al*. 2007, Patterson *et al*. 2008). Moreover, delaying post-weaning ovulation increases the preovulatory size of the largest follicles by nearly 1 mm (Wellen *et al*. 2007, van Leeuwen *et al*. 2009). Whether a smaller ovulatory size reflects a reduced maturity and lower follicle and oocyte quality remains to be determined.

Role of body reserves

There is evidence that maternal body reserves interact with feed or nutrient intake during lactation to influence post-weaning performance of reproduction.

We demonstrated that greater body weight at farrowing (and at weaning) played a protective role against the detrimental impact of a protein restriction in primiparous sows (Quesnel *et al*. 2005b). The protein restriction impaired the return to oestrus and reduced ovulation rate after weaning in sows weighing 180 kg at farrowing but not in sows weighing 240 kg. Consistently, sows weighing 165 kg at farrowing had less developed ovaries at weaning than sows weighing 190 kg, despite a similar protein deficiency during lactation (Clowes *et al*. 2003a). Large body reserves could partly prevent negative nutritional effects by providing energetic substrates and thereby attenuating metabolic and physiological perturbations.

Conclusions

Lactation inhibits LH secretion by inhibiting GnRH secretion and reducing pituitary response to GnRH. As lactation progresses, LH secretion increases, which allows resumption of folliculogenesis. The nutritional deficiency associated with mobilization of body reserves constitutes an additional inhibitory factor, which affects LH secretion and follicular maturation during lactation and after weaning. The lactational and nutritional effects at the different levels of the reproductive axis are likely to influence the ovarian response to lactation management strategies (e.g. short lactation duration, split-weaning, interrupted suckling), as reviewed by Soede *et al*. (2009). The metabolic mechanisms that affect folliculogenesis are not fully understood (Fig. 3). Glucose, insulin and leptin are the most likely signals at the hypothalamic level, yet their roles have not been definitely established. At the ovarian level, folliculogenesis is likely to be altered by the reduction in insulin and IGF-I concentrations induced by the nutritional deficiency. However, more knowledge on nutritional effects on intrafollicular characteristics, such as IGF, glucose/insulin and leptin systems, could provide a better understanding on nutritional effects on follicular development, occyte quality and embryo development.

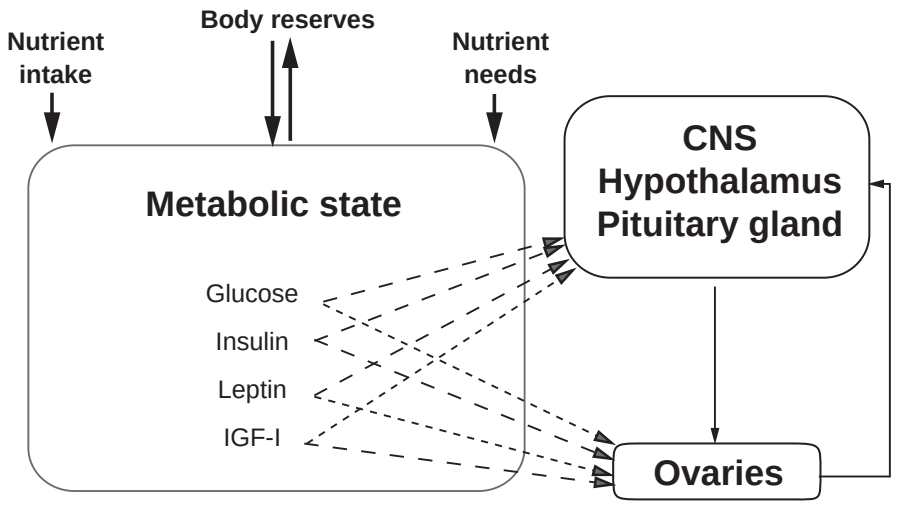


Fig. 3 Putative sites of action for metabolic substrates and metabolic hormones in the reproductive axis in the sow submitted to feed restriction.

Interrupted line: the potential stimulatory effect is reduced in situation of feed restriction.
Dotted line: poor evidence of the effect in swine.

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Appearance, fate and utilization of abnormal porcine embryos produced by in vitro maturation and fertilization

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In vitro production (IVP) including in vitro maturation (IVM) and fertilization (IVF) is now an important technology for obtaining live piglets. However, there are still two significant obstacles to the efficient production of viable porcine embryos: (1) polyspermy and (2) fertilization of oocytes arrested at the immature stage. These phenomena relate to production of embryos with abnormal ploidy (polyploidy). To avoid these problems, careful selection of mature oocytes for IVF, and regular monitoring of normal and abnormal fertilization (polyspermy and/or lack of male pronucleus formation) are very important. In our recent studies, however, we have confirmed that some oocytes with abnormal ploidy after polyspermy can develop into diploid embryos with potentially normal developmental ability. The mechanism by which such fertilized polyploid oocytes develop to a normal state during embryo development is still not well understood. Attempts to clarify this mechanism would hopefully reveal data that are very useful for not only IVP but also other technologies such as the production of transgenic or cloned animals using IVM oocytes, including other species, also for human reproductive manipulation. In this review, we focus on studies of normality of IVM oocytes and ploidy of IVP embryos, and try to suggest practical ways of solving the problems mentioned above in pigs.

Normality of porcine zygotes produced in vitro

The in vitro developmental competence or viability of porcine in vitro-matured (IVM)–in vitro-fertilized (IVF) oocytes to the blastocyst stage was first confirmed and reported by Mattioli *et al*. (1989). Since then, live-born piglets have been obtained from IVM–IVF embryos after in vitro culture (IVC) to the 2- to 4-cell stages (Mattioli *et al*. 1989; Yoshida *et al*. 1993; Funahashi *et al*. 1996; Funahashi & Day 1997). Viable piglets have also been generated by transfer of in vitro-produced (IVP = IVM, IVF and IVC) embryos at the blastocyst stage (Marchal *et al*. 2001; Kikuchi *et al*. 2002). Over the last few years, IVC procedures have been improved, but IVM and IVF systems are still hampered by problems resulting in poor developmental ability, and low quantity and quality of the embryos produced. This leads to embryo loss even after their transfer to recipients. One of the major causes of this problem is abnormal ploidy of IVP embryos due to (1) polyspermy during IVF and (2) fertilization of oocytes arrested at the

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immature stage. In addition, aneuploidy during oocyte maturation should be also noticed as a cause of embryo abnormality (Lechniak *et al.* 2007). It is essential to make every effort to achieve normality in IVP embryos after IVM and IVF. However, it is basically impossible to prevent precocious meiotic arrest or polyspermy in all cultured oocytes even using currently established technologies. Another approach for obtaining a good result (live offspring) under these conditions is to select fertilized oocytes or embryos that guarantee the developmental competence to piglets. It is generally accepted that only monospermic fertilized oocytes that have matured meiotically to the metaphase-II (M-II) stage can be guaranteed to show normal embryonic development. On the other hand, in comparison with sperm, oocyte sources are limited, especially those from rare genetic resources. To apply in vitro reproductive technologies in such animals, it is important to utilize as many oocytes as possible from a limited number of females. For this propose, assuming some ability to develop to term, abnormally generated embryos may be used to generate offspring. For half a century, mammalian embryos with abnormal ploidy have been known to be capable of surviving even into the post-implantation period (Piko & Bomsel-Helmreich 1960; Bomsel-Helmreich 1971; Han *et al.* 1999a). However, the details of the mechanisms involved have remained unclear.

In the present review article focusing on porcine IVP systems, we summarize the status and consequences of generating abnormal embryos obtained as a result of polyspermy and also by fertilization before completion of meiotic maturation. We also consider the use of abnormally generated embryos to improve porcine IVP efficacy.

Polyspermy

Status of efforts to reduce polyspermy

Polyspermy occurs as a result of simultaneous penetration of an oocyte by two or more spermatozoa. The problem of polyspermy in IVP porcine systems is significant, and has remained unsolved for many years. The main reason for polyspermic fertilization seems to be the presence of a large number of spermatozoa at the site of IVF in the absence of a regulatory effect of the female reproductive tract to control the quantity and quality of spermatozoa. An optimal sperm concentration and time interval for IVF are basic requirements for avoiding an extremely high incidence of polyspermy (Nagai *et al.* 2006). In our laboratory, we have chosen batches of frozen-thawed epididymal sperm showing good penetration ability to conduct IVF by co-incubation of 1×10^5 sperm/ml with oocytes for 3 h. Under these conditions, we are able to achieve reproducible sperm penetration beginning at 2 h post-insemination, the penetration rate reaching a plateau of around 80% at 4 h with a 60% polyspermy rate and an average of 3 spermatozoa per oocyte (Kikuchi *et al.* 2002, 2006). This means that only 20% of the oocytes subjected to IVF are monospermic. Another reason for polyspermy is considered to be the insufficient ability of IVM oocytes to block polyspermy due to a delayed or incomplete zona reaction and the imperfect characteristics of the zona caused by lack of exposure to oviduct fluid (Funahashi 2003).

Up to now, many attempts to decrease polyspermy have been reported. There have been several attempts to imitate in vivo conditions during IVF in order to regulate the number of spermatozoa near the oocytes. Some procedures have been based on the concept of allowing a small number of functional or capacitated spermatozoa to approach the oocytes, and these include the climbing-over-a-wall (COW) method (Funahashi & Nagai 2000), straw IVF (Li *et al.* 2003) and the biomimetic microchannel IVF system (Clark *et al.* 2005). Other methods have aimed at improving the characteristics of oocytes (more specifically the zona pellucida: ZP), such as treatment of oocytes with oviductal glycoproteins before IVF (Kouba *et al.* 2000; McCauley

et al. 2003a), and/or modulation of sperm capacitation, acrosome status or sperm binding to the ZP by techniques such as exposure of spermatozoa and oocytes to oviductal cells (Nagai & Moor 1990), oviductal fluid (Kim *et al*. 1997), oviduct-specific glycoproteins (Kouba *et al*. 2000; McCauley *et al*. 2003a), follicular fluid (Funahashi & Day 1993) or hyaluronan (Suzuki *et al*. 2000) before or during IVF. Although these approaches have been able to moderate the frequency of polyspermy, they could not eliminate it completely. Furthermore, some of these methods require special equipment (e.g. the biomimetic microchannel) or involve undefined factors (e.g. co-culture systems), and thus cannot be applied routinely to porcine IVP systems. As a consequence, in currently used IVF systems, the incidence of polyspermic fertilization is still very high. Thus, polyspermy is still considered to be a major problem that reduces the productivity of IVP systems in the pig.

Selection of monospermic oocytes among IVF oocytes

The most reliable method for selecting monospermic oocytes is to observe a single penetrated sperm head or male pronucleus in the ooplasm directly. Observation of a pronucleus(ei) is quite reliable, because oocytes with two (male and female) pronuclei are normally the result of monospermy, whereas oocytes with three or more pronuclei are the result of polyspermy. Unfortunately, the ooplasm in large animals such as pigs and cattle contains a large number of lipid droplets that make direct observation of organelles difficult. Pronuclei in such oocytes can be visualized by either nuclear staining or centrifugation. Vital nuclear staining is usually performed using fluorochromes such as Hoechest 33342, but this has been proven to have a detrimental effect on embryo development (Ebert *et al*. 1985; Tsunoda *et al*. 1988; Smith 1993; Yang *et al*. 1990).

Centrifugation is an effective way to polarize lipid droplets and thus to visualize pronuclei (Cran 1987), and seems to be safer than nuclear staining with fluorescent dye. To study the effects of centrifugation on IVP zygotes, we have evaluated the survival and developmental ability of fertilized oocytes after centrifugation (Somfai *et al*. 2008). In pigs, pronucleus formation is evident from 5 h post-insemination and reaches a plateau after 8 h (Kikuchi *et al*. 2006). Oocytes emitting 2 polar bodies, and thus considered to be mature and activated by sperm penetration, were centrifuged 10 h after insemination at 10,000 $\times$ *g* for 20 min. This method rendered pronuclei visible even under a stereo microscope, and also allowed the oocytes to remain viable. They were then cultured in vitro for 6 days. The cleavage rates on Day 2 (Day 0 = the day of IVF), and the rates of viable blastocysts and average numbers of cells within them on Day 6 did not differ significantly between the centrifugation and control groups. These results indicate that centrifugation itself does not affect developmental ability. After centrifugation, oocytes with 1, 2 or more pronuclei and unfertilized oocytes were separable under a stereo microscope by direct observation of pronuclei (Fig. 1). The efficacy of selection was verified by fixation and staining, and we found that, in most (75.0–82.8%) of the selected oocytes, the number of pronuclei assessed by direct observation matched that assessed by nuclear staining (Somfai *et al*. 2008). We therefore considered that the selection of mono- and polyspermic oocytes by centrifugation followed by direct nuclear observation at the pronucleus stage was acceptable for further experiments.

Development after polyspermic fertilization

When zygotes with different numbers of pronuclei were selected and cultured separately, we found that the cleavage rates of oocytes with one or two pronuclei (considered to be

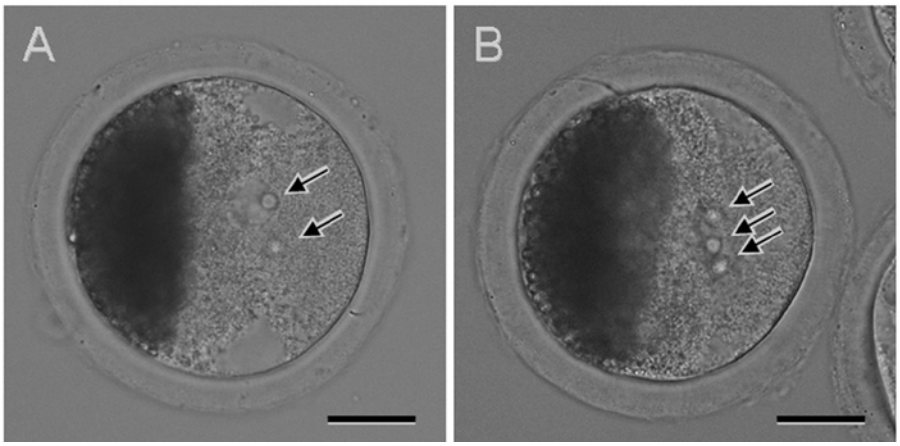


Fig. 1 Porcine oocytes after centrifugation treatment 10 h after IVF. Location and number of pronuclei are determined by the presence of nucleolus precursor bodies (arrows). A) Zygote with two pronuclei. B) Zygote with three pronuclei. Scale bar represents 30 μm.

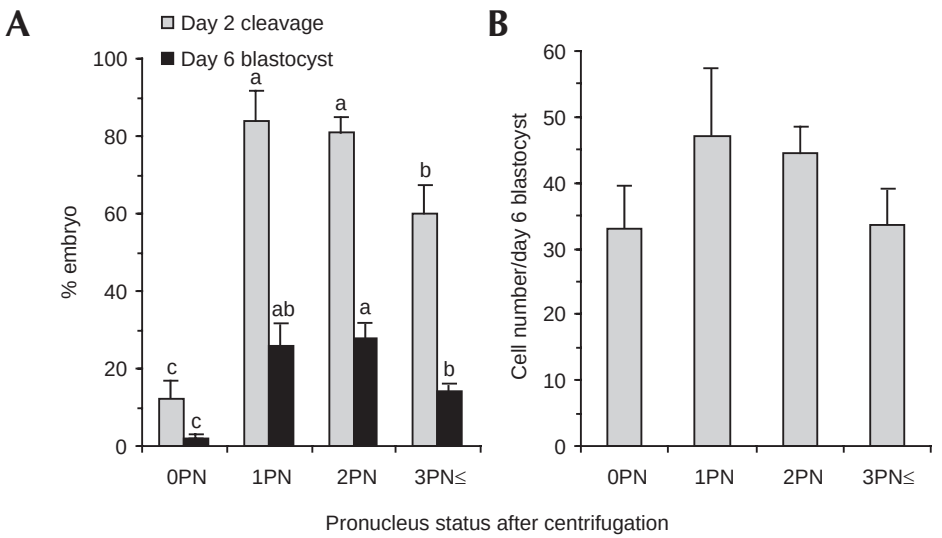


Fig. 2 In vitro development of in vitro-produced porcine zygotes with different numbers of pronuclei visualized by centrifugation. (A) Rates of cleaved embryos on Day 2 and blastocysts on Day 6. (B) Average cell number of blastocysts on Day 6. 0PN; oocytes without pronucleus, 1PN; oocyte with one pronucleus, 2PN; oocytes with two pronuclei, and 3PN $\leq$; oocytes with three or more pronuclei. Mean $\pm$ SEM are presented. $^{a\text{-}c}$ Percentages with different superscripts differ in each category ($P < 0.05$). Data is from Somfai *et al*. (2008).

parthenogenetic or monospermic, respectively) were significantly higher than those of oocytes with three or more pronuclei (considered to be polyspermic) (Fig. 2) (Somfai *et al*. 2008). The rate of development to the blastocyst stage was higher for zygotes with two pronuclei (monospermy) than for those without any pronucleus or with three or more pronuclei (unfertilized oocytes

or polyspermy, respectively), although it was not significantly different from that of zygotes with one pronucleus (parthenotes). The numbers of cells did not differ significantly among the oocytes thus classified. We analyzed the ploidy of blastocysts that had developed from putative monospermic and polyspermic zygotes (Table 1), and found that 73.8% of blastocysts that developed from monospermic oocytes were diploid (2n) whereas 12.5% of them were mixoploid containing a diploid cell(s). Thus, after calculation, 86.3% of the blastocysts that had developed from monospermic oocytes were found to contain diploid cells. On the other hand, 31.3% and 14.5% of polyspermic oocytes were found to develop into diploid blastocysts and mixoploid blastocysts containing a diploid cell(s), respectively. Calculation revealed that 45.8% of the blastocysts from polyspermic oocytes contained a diploid cell(s). These results suggest that not only monospermic oocytes but also a proportion of polyspermic oocytes may have the ability to develop to diploid embryos or fetuses.

Table 1. Ploidy of in vitro-cultured porcine blastocysts developing from zygotes bearing two or multiple pronuclei.

Pronuclear status after centrifugation	% ploidy of blastocyst							% blastocyst with 2n cells
	n	2n	3n	4n	4n <	Mixoploid with 2n cells	Mixoploid without 2n cells	
2PN	8.9 ± 4.4	73.8 ± 11.9[a]	0	4.7 ± 4.7	0	12.5 ± 12.5	0	86.3 ± 0.5[a]
3PN ≤	17.0 ± 11.9	31.3 ± 10.1[b]	12.1 ± 6.5	6.0 ± 3.2	3.7 ± 3.7	14.5 ± 7.2	15.1 ± 2.6	45.8 ± 9.4[b]

PN; pronuclei.
Mean ± SEM are presented.
[a,b] Percentages with different superscripts differ in each column ($P < 0.05$).
Table is modified from Somfai *et al*. (2008).

Our data agree with previous studies that found high frequencies of chromosomal abnormalities (mainly polyploidy) in IVP porcine embryos, which were thought to have resulted from polyspermic fertilization (McCauley *et al*. 2003b; Somfai *et al*. 2005). Polyspermic porcine embryos reportedly have an ability to develop to the blastocyst stage in vitro (Han *et al*. 1999a). This suggests that a considerable proportion of IVP blastocysts in those studies resulted from polyspermic fertilization. It has also been reported that, when polyspermic zygotes are transferred into recipients, most of the fetuses on Day 40 are diploid. This indicates that, in many polyspermic embryos, the effect of abnormally high numbers of penetrating spermatozoa on embryo ploidy is neutralized during in vivo development (Han *et al*. 1999b).

Possible mechanism of ploidy correction in polyspermic embryos

The mechanisms involved in the correction of polyploidy to a normal diploid status in polyspermic oocytes are still unclear. The ploidy of porcine embryos resulting from polyspermic fertilization is thought to depend on the location of the pronuclei before the first cleavage (Han *et al*. 1999b). This phenomenon is believed to be related to the different cleavage patterns of polyspermic embryos (including abnormal tripolar and tetrapolar cleavage), which can result in diploid, polyploid and also mixoploid embryos with a wide variety of blastomere ploidy after the first cell cleavage, including polyploids, diploids, and aneuploids (Han *et al*. 1999b; Funahashi 2003). It is believed that, during early development of embryos, blastomeres with abnormal ploidy fail to develop to the fetal stage whereas those with diploid blastomeres are able to do so. Thus, mosaic embryos having diploid cells may have the ability to develop to term (Han *et al*. 1999b, Funahashi *et al*. 2003). Other authors have suggested the involvement

of a different mechanism; Kola et al. (1987) found that tripolar cleavage of polyspermic human oocytes always resulted in abnormal numbers of chromosomes, whereas the appearance of a protrusion in 2 cell embryos was associated with diploid status in polyspermic oocytes. Similar results were obtained recently in in vitro-produced bovine embryos (Somfai *et al.* 2009). Funahashi (2003) has mentioned another possible mode of ploidy correction, when the chromosome compartment (a male pronucleus or a decondensed sperm head) from one sperm does not contribute to syngamy and the formation of the mitotic metaphase. Such a male nucleus may remain in the cytoplasm of one of the blastomeres of the resulting 2-cell embryo, and later this extra chromatin is disrupted and disappears through a mechanism that involves lysosomes. In current IVP systems, a reasonable proportion (15–30%) of porcine embryos can develop to the blastocyst stage, but with a wide variety of morphological appearances including partially fragmented embryos characterized by both nuclear and anuclear blastomeres (Kikuchi *et al.* 2002; Somfai *et al.* 2005; Somfai *et al.* 2008). Our recent study (Somfai *et al.* 2008) has also revealed that at 36 h after IVF a higher rate of cleaved monospermic than polyspermic embryos developed to the two-cell stage, whereas the proportion of embryos containing four or more blastomeres was significantly higher in the polyspermic than in the monospermic group. Nuclear staining revealed that the frequency of embryos containing bi- or multi-nuclear blastomere(s) did not differ between the monospermic and polyspermic groups, whereas the proportion of embryos containing at least one blastomere without any nucleus (considered as a sign of a fragmented cytoplasm) was significantly higher in polyspermic than in monospermic embryos. The mean number of blastomeres was significantly higher in polyspermic than in monospermic embryos, whereas the mean number of nuclei per embryo did not differ between the two groups (Table 2). These results suggest that polyspermic fertilization is associated with partial embryo fragmentation; however, it is not clear if this phenomenon is related to ploidy correction during early embryonic development.

Table 2. Ploidy of in vitro-cultured porcine blastocysts developing from zygotes bearing two or multiple pronuclei.

Pronuclear status after centrifugation	%[a] cleaved	%[b] embryos by microscopic evaluation			Nuclear staining			
		2 cell	3 cell	4 cell ≤	%[b] embryos with multiple nuclei	%[b] embryos with anuclear blastomere	Mean no. blastomeres per embryo	Mean no. nuclei per embryo
2PN	77.7 ± 6.0[c]	56.0 ± 6.0[c]	22.0 ± 6.1	24.9 ± 7.0[c]	6.9 ± 3.4	34.4 ± 3.1[c]	2.8 ± 0.1[c]	2.3 ± 0.1
3PN ≤	59.7 ± 6.4[d]	26.7 ± 4.1[d]	25.8 ± 3.9	47.7 ± 6.6[d]	13.9 ± 1.3	57.6 ± 3.8[d]	3.4 ± 0.1[d]	2.5 ± 0.1

PN; pronucleus(ei).
All embryos were evaluated or fixed at 36 h after onset of IVF
[a] Percentage to the examined embryos
[b] Percentage to the cleaved embryos
Mean ± SEM are presented.
[a,b]Percentages with different superscripts differ in each column ($P < 0.05$).
Table is modified from Somfai et al. (2008).

Fertilization before completion of meiotic maturation

Cytoplasmic maturity of "metaphase-I-arrested" oocytes

In our previous IVM system (Kikuchi *et al.* 1999a), matured oocytes at the M-II stage appeared at 30 h and the maturation rate became maximal after 36 h of IVM. About 35% of the cultured oocytes failed to reach the M-II stage and about 25% of them were arrested while still immature

(Fig. 3). These oocytes were characterized by a typical metaphase plate without a first polar body. This phenomenon is often referred to as "metaphase-I (M-I) arrest". In general, meiotic arrest at the M-I stage can be caused by a number of factors, such as insufficient meiotic competence because of an inadequate follicular phase or oocyte diameter (Szybek 1972; Sorensen & Wassarman 1976; Motlik & Fulka 1986; Eppig *et al.* 1994) or stress caused by inappropriate culture conditions, such as the use of inadequate isolation or culture media (Bae & Foote 1980; Bagger *et al.* 1987; Kikuchi *et al.* 1999b). Intracellular calcium regulation reported to affect this phenomenon (Lechniak *et al.* 2005). As well as in pigs, in certain mouse strains (LT/Sv and LT-related strains), it is known that oocytes undergo meiotic arrest during the M-I stage (Hirao & Eppig 1997; 1999).

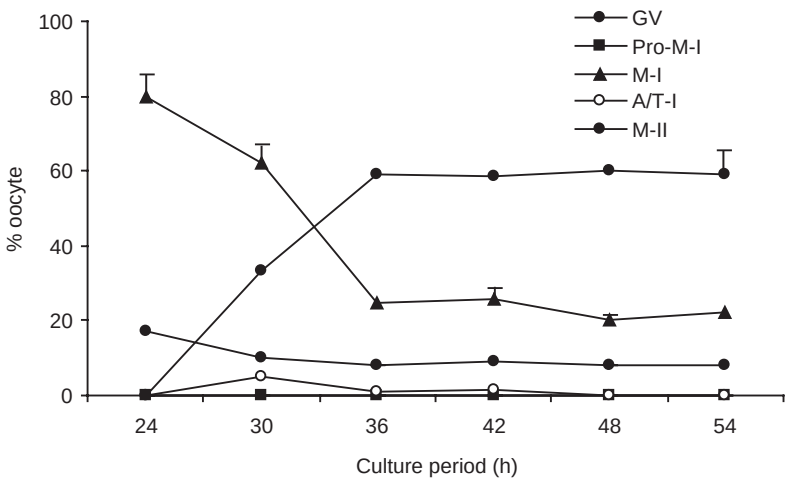


Fig. 3 Nuclear transition during in vitro maturation of porcine follicular oocytes. Oocytes were cultured and their nuclear status examined every 6 h from 24 to 54 h. Data are presented as means ± SE. GV, germinal vesicle; Pro-M-I, pro-metaphase-I; M-I, metaphase-I; A/T-I, anaphase-I or telophase-I; M-II, metaphase-II. The figure is modified from Kikuchi *et al.* (1999a).

Mature (M-II) oocytes can be distinguished from immature ones by direct observation of a first polar body with minimum error [less than 6% in the polar body (PB) (+) group, Kikuchi *et al.* 1999b]. Comparing with selection after staining with a fluorescent dye, this method is very easy to perform and does not seem to affect developmental ability (Ebert *et al.* 1985; Tsunoda *et al.* 1988; Smith 1993; Yang *et al.* 1990). Our previous study (Kikuchi *et al.* 1999b) showed that "M-I-arrested" oocytes completed their cytoplasmic maturation just like M-II oocytes, and were capable of activation to form male and female pronuclei after IVF and also the female pronucleus after parthenogenetic stimulation. Mature oocytes having a first polar body emitted a second polar body after oocyte activation, whereas "M-I-arrested" oocytes without a polar body were activated with emission of a single polar body (Table 3). The pronucleus status (timing of its formation and morphological features) did not differ between activated "M-I-arrested" and M-II oocytes (Somfai *et al.* 2005). These results suggest that cytoplasmic maturation for oocyte activation can be completed after 48 h, even when nuclear maturation has not completed the M-II stage. On the other hand, maturing oocytes with insufficient cytoplasmic maturity [PB(–) oocytes after culture for 24 h, considered to be "fresh" M-I] did not respond to parthenogenetic stimulation and only a few of them formed pronuclei after IVF. This difference was explained by a high level of maturation-promoting factor in "fresh" M-I oocytes and a reduced level of

this factor in oocytes that were cultured for 48 h, regardless of the existence of a polar body (Kikuchi *et al*. 1999b). In fact, sperm heads that had penetrated into the immature cytoplasm of "fresh" M-I oocytes did not undergo transformation to a male pronucleus, but recondensed or transformed to metaphase-like chromosomes (Fig. 4). This also confirms the insufficient cytoplasmic ability of M-I oocytes at an earlier maturational stage. These results suggest that, unlike maturing oocytes, oocytes arrested at an immature stage after germinal vesicle breakdown undergo cytoplasmic maturation that allows oocyte activation and male pronucleus formation after IVF, similar to the case of matured (M-II) oocytes.

Table 3. Activation of porcine oocytes classified on the basis of the presence of a polar body.

Culture [h]	Polar body*	Treatment	% oocytes	% activated oocytes	
				With one polar body	With two polar bodies
24	–	IVF	6.2 ± 1.5[b]	80.0 ± 16.7[a]	20.0 ± 16.7[b]
48	–	IVF	68.6 ± 10.8[a]	90.0 ± 1.7[a]	10.0 ± 1.7[b]
48	+	IVF	61.8 ± 13.8[a]	5.2 ± 2.3[b]	94.9 ± 2.3[a]
24	–	ES	0[b]	–	–
48	–	ES	52.9 ± 5.0[a]	73.0 ± 9.7[a]	13.5 ± 4.7[a]
48	+	ES	80.5 ± 4.8[a]	8.9 ± 2.6[b]	79.0 ± 6.7[b]

*Examined by Nomarski differential interference contrast microscopy before treatment.
IVF; in vitro fertilization, ES; electrical stimulation
[a,b] Percentages with different superscripts differ in each treatment (IVF or ES) ($P < 0.05$)
Table is modified from Kikuchi et al. (1999b).

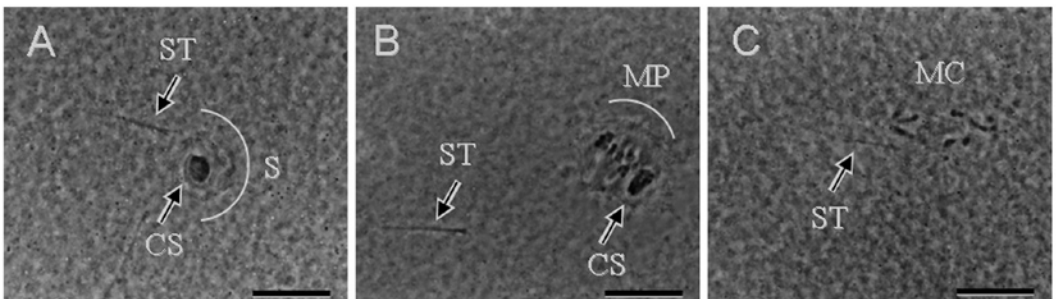


Fig. 4 Sperm heads penetrating an immature porcine oocyte at the metaphase-I stage. Oocytes were cultured for 24 h, fertilized in vitro and subsequently cultured for up to 36 h. Arrows indicate the tails of the penetrated spermatozoa. After initial decondensation, sperm heads were recondensed into a mass (Cs), being surrounded by a spindle (S) or incorporated into the maternal metaphase plate (Mp) (A and B, respectively), or transformed into metaphase-like chromosomes (C). Scare bars represent 10 μm. The figure is modified from Kikuchi *et al*. (1999b).

Developmental competence of "M-I-arrested" oocytes

"M-I-arrested" porcine oocytes seem to have some developmental ability, as they were shown to develop to the blastocyst stage after IVF, similarly to M-II oocytes (Somfai *et al*. 2005). In mice also, "M-I-arrested" oocytes have been shown to be capable of developing to the blastocyst stage (Eppig *et al*. 1994). The developmental competence of meiotically arrested porcine oocytes is rather poor, resulting in low rates of formation of blastocysts, which contain low numbers of cells (Table 4). Chromosomal analysis of blastocysts resulting from IVF of "M-I-

arrested" porcine oocytes has revealed a high frequency of chromosome numerical abnormality in blastomeres, especially an increased rate of triploidy (Table 5). Similar results have already been reported in mice (Eppig *et al.* 1994). Fertilization of "M-I-arrested" oocytes is suggested to result in the development of digynic triploid embryos. As reported by Kaufman *et al.* (1989), triploid diandric mouse embryos appear morphologically normal, but are smaller than normally fertilized diploid embryos.

Table 4. Blastocyst formation after IVF of porcine oocytes with/without a polar body after culture for 44 h.

Polar body*	% blastocysts	% blastocysts categorized morphologically as			Cell number per blastocyst
		Excellent	Good	Poor	
–	20.7 ± 2.8[b]	60.0 ± 5.1	20.0 ± 8.5[b]	20.0 ± 2.0[a]	29.1 ± 1.4[b]
+	34.6 ± 2.4[a]	68.1 ± 5.1	23.0 ± 3.8[b]	8.7 ± 1.4[b]	52.0 ± 2.5[a]

*Examined by stereo microscopy before IVF.
IVF; in vitro fertilization, ES; electrical stimulation
[a,b] Percentages with different superscripts differ in each column ($P < 0.05$).
Table is modified from Somfai *et al.* (2005).

Table 5. Chromosome analysis of blastomeres in Day 6 blastocysts generated from porcine oocytes with/without a polar body.

Polar body	No. of blastocysts examined	No. of metaphases prepared (per blastocyst)	No. (%) of metaphases analyzed as				
			Total[a]	Haploid[b]	Diploid[b]	Triploid[b]	Tetraploid[b]
–	66	132 (2.0)	100 (78.8)[c]	16 (16.0)	44 (44.0)[d]	34 (34.0)[c]	6 (6.0)
+	122	241 (2.0)	155 (64.3)[d]	29 (18.7)	108 (69.7)[c]	13 (8.4)[d]	5 (3.2)

[a] Percentage relative to the number of prepared metaphases
[b] Percentage relative to the number of analyzed metaphases
[c,d] Percentages with different superscripts differ in each column ($P < 0.05$)
Table is modified from Somfai et al. (2005).

The ploidy of porcine embryos, as suggested previously, might be related to their developmental competence. The existence of a ploidy correction mechanism like that operating in polyspermy has not yet been confirmed in zygotes or embryos from "M-I-arrested" oocytes. Nevertheless, diploid cells have been found in blastocysts that have developed from "M-I-arrested" oocytes, suggesting that the embryos are diploid, or are mixoploid but contain diploid cells. Detailed chromosome analysis will be needed to clarify the nuclear competence of such embryos.

Possible mechanism of "M-I arrest" before completion of oocyte maturation

We have compared the chromosome configurations of "M-I-arrested" oocytes with those of M-I oocytes at 33 h of IVM ("real M-I" oocytes) and with those of M-II oocytes at 48 h of IVM (Somfai *et al.* 2006) (Fig. 5). In a series of experiments, we matured porcine oocytes in the presence of the actin polymerization inhibitor cytochalasin-B (CB), a drug that inhibits actin filament polymerization. This drug has been reported to induce meiotic arrest of mammalian oocytes at the M-I stage (Wassarman *et al.* 1976). Constant tracking of nuclear progression during maturation revealed that segregation of homologous chromosomes did, in fact, occur in CB-treated oocytes; however, extrusion of the first polar body failed. This caused the formation of two sets of segregated homologous chromosomes inside the oocyte. Later, these sets united and formed a single, tetraploid metaphase plate (Somfai *et al.* 2006) (Fig. 6). Similar observations

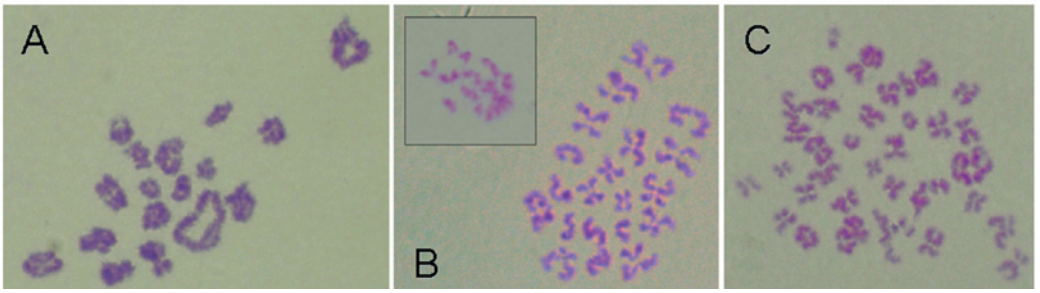


Fig. 5 Morphology of porcine oocyte chromosomes. (A) chromosomes prepared from oocytes at the metaphase-I (M-I) stage (33 h IVM), where 19 ring-shaped, symmetrical complexes of the homologous chromosomes from bivalents are observed; (B) chromosomes prepared form oocytes at the metaphase-II (M-II) stage (44 h IVM). Insert: chromosomes incorporated by a first polar body; and (C) chromosomes prepared from oocytes cultured in the presence of cytochalasin-B (44 h IVM). Both M-II- and CB-treated oocytes displayed similar separate chromosomes with two chromatids; M-II oocytes had 19 chromosomes, whereas the number of chromosomes in the CB-treated oocytes was double (38) compared with M-II oocytes. The figure is modified from Somfai *et al.* (2006).

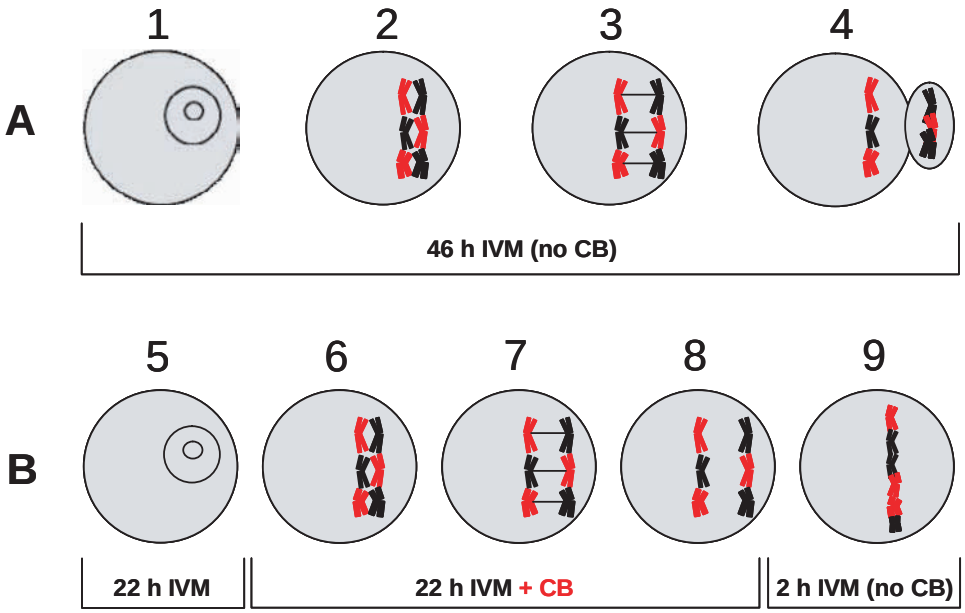


Fig. 6 Scheme of meiotic maturation without (A) or with (B) cytochalasin-B (CB). (A) Germinal vesicle (GV) oocytes (1) are cultured in vitro without CB for 46 h. After the GVBD (around 33 under these culture conditions), the homologous paternal and maternal chromosomes form bivalents [metaphase-I (M-I) stage (2)]. At this stage, the oocyte is tetraploid. Later segregation of the homologous chromosomes occurs (3) with extrusion of the first polar body [metaphase-II (M-II) stage (4)], so the oocyte becomes diploid. (B) After culture of GV oocytes for 22 h in CB-free medium (5), they are subsequently matured in the presence of CB for 22 h. The M-I stage (6) is followed by segregation of homologous chromosomes (7), but extrusion of the polar body is inhibited, so that all chromosomes remain in the oocytes (8). During the last phase of maturation culture, the segregated chromosomes undergo rearrangement into an M-I-like metaphase plate (9). The figure is modified from Somfai *et al.* (2006).

were published previously by Kubiak *et al*. (1991), who cultured mouse oocytes in the presence of cytochalasin-D, a drug similar to CB. The metaphase plate of porcine oocytes after treatment with CB contained 2n (38) segregated chromosomes, unlike those of "real M-I" oocytes that contained 19 pairs of homologous chromosomes forming bivalents and those of M-II oocytes that contained n (19) segregated chromosomes with 2 chromatids. Therefore the structure of the metaphase plates in "M-I-arrested" oocytes generated by CB treatment appears similar to that of M-II oocytes; however, it contains a double (diploid) set of 2 chromatids. Therefore such a nuclear stage cannot be considered a "real M-I" stage, but rather an "M-I-like" stage.

Previously, we compared the chromosome complements of CB-treated oocytes with those of "M-I-arrested" oocytes that were blocked spontaneously during IVM, and found exactly the same chromosome configurations (Fig. 7) (Somfai *et al*. unpublished results). This suggests that, during meiotic progression from the M-I to M-II stages, an as-yet unclarified mechanism gives rise to a similar actin-depolymerization-related phenomenon of chromosome reunion and rearrangement, even after successful segregation of the homologous chromosomes. The resulting nuclear stage is characterized by a single metaphase plate without any polar body, and has been referred to as "M-I arrest" in our previous papers (Kikuchi *et al*. 1999b; Somfai *et al*. 2005). This abnormality during porcine oocyte maturation occurs with increased frequency as culture is prolonged (Sosnowski *et al*. 2003). This suggests the malfunctions of the spindle during maturation culture. Further studies of these phenomena will be important not only in porcine IVP system, but also in human reproduction, because incompletion of oocyte maturation before the M-II stage is considered to be an important cause of human infertility (Mrazek & Fulka 2003).

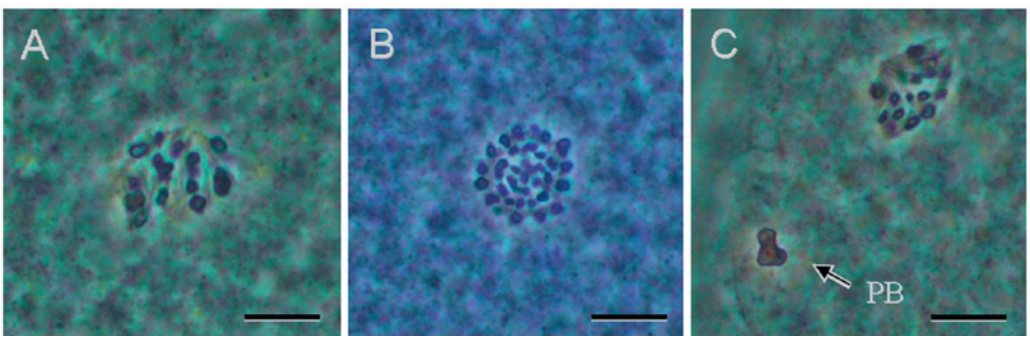


Fig. 7 Chromosome configurations of (A) metaphase-I (cultured for 33 h), (B) "metaphase-I-arrested" (for 48 h) and (C) metaphase-II porcine oocytes. Specimens were prepared after fixation and staining with aceto-orcein, and examined by phase-contrast microscopy. Scale bars represent 10 μm.

Conclusion

Our results have clearly revealed that development to the blastocyst stage is not a perfect indicator of embryo quality in porcine IVP systems, since polyploid embryos can develop to the blastocyst stage. Careful selection of M-II oocytes for IVF, and regular monitoring of fertilization and polyspermy rates are very important in order to obtain reliable results, not only in porcine IVP systems but also in other technologies using IVM oocytes. On the other hand, it is necessary to know more about the mechanism by which fertilized oocytes with abnormal ploidy can develop to a normal state during embryo development. Further improvements of IVM-IVF systems are necessary to increase the degree of nuclear and cytoplasmic maturity and to reduce the incidence of polyploidy in porcine IVP systems.

Acknowledgments

The authors would like to thank Drs. N. Kashiwazaki, H. Kaneko, J. Noguchi, M. Fahrudin, NWK Karja, M. Ozawa, N. Maedomari, for completion of a series of experiments, and also thank Ms. T. Aoki and Mr. Y. Funakubo for technical assistance

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Intra-follicular regulatory mechanisms in the porcine ovary

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The mechanisms controlling the follicular growth continuum in the pig involve the interaction between local growth factors which are expressed throughout development and extra-follicular factors such as gonadotrophins. A large number of follicular growth factors, many belonging to the transforming growth factor-β (TGF-β) superfamily, have been identified in the somatic cells and in the oocyte. The relative importance of these intra-follicular factors varies with stage of development. The initiation of follicular growth and early preantral development is controlled locally (by factors including c-kit-kit ligand, members of the bone morphogenetic family (e.g BMP-15) and growth differentiation factor-9 (GDF-9)) and gonadotrophins are not thought to be involved until later. During antral follicle development, the oocyte secretes factors that stimulate porcine granulosa cell proliferation and differentiation, modulate apoptosis and suppress progesterone production, thereby preventing premature luteinisation. Likely candidates for mediating these effects include BMP-6, -15 and GDF-9 that are critical for fertility and ovulation rate in several mammals. There are also paracrine interactions between the somatic cells, with theca derived transforming growth factor β (TGF-β) playing a key role in regulating antral follicle maturation. Finally, during the periovulatory period, members of the EGF family from the granulosa cells stimulate cumulus expansion and oocyte maturation. Evidence indicates that some of these local factors may also influence oocyte developmental potential, emphasizing further the complexity, and importance, of these intra-follicular interactions.

Introduction

In many mammalian species including the pig, primordial follicle growth, once initiated, continues until the follicle either becomes atretic (>99.9%) or proceeds to ovulation. Although the gonadotrophic regulation of antral follicle development has been studied in some detail in the pig, the role of local factors is less well known. In recent years however, significant progress has been made in understanding the complex intraovarian control mechanisms. Within the ovarian follicle, oocyte growth and differentiation depends upon an intimate association between the somatic follicular cells and the developing germ cells. An abundance of follicular growth factors, many belonging to the transforming growth factor-β (TGF-β) superfamily, have

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been identified in the somatic cells and in the oocyte and are thought to be involved in the recruitment, selection and growth of follicles from the primordial stage through to ovulation and corpus luteum formation. This review will focus on the accumulating evidence that intrafollicular factors are key regulators of follicle development in the pig. Although much of the information generated in this field has come from rodent models and ruminants, we will emphasise pig data where available and integrate the available information to form conclusions relevant to porcine follicular development.

The initiation of follicle growth and preantral development

The ovarian reserve of primordial follicles is established before birth in the pig and the recruitment of resting primordial follicles into the growing pool begins during fetal life. The activation of oocytes in primordial follicles causes transformation of their surrounding granulosa cells to a cuboidal shape. Throughout gestation and post-natally there is a gradual shift in the proportions of oogenic structures within the porcine ovary – initially egg nests predominate but they decline from around day 60 of gestation whilst primordial follicles increase. Primary follicles appear from approximately day 70, secondary follicles from birth and antral follicles from around day 60 post partum (Oxender *et al*. 1979). Evidence suggests that gonadotrophins are unlikely to be a critical factor for initiating primordial follicle growth (Yuan *et al*. 1996). The precise mechanisms controlling the initiation and the number of primordial follicles that start to grow and avoid regression are still unclear, although several growth factors, particularly members of the TGF-β superfamily have been implicated in other species (Visser & Themmen 2005, Knight & Glister 2006). The development of primary follicles to the late preantral stage involves oocyte growth, extensive granulosa cell proliferation, formation of the basal lamina and theca layer. The following sections will review the role of some of the key growth factors in early follicle development in the pig.

c-kit/Kit Ligand Interactions

The c-kit-Kit ligand (KitL) complex is a pleiotropic receptor–growth factor complex active in diverse cell systems in both the adult and the embryo. C-kit receptor is a type III transmembrane tyrosine kinase receptor expressed in the oocyte and granulosa cells, while its ligand, KitL also known as stem cell factor, is a proto-oncogene product of the granulosa cells and is expressed in the pre-granulosa cells that surround the oocyte in primordial follicles. In the fetus, it has been proposed that the interaction between c-kit and its ligand is necessary for germ cell migration to the developing gonadal ridge in mice (Keshet *et al*. 1991). Postnatally in the mouse, the receptor-ligand interaction may aid follicle recruitment into the pool of growing follicles (Parrott & Skinner 1999) and in the recruitment and proliferation of theca cells from the surrounding stromal tissue (Nilsson & Skinner 2004). Furthermore, in ovine follicles, c-kit is expressed before the follicle stimulating hormone (FSH) receptor, suggesting that c-kit may be one of the regulatory factors preceding the actions of FSH on early follicle growth (Clark *et al*. 1996). *In vitro* studies in mice have shown that the addition of KitL to culture medium accelerates oocyte growth, and also that the oocyte can regulate KitL expression. In addition to its role in the survival of fetal germ cells and initiation of follicle growth, there is increasing support for the importance of KitL/c-kit activity for oocyte growth during preantral development (Packer *et al*. 1994) which may be modulated by the presence of gap junctions (Klinger & De Felicic 2002). Evidence suggests that the c-kit ligand complex has a similar role

in the porcine ovary. C-kit expression has been detected in all oocytes from primordial to large antral follicles, in the theca cell layer and endothelial tissue, and mRNA for KitL was found in granulosa cells of preantral and antral follicles (Brankin *et al*. 2004, Moniruzzaman & Miyano 2007). Furthermore, exposure of porcine cortical tissue to KitL prior to xenografting increased oocyte survival (Moniruzzaman & Miyano 2007). Thus it is likely that the c-kit-KitL interaction is necessary for early follicle development in the pig, as in other species, and is probably also involved in a complex regulatory loop with oocyte factors and FSH (Thomas & Vanderhyden 2006; see below).

TGF-β superfamily

The bone morphogenetic proteins (BMPs)

The BMP subfamily represents a relatively large subset of the TGF-β superfamily. Bone morphogenetic proteins are synthesized and secreted as prepropeptides and are proteolytically cleaved to form mature, activated disulphide-linked dimmers (Elvin *et al*. 2000). The subfamily is comprised of at least 8 ligands namely BMP-2, -3, -4, -5, -6, -7, -8, and -15 and, with the exception of BMP-8, their expression has been reported in ovarian follicles of several species (Knight & Glister 2006). These ligands are known to interact with at least 7 different receptors that can be separated into type 1 receptors including ActRIA (or ACVRI, ALK2), BMPR1A (or ALK3), BMPR1B (or ALK6), TGFβR1 (or ALK5) and type II receptors including BMPRII, ActRII and ActRIIB (Shimasaki *et al*. 2004, Juengel & McNatty 2005). For the purpose of this review, GDF-9 and its known receptors (TGFβR1 and BMPRII) will also be included in this section.

Transduction of the BMP and GDF-9 signal requires the formation of a hetero-oligomeric complex of a type II and a type I receptor, followed by a signalling cascade that involves the Smad proteins (see review by Miyazono *et al*. 2001). What makes the BMP family of such interest is that both induced and naturally occurring genetic mutations in BMP family members in mammals such as mice and sheep profoundly affect very early follicle development and ovulation rate (Dong *et al*. 1996, Galloway *et al*. 2000).

Oocyte derived BMPs

Particular focus has been given to BMP-6, BMP-15 and GDF-9 since they have been shown to be primarily derived from the oocyte and can regulate somatic cell differentiation and proliferation (Juengel & McNatty, 2005, Gilchrist *et al*. 2008). There appear to be crucial species differences in the timing of expression and importance of some members of the BMP family. For example, GDF-9 is expressed in primary and later follicles in mice (Dong *et al*. 1996) but in contrast, is expressed by primordial follicles in sheep and cattle (Bodensteiner *et al*. 1999). The function of GDF-9 in primordial follicle growth remains somewhat obscure. Mice deficient in GDF-9 show an arrest in follicle development at the primary stage, the granulosa cells are abnormal with increased expression of KitL and they fail to acquire a theca layer, although oocyte growth is accelerated. Ewes with naturally occurring mutations in *GDF-9* also suffer from primary ovarian failure and these results indicate a paracrine role of GDF-9 in early folliculogenesis, by regulating granulosa cell proliferation and recruitment of theca cells, while limiting growth of the oocyte (Dong *et al*. 1996, Nilsson & Skinner 2002). Little is currently known about GDF-9 in early porcine follicle development, although GDF-9 expression has been detected in fetal and neonatal ovaries, albeit not to specific structures (Shimizu *et al*. 2004a). Evidence for a functional role for GDF-9 in the pig ovary was provided by the studies of Shimizu *et al*. (2004b) who injected *GDF-9* gene fragments into the ovaries of prepubertal gilts and found that this increased the number of primary, secondary and tertiary follicles, concomitant with a

decrease in the number of primordial follicles. Thus GDF-9 can promote early folliculogenesis in the porcine ovary, but whether it is obligatory or not remains unclear.

Two other TGF-B superfamily members, BMP-15 (also known as GDF-9B) and BMP-6 are selectively expressed by oocytes from early-stage mouse and sheep follicles, as for GDF-9 (Elvin *et al*. 2000; McNatty *et al*. 2005). However, unlike GDF-9, knockout of the *BMP-6* gene in mice has little effect on ovarian function and *BMP-15* null mice express only minor histopathological defects and are subfertile (Yan *et al*. 2001). Thus they do not appear to be crucial for fertility in the mouse. However, again there are species differences, as naturally occurring mutations of either the *BMP-15* or *GDF-9* gene have profound effects on fertility in sheep (McNatty *et al*. 2005). It is hypothesized that these mutations decrease production of the mature protein or interfere with binding to cellular receptors. Ewes that are heterozygous for either of these mutations show an increase in ovulation rate, while homozygotes are infertile with follicles failing to develop beyond the primary stage due to lack of granulosa cell proliferation; on a histological level, ovaries from homozygotes show many of the same features as GDF-9 null mice ovaries (McNatty *et al*. 2005). Collectively, this evidence suggests that oocyte-derived GDF-9 (rodents) or both GDF-9 and BMP-15 (sheep) have critically important effects on follicular somatic (pre-granulosa and/or granulosa) cells of primordial and/or primary follicles that are essential for further follicle progression. The precise mechanisms through which this is achieved remain unclear, although it has been postulated that upregulation of KitL expression may be involved (Knight & Glister 2006).

Although the roles of BMPs and GDF-9 in early porcine follicle development have not yet been examined in detail, work from our laboratory and others has localised BMP-15 and BMP-6 to the porcine oocyte (Quinn *et al*. 2002, Brankin *et al*. 2005a, Zhu *et al*. 2008) in pre-antral and antral follicles, but it is unknown how early in development expression starts and this clearly requires investigation. BMP receptors (BMPR-IA, -IB and –II) have been identified in porcine fetal ovaries, specifically in the fetal egg nests and oocytes of primordial follicles (Fig.1: Quinn *et al*. 2004a) and BMP-4, -5, and -6 have been detected in neonatal ovaries (Shimizu *et al*. 2004a). These data indicate that there is an active BMP system in the porcine ovary from the fetal stage onwards and indicate the potential involvement of BMPs in the formation, activation and early development of pig follicles.

Somatic cell derived BMPs

In rodents, BMP-4 and BMP-7, are expressed in theca cells from the primary/secondary stage onwards and have been shown to reduce the number of primordial follicles whilst increasing the number of primary, preantral and antral follicles (Lee *et al*. 2001, Nilsson & Skinner 2003). This suggests a positive paracrine action of these BMPs on the growth of preantral follicles, as there is in antral follicles (see below). Again data on early follicle development in pigs is lacking, although as mentioned above, BMP-4 and -6 and their receptors are expressed early in development (Quinn *et al*. 2004a, Shimizu *et al*. 2004a).

Anti Mullerian Hormone (AMH)

There is compelling evidence from a number of species, particularly the mouse, that another TGF-β superfamily member, AMH, plays an inhibitory role in the initiation of primordial follicle growth (Durlinger *et al*. 2002). In addition to its role in the differentiation of the male reproductive tract, AMH is expressed by the granulosa cells of the female gonad and exposure of neonatal mouse ovaries to AMH halved the number of growing follicles, whereas deletion of

the AMH gene increased the rate of recruitment of primordial follicles (Durlinger *et al*. 2002, Nilsson *et al*. 2007). It seems likely that AMH acts at the level of the pre-granulosa/granulosa cells, as the AMH type II receptor has been localised to these cells, but not to the oocyte. AMH continues to be expressed in granulosa cells until the mid-antral stage in humans or pre-ovulatory in sheep (Visser & Themmen 2005), implying a continuing role in follicle development beyond the primordial to primary stage. Currently, there is a paucity of information on AMH expression and its role in the growth of porcine primordial follicles.

Antral follicle development

Antral follicle development starts with the formation of the antrum, which then leads to the differentiation of the granulosa cell population into the mural granulosa cells that remain in close contact with the follicle wall, and also the cumulus granulosa cells that closely surround the oocyte. These two granulosa cell populations differ greatly in their ability to proliferate and in their steroidogenic activities. While the mural granulosa cells are actively proliferating and highly steroidogenic, the cumulus cells are in close contact with the oocyte, providing it with metabolites and other important factors necessary for the acquisition of meiotic and developmental competence. The theca cell layer, formed during the late preantral stage, is also important for the steroidogenic activity in the follicle. The following sections will review the role of key local growth factors in the regulation of antral follicle development in the pig.

TGF-ß superfamily

The bone morphogenetic proteins (BMPs)

Oocyte derived BMPs

Early studies showed that removal of the oocyte from rabbit antral follicles led to premature luteinisation of the follicle and oocyte secreted factors have now been clearly identified as negative modulators of cumulus and mural granulosa cell progesterone production (Gilchrist *et al*. 2008). However, the role of oocyte-secreted factors is not limited to controlling luteinisation or progesterone production but rather seems to extend to steroidogenesis in general. In pig mural granulosa cell, the oocyte was shown to stimulate FSH-induced oestradiol production while the converse was observed in their cumulus cells (Coskun *et al*. 1995, Brankin *et al*. 2003a). In addition, similar to other species, porcine oocytes stimulate cell proliferation but also increase granulosa and theca cell viability (Hickey *et al*. 2005, Brankin *et al*. 2003a). Moreover, mouse oocytes also control cumulus cell metabolism (Eppig *et al*. 2005). Finally, but not least, bovine and murine oocyte secreted factors have been shown to enhance oocyte developmental competence (Hussein *et al*. 2006; Gilchrist *et al*. 2008). The latter two oocyte functions have yet to be described in a porcine model but the potential for similar roles in porcine cumulus cell metabolism and oocyte developmental competence should be investigated.

BMP-6, BMP-15 and GDF-9 have become central players in studies on the regulation of ovarian follicle growth, although it is cannot be ruled out that other oocyte factors also play a role. In the pig, similar to the other species, BMP-6, BMP-15 and GDF-9 are primarily expressed in the oocyte and are extremely abundant (Prochazka *et al*. 2004, Brankin *et al*. 2005a, Zhu *et al*. 2008, Lee *et al*. 2008, Paradis *et al*. 2009). Furthermore, mRNA for all three ligands and/or protein have also been detected, albeit at much lower levels, in ovarian somatic cells indicating some species differences and also the potential for somatic cells interactions. Their receptors, BMPR1A, BMPR1B, TGFβR1 and BMPRII are expressed in all cell types in the antral follicle

(Fig.1) indicating that these ligands may modulate the function of the mural granulosa, theca and cumulus cells (Quinn *et al*. 2004a, Zhu *et al*. 2008 and Paradis *et al*. 2009). In addition, Paradis *et al*. (2009) showed that oocyte derived mRNA for BMP-6, BMP-15 and GDF-9 as well as BMP-15 protein are expressed at constant levels throughout the follicular phase, however the expression of the mRNA for their receptors is temporally regulated (Fig.3). Interestingly, *BMPR1B* mRNA abundance was positively correlated with plasma oestradiol concentrations, suggesting that the receptors may be hormonally regulated.

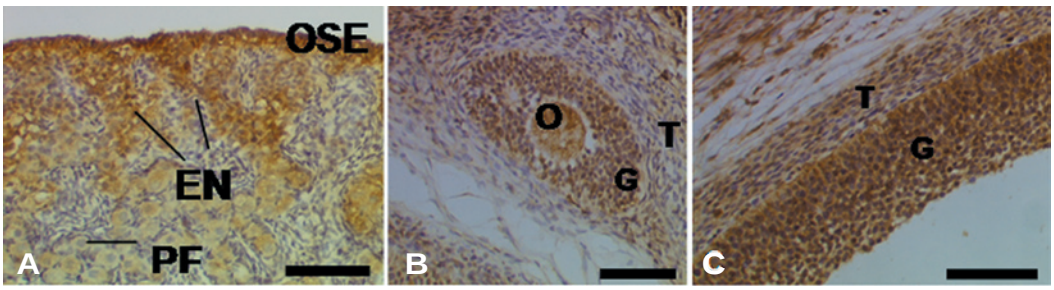


Fig. 1 Immunohistochemical localisation of the BMP receptor 1A in A) fetal ovary; B) preantral follicle and C) antral follicle. PF, primordial follicle; EN, egg nests; OS, surface epithelium; O, oocyte; G, granulosa cells; T, theca cells. Scale bars = 100 μm.

Moreover BMP-6 and BMP-15 regulate porcine granulosa and theca cell steroidogenic activity *in vitro* in a manner very similar to that of the oocyte (Fig. 2; Brankin *et al*. 2003b, Quinn *et al*. 2004b, Brankin *et al*. 2005a, b). For example, in the granulosa cells, BMP-6 and BMP-15 consistently inhibited progesterone while increasing oestradiol production (Brankin *et al*. 2003b, Brankin *et al*. 2005a). The mechanism of action of BMP-6 on progesterone is via inhibition of the second messenger cAMP, and reduction in protein expression of steroid acute regulatory protein (StAR) and 3β-hydroxysteroid dehydrogenase (3β-HSD; Brankin *et al*. 2005a). On the other hand, BMP-6 suppressed androstenedione and progesterone production by the theca cells while BMP-15 increased their progesterone production (Quinn *et al*. 2004b, Brankin *et al*. 2005b). Recombinant GDF-9 also decreased FSH-stimulated progesterone production in porcine granulosa cells and its effect was enhanced by androgen (Hickey *et al*. 2005). BMP-6 and BMP-15 have been shown to increase theca cell viability while BMP-6 and GDF-9 were found to respectively stimulate theca and granulosa cell proliferation (Quinn *et al*. 2004b, Brankin *et al*. 2005a, Brankin *et al*. 2005b, Hickey *et al*. 2005). Finally, although it still remains to be shown in the pig, GDF-9 increased bovine oocyte developmental competence (Hussein *et al*. 2006). Overall, the oocyte and oocyte-derived ligands appear to dictate the steroidogenic ability of the cumulus and mural granulosa cells and clearly prevent precocious luteinisation of the follicle. Moreover, their effects on cell proliferation and viability suggest that the oocyte also modulates the growth of healthy antral follicles.

Somatic cell derived BMPs

Evidence also supports a role for the somatic cell derived BMPs including BMP-2, -4, and -7 in antral follicle development but the information available in mammals and in pigs is limited. Their localisation in the ovary is perhaps the first clue that these molecules act as paracrine and autocrine modulators of ovarian functions. In the porcine follicle, BMP-2 mRNA and protein was found to be expressed by the granulosa and theca cells throughout the follicular phase (Brankin *et al*. 2005a, Paradis *et al*. 2009). In addition, BMP-4 and BMP-7

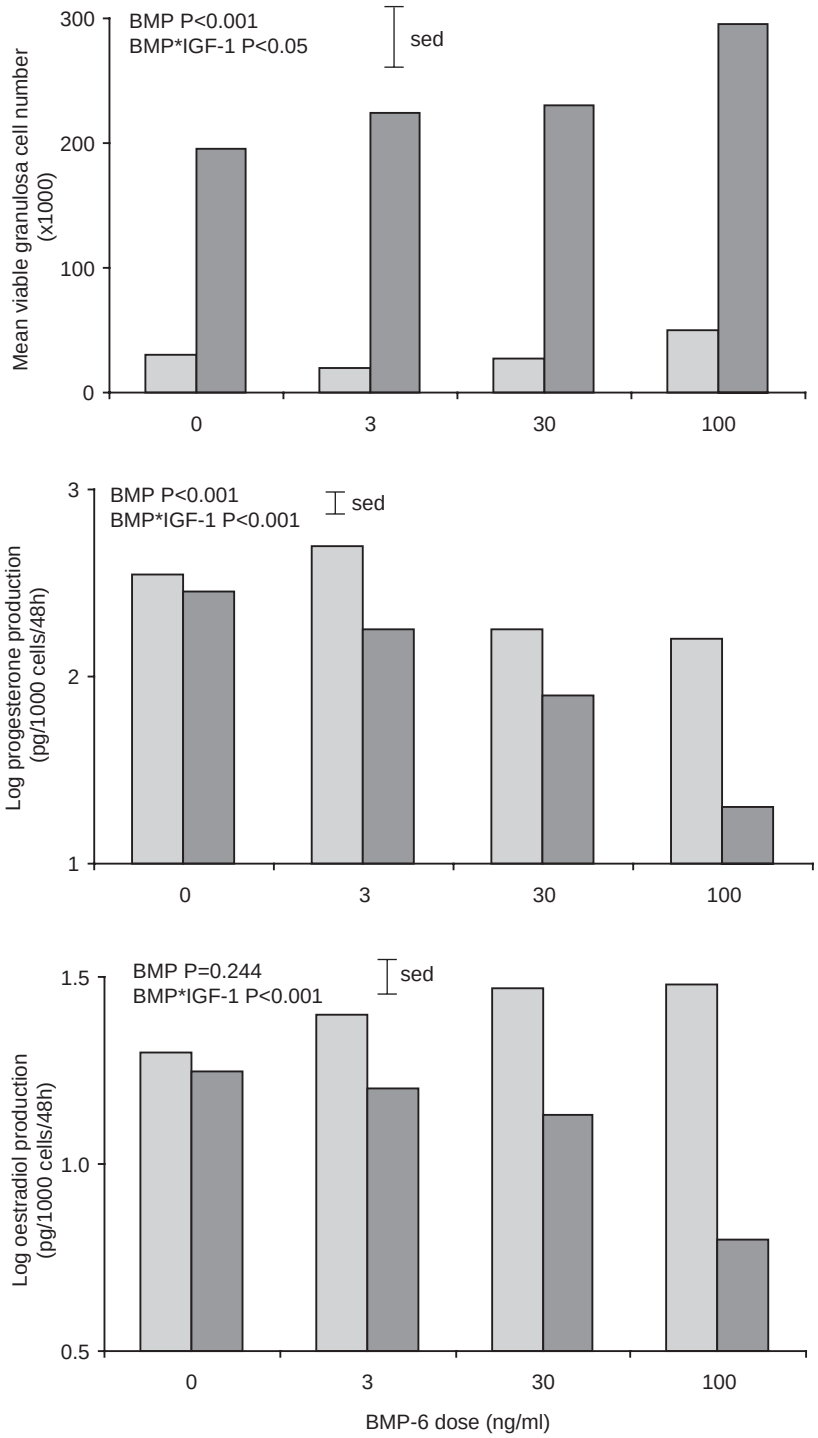


Fig. 2 The effect of BMP-6 on (**a**) mean (± sed) viable granulosa cell number (**b**) mean (± sed) $\log_{10}$ progesterone production by granulosa cells and (**c**) mean (± sed) $\log_{10}$ oestradiol production by granulosa cells after 144 hours in serum free culture in the absence (□) and presence (■) of 100 ng/ml IGF-1. Values are from 3 independent cultures.

were reported to be expressed exclusively by the theca cells of rat and bovine antral follicles (Erickson & Shimasaki 2003, Glister *et al*. 2004). As for the oocyte derived BMPs, every cell type in the follicle is a potential target for BMP-2, -4, and 7 since they signal through the same type I and type II receptors.

In the porcine antral follicle, only BMP-2 has been tested for its ability to promote granulosa and theca cell steroidogenesis, and cell proliferation and viability (Brankin *et al*. 2005a,b). Its action on the theca cells is remarkably similar to that of BMP-6, as it reduces progesterone, androstenedione and oestradiol production. It also suppressed granulosa cell progesterone production in a dose-dependent manner. Finally, perhaps of more interest is the action of the theca cell specific BMP-4 and BMP-7 on granulosa cell and theca cell function. In bovine theca cells, BMP-4 and BMP-7 decreased androstenedione production while they increased oestradiol, inhibin A, activin A and follistatin secretion by the granulosa cells (Glister *et al*. 2004, 2005). This is particularly interesting because follistatin, which is known to bind activin with high affinity, was also found to counteract the effect of many BMPs (Glister *et al*. 2004, 2005). Other binding proteins including chordin and gremlin have also been reported to counteract the effect of specific BMP proteins (Glister *et al*. 2005, Juengel & McNatty 2005). This observation sheds some light on how the autocrine and paracrine BMP signalling pathways are regulated in the ovarian follicle but also creates another level of complexity, demonstrating that the different TGF-β subfamilies interact together and also with other growth factor families (such as IGF-1, c-kit-KitL).

It is interesting to note that the functions of the BMPs on steroidogenesis and cell proliferation (Fig. 4) are somewhat redundant and they clearly act in concert to prevent precocious luteinisation of the follicle. They also promote cell proliferation, but whether they have a role in regulating ovulation rate in the pig, as in sheep, is currently unknown. The observation that the oocyte appears to directly control the steroidogenic and metabolic activity of the cumulus cells suggests that the gradient of oocyte-derived BMPs may be involved in establishing the cumulus cell versus mural granulosa cell phenotype.

TGF-ß and associated receptors

The TGF-ß subfamily is comprised of three ligands namely TGF-ß1, -ß2 and -ß3 and two associated receptors, TGFßRI (also known as ALK5) and TGFBRII (ten Dijke & Hill 2004, Juengel & McNatty 2005). Betaglycan (also known as TGFßRIII) has also been shown to be necessary for binding of TGF-ß2 in many cell types (ten Dijke & Hill 2004). In porcine follicles, the theca interna appears to be the primary source of TGF-ß and TGF-ß1 accounts for most of the TGF-ß bioactivity on cell proliferation (May *et al*. 1996). Similarly, TGF-ß3 appears most prominent in the theca cells of large antral follicles, but its functions have yet to be established (May *et al*. 1996, Steffl *et al*. 2008). The expression of the TGF-ß receptors in the porcine ovary has been less extensively studied than their ligands but Goddard *et al*. (1995) reported that cultured granulosa cells expressed *TGFßRI*, *TGFßRII* and *TGFßRIII* mRNA and produced the protein for TGBßR1 and TGFßRII. However, since granulosa cells tend to luteinize in culture, particularly in presence of serum, these results may reflect the ability of luteal, rather than follicular, cells to respond to the TGF-ß ligand. Nevertheless, we detected the expression of *TGFßR1* mRNA in antral follicles throughout the follicular phase and also in the final preovulatory population pre- and post-LH surge. Our findings confirmed that *TGFßR1* mRNA is present in the oocyte, granulosa cells and theca cells of antral follicles at all stages studied (Paradis *et al*. 2009). Our results also showed that both granulosa and theca cell derived *TGFßR1* mRNA increased during the mid-selection phase and remained constant until the preovulatory LH surge (Fig. 3),

suggesting the involvement of one of its ligands in follicle selection and in the maintenance of the preovulatory follicle population.

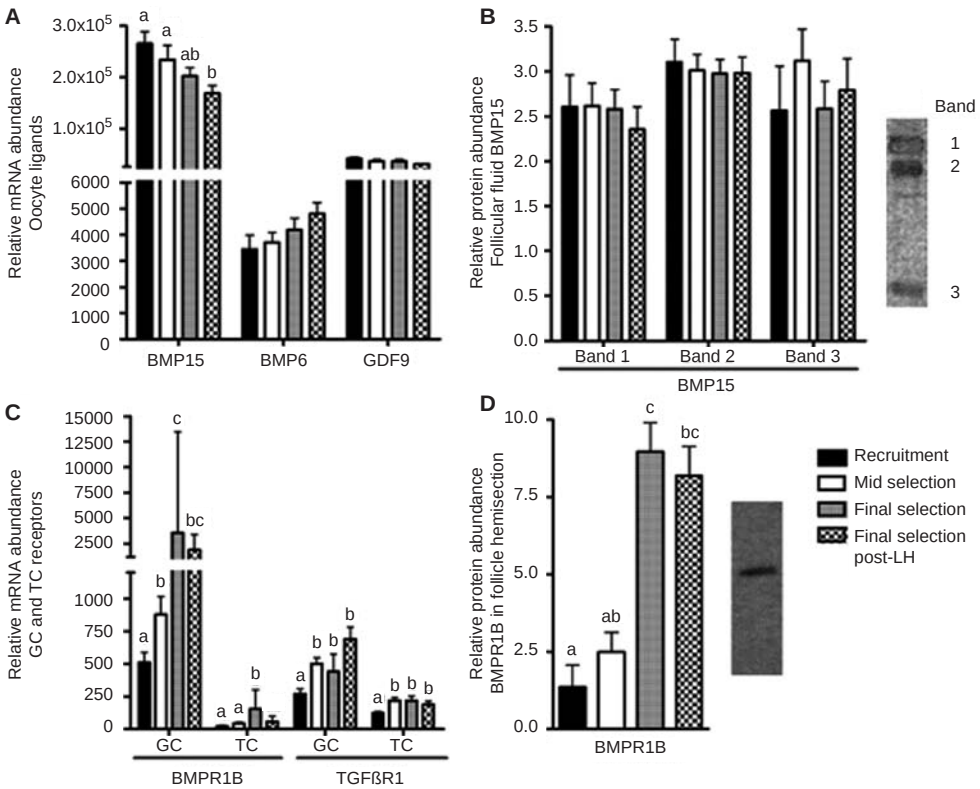


Fig. 3 Temporal changes in the mRNA abundance of (A) oocyte-derived ligands and (C) their corresponding receptors in granulosa (GC) and theca cells (TC) during the porcine follicular phase. (B) represents the corresponding changes in protein abundance of BMP15 in the follicular fluid and (D) of BMPR1B in follicle hemisections. Data are expressed as relative abundance ± SEM. Different superscripts within gene represent significant differences between phases ($P \leq 0.05$).

The effects of the TGF-ß proteins on ovarian cells have been mainly associated with cell proliferation and differentiation (ie. steroidogenesis). TGF-ß1 and TGF-ß2 both inhibited basal- and growth factor-stimulated porcine granulosa cell proliferation while having little effect on theca cells (Mondschein *et al*. 1988). In addition, TGF-ß1 decreased both basal and FSH-stimulated aromatase activity while the converse was observed in theca cells (Caubo *et al*. 1989, Goddard *et al*. 1995). TGF-ß1 also inhibited both basal and FSH or LH -stimulated progesterone production in cultured granulosa or theca cells respectively (Mondschein *et al*. 1988, Caubo *et al*. 1989, Engelhardt *et al*. 1992). The inhibitory effect observed on theca cell progesterone production is believed to occur upstream of P450scc (Engelhardt *et al*. 1992) and interestingly, TGF-ß1 and TGF-ß2 inhibited hCG binding to granulosa cells (Gitay-Goren *et al*. 1993). These observations suggest that the TGF-ß subfamily may mediate the steroidogenic activity of the granulosa cells and potentially of the theca cells (Fig. 4) through direct regulation of the gonadotrophin receptors. On the other hand, TGF-ß1 has also been shown to interact

with the IGF system in granulosa cells (Mondschein *et al*. 1990) which in turn could indirectly influence the steroidogenic activity of those cells. Collectively, these observations suggest that the TGF-ß subfamily is important for modulating the rate of follicle growth while preventing precocious luteinisation of the follicle (Fig. 4).

The periovulatory period

The LH surge triggers a series of events leading to ovulation, luteinisation of the mural granulosa cell and theca cells, cumulus cell expansion and oocyte meiotic resumption (Richards *et al*. 2002). Although gap-junctional communication has been postulated to propagate the signal from the LH responsive mural granulosa cells to the cumulus cells and oocyte, increasing evidence suggests that paracrine signalling from the mural granulosa cells is also important. This section will address the roles of the BMPs and the EGF/EGF-like ligands during the periovulatory period.

The bone morphogenetic proteins (BMPs)

Cumulus cell expansion is a necessary event for ovulation (Richards *et al*. 2002) and in the mouse, the oocyte is required for FSH to stimulate cumulus expansion and hyaluronic acid synthesis (Salustri *et al*. 1990). The factor(s) responsible for cumulus cell expansion, named cumulus expansion-enabling factor (CEEF) is also produced by porcine oocyte (Singh *et al*. 1993) but appears dispensible for porcine cumulus cells expansion and production of hyaluronic acid (Prochazka *et al*. 1991, Singh *et al*. 1993, Nagyova *et al*. 1999). Interestingly, Prochazka *et al*. (1998) demonstrated that porcine cumulus and mural granulosa cells also produce the CEEF.

The identity of the CEEF still remains a controversial topic however, BMP-15 and GDF-9 represent prime candidate to fulfill this function. First of all, oocytes deficient in GDF-9 fail to stimulate cumulus expansion and the genes necessary for that process (Vanderhyden *et al*. 2003) and recombinant GDF-9 has been shown to promote mouse cumulus expansion and induce hyaluronan synthase 2 (HAS2) expression (Elvin *et al*. 1999). On the other hand, *Bmp-15* null mice have a decreased ovulation rate while the *Bmpr1b* null mice exhibited defective cumulus expansion (Yan *et al*. 2001, Yi *et al*. 2001, Su *et al*. 2004). In addition, the mature form of BMP-15 appears during the periovulatory period in the mouse, following gonadotrophin stimulation (Yoshino *et al*. 2006, Gueripel *et al*. 2006). Unfortunately, recombinant BMP-15 has yet to be tested for its ability to promote cumulus expansion, and hyaluronic acid synthesis and its related genes. In the pig, both *BMP-15* and *GDF-9* mRNA and protein were detected in the oocyte, cumulus and mural granulosa cells, convincingly demonstrating the potential for those cell types to promote cumulus cell expansion (Prochazka *et al*. 2004, Lee *et al*. 2008, Paradis *et al*. 2009). Although no concomitant increase in *GDF-9* mRNA was observed during porcine cumulus cell expansion, it does not preclude the possibility that post-translational modification or processing could regulate the bioactivity of GDF-9 (Prochazka *et al*. 2004). Similarly, Paradis *et al*. (2009) showed that although the abundance of BMP-15 did not change dramatically during follicle development, the expression of BMPR1B in the granulosa cells (combined cumulus and mural granulosa cells) increased in the periovulatory period (Fig. 3) suggesting a role for this ligand in preparation for ovulation. The direct effect of recombinant GDF-9 or BMP-15 on porcine cumulus expansion has yet to be reported but could shed the light on how the ovulatory process is regulated in the porcine follicle.

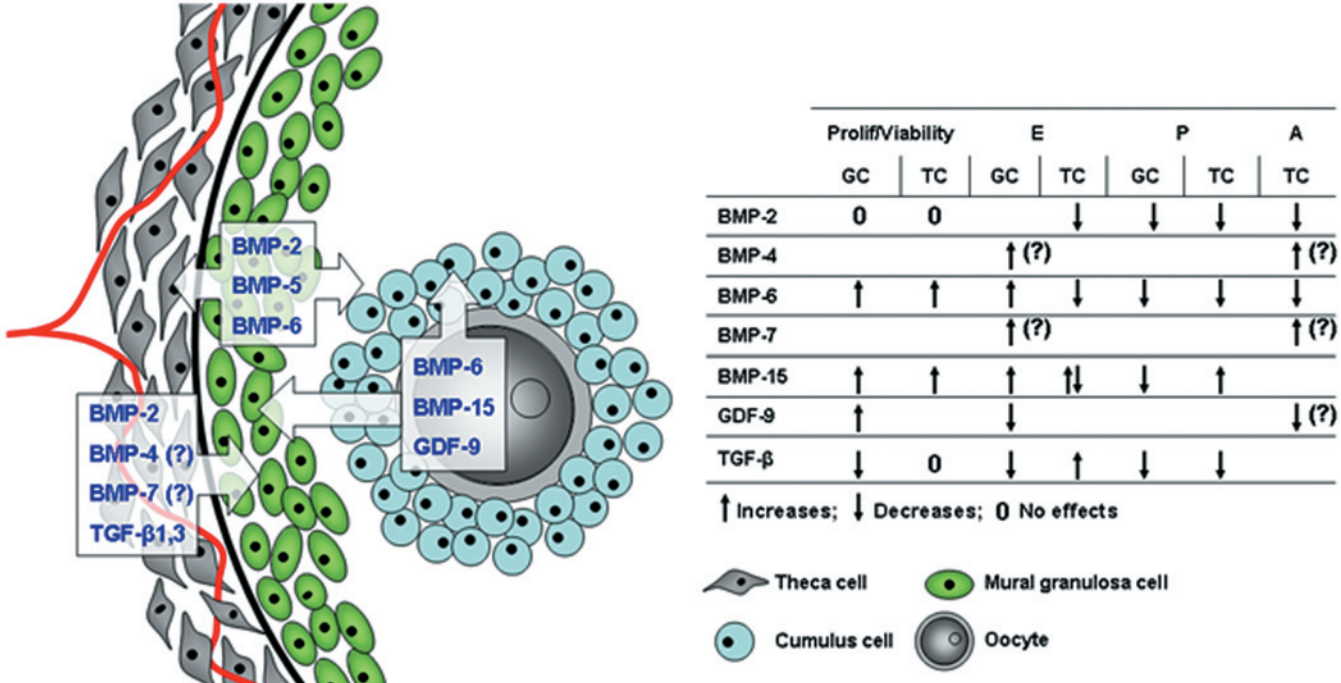


Fig. 4 Schematic representation of the localization and actions of TGF-ß superfamily members in porcine antral follicles. The table lists the known autocrine and paracrine functions on granulosa (GC) and theca cell (TC) proliferation and viability (Prolif/Viability) and oestradiol (E), progesterone (P) and androgen (A) synthesis. (?) indicates the possible localisation and functions in porcine antral follicles based on observations from other species.

EGF and EGF-like peptides

The epidermal growth factor family includes many closely related proteins including EGF, amphiregulin, epiregulin and betacellulin (Conti *et al*. 2006). These ligands signal through the EGF receptors, including EGFR (ErbB1), belonging to tyrosine kinase receptor family. The effect of EGF on cumulus and oocyte function in culture has been well studied in several species and the roles of amphiregulin, epiregulin and betacellulin are becoming evident.

In porcine follicles, EGF appears to be predominantly expressed in the mural granulosa cells although it has been also detected at much lower level in the oocyte and cumulus cells (Singh *et al*. 1995). The EGFR was detected in the cumulus and mural granulosa cells as well as in the theca cells, suggesting that most effects on the oocyte occur indirectly through the cumulus cells. EGF potently stimulates porcine cumulus cell expansion *in vitro* and interestingly, the ability of the porcine cumulus cells to undergo expansion increases with follicle size and is linked to the FSH-enhanced ability of those cells to activate the EGF receptor (Prochazka *et al*. 2000, 2003). Furthermore, EGF supplementation enhances oocyte nuclear maturation and oocyte developmental competence *in vitro* (Reed *et al*. 1993, Prochazka *et al*. 2000).

More recently, a group of EGF-like ligands namely amphiregulin (AREG), epiregulin (EREG) and betacellulin (BTC) have been found to mediate LH action in the mouse follicle (Park *et al*. 2004). All three factors stimulated oocyte meiotic resumption in a whole follicle culture system in a manner similar to LH, although their action appeared to be downstream of LH (Park *et al*. 2004). Interestingly, LH- but not AREG-, EREG- or BTC-stimulated cumulus cell expansion was abolished when cumulus-oocyte complexes rather than whole follicles were used, suggesting that LH regulation of cumulus cell expansion and oocyte meiotic resumption occurs indirectly through mural granulosa cells. Moreover, the effects of the EGF-like ligands on the COC are believed to occur via the cumulus cells, as denuded oocytes did not resume meiosis. Further convincing evidence for the involvement of these three factors in cumulus expansion was that all three EGF-like ligands induced *HAS2* mRNA in the same way as LH (Park *et al*. 2004, Shimada *et al*. 2006). Finally, the effect of AREG, EREG and BTC was shown to occur through the EGFR since the EGFR kinase inhibitor AG1478 completely prevented their effects. Similar observations have been made in porcine COC (Yamashita *et al*. 2007, Chen *et al*. 2008). Another interesting aspect is that AREG and EREG are expressed as transmembrane precursors and require metalloprotease activity to be released and to activate the EGFR on the target cells (Dong *et al*. 1999). In cultured porcine COC, mRNA expression and activity of TACE/ADAM17 metalloprotease was induced by FSH and LH in similar manner to that of AREG and EREG (Yamashita *et al*. 2007). Metalloprotease inhibitor prevented cumulus expansion while EGF completely reversed this inhibitory effect. From these results, the authors concluded that TACE/ADAM17 metalloprotease is required for the release of the membrane bound EGF-like ligand and EGFR-mediated activation of cumulus cell expansion and oocyte meiotic maturation (Yamashita *et al*. 2007). Finally, it remains to be established whether there is an interaction between the TGF-ß superfamily and the EGF system in the regulation of the periovulatory events leading to ovulation.

Conclusions

It is clear that follicle development (from primordial to pre-ovulatory follicle) and acquisition of oocyte developmental competence involves the interaction of an ever increasing number of local growth factors. Paracrine interactions occur within the somatic cells and also between the somatic cells and the oocyte in a bi-directional manner, all of which are essential for normal follicular development. These local factors, along with endocrine hormones, act to

regulate steroidogenesis, proliferation, differentiation, apoptosis, cumulus expansion, oocyte maturation and developmental potential. Although our knowledge of these intra-follicular regulatory factors, particularly members of the TGF-ß superfamily, has increased dramatically in recent years, there are still unanswered questions including how the temporal and spatial expression of these factors is regulated, why there appears to be redundancy of some of the ligands and precisely how the various growth factor families interact in the growing follicle. Furthermore, much of the information discussed in this review has been generated from *in vitro* or localisation studies, or by extrapolation from other species and whilst such information is extremely valuable, particularly from a mechanistic viewpoint, there is still a need to understand precisely how these factors interact *in vivo*. Therefore the testing of some of the hypothesised roles of these paracrine hormones in animal models should be a high priority to help unravel their relative importance in regulating follicle development in the pig. It is only through increased understanding of the precise roles of these intra-follicular factors, that further improvements in reproductive efficiency will be made.

Acknowledgements

MGH acknowledges funding from BBSRC (42/S17232). FP acknowledges funding from NSERC and Alberta Ingenuity.

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Transcriptional, post-transcriptional and epigenetic control of porcine oocyte maturation and embryogenesis

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Embryogenesis is a complex process that is controlled at various levels. As new discoveries are made about molecular mechanisms that control development in other species, it is apparent that these same mechanisms regulate pig embryogenesis as well. Methylation of DNA and modification of histones regulate transcription, and mechanisms such as ubiquitinization, autophagy and microRNAs regulate development post-transcriptionally. Each of these systems of regulation is highly dynamic in the early embryo. A better understanding of each of these levels of regulation can provide tools to potentially improve the reproductive process in pigs, to improve methods of creating pig embryos and cloned embryos *in vitro*, and to provide markers for predicting developmental competence of the embryo.

Introduction

Development of the embryo and the relative likelihood that it will give rise to a viable offspring is dependent upon factors that include, but are not limited to, the general health status of the sire and dam during gametogenesis and their genetic contributions. These factors set the stage for the oocyte to mature with the proper stores of information to support complete development. Factors that regulate development will be presented with a focus on transcriptional, post-transcriptional and epigenetic regulation of development in the pig. Finally, examples of how this information can be used to our advantage will be presented.

Overview of embryogenesis

An oocyte competent to develop to term is the result of adequate growth of the oocyte, and subsequent nuclear and cytoplasmic maturation. During this time the information necessary for early development is transcribed and stored in the form of RNA, or translated and stored in the form of protein. Upon germinal vesicle breakdown (GVBD) transcription ceases and nuclear maturation progresses until arrest at metaphase II of meiosis. Fusion of the sperm and oocyte plasma membrane results in the deposition of the sperm chromatin in the ooplasm, and resumption and completion of meiosis of the maternal chromatin. The maternally- and paternally-derived chromatin each forms a separate pronucleus that migrates to the center of the oocyte. At first mitosis the pronuclear envelopes breakdown and for the first time the maternally- and paternally-derived chromatin mix as the chromosomes align on the metaphase plate and subsequently segregate to the two poles prior to cytokinesis. In the pig, after the first mitosis the embryo immediately begins DNA synthesis without a G1 phase. After DNA

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synthesis is completed (about 12 hours (Prather *et al*. 1996)) the embryo enters directly into mitosis without an intervening G2 phase. Entry into the 4-cell stage is the first time that a G1 phase is inserted and corresponds with the onset of transcription (Schoenbeck *et al*. 1992). The length of the 4-cell stage is over twice that of the 2-cell stage; the difference being the result of a short G1 phase and a long G2 phase (Anderson *et al*. 2001). When the chromatin is not encumbered with DNA polymerases, i.e. G1 and G2, the transcriptional machinery can gain access to the chromatin and RNA can be synthesized (Prather 1993).

Since there is little or no transcription between GVBD and the 4-cell stage, it is imperative that the germinal vesicle stage oocyte (GV) be poised to control development for the first few divisions. Thus, the information stored in the oocyte must be sufficient to direct the first few cellular divisions and establish the correct chromatin configuration so that the symphony of gene expression can begin. If the chromatin configuration is not correct, the embryo may develop to varying degrees, but it may not have normal transcription (Tian *et al*. 2009) or it may not thrive and reproduce - as observed with some cloned animals (Carter *et al*. 2002). Thus it is imperative that the correct developmental pattern of gene expression be established during the first few cleavage divisions.

Upon successful activation of the embryonic genome (ZGA) the pig embryo continues to divide to the 8-, 16- and subsequently the 32-cell stage. Compaction of the blastomeres occurs during this time and the embryo continues the differentiation process by forming a blastocyst with two distinct cell types, each with its own transcriptional repertoire. Dramatic changes in mRNA abundance occur between the oocyte and 4-cell stage and blastocyst stage and these changes are driven by both transcriptional and post-transcriptional regulation (Whitworth *et al*. 2005).

Transcriptional regulation

Mammalian oogenesis, oocyte maturation and early embryo development are distinct processes, each carefully coordinated, and bound by a common thread: the need to supply an appropriately programmed genome that permits proper gene expression in time and space. In addition to transcription factor binding to promoters, regulation of transcription early in development is achieved via epigenetic mechanisms. The term epigenetics, as used and accepted today, is defined as "heritable changes in gene function that cannot be explained by changes in DNA sequence" (Russo *et al*. 1996). Epigenetic mechanisms in the oocyte and early embryo include DNA methylation, histone modification, chromatin remodeling, and non-coding RNAs (ncRNA) (Li 2002; Morgan *et al*. 2005). Space limitations prevent a thorough treatment of each of these topics. Rather a brief summary of the status of our collective understanding of how these mechanisms are/could be involved in controlling transcription in the mammalian oocyte and early embryo will be provided. Most of the information will focus on porcine embryo development.

Methylation of the cytosine of CpG dinucleotides plays a central role in transcriptional regulation in mammals. In most situations, methylation of DNA in and near genes is associated with repression of transcriptional activity of those genes (Iager *et al*. 2008). DNA methyltransferases are responsible for establishing (DNMT3a and DNMT3b) and maintaining (DNMT1) methylation patterns within DNA (Li 2002). Interestingly, mRNA levels for DMNT1 are 20-fold higher in the pig GV oocyte to 4-cell stage as compared to the blastocyst stage, whereas DMNT3b levels are not different (Whitworth *et al*. 2005). Four general mechanisms have been proposed for the methylation-mediated silencing of gene expression: 1) direct inhibition of transcription factor binding of methylated gene promoter regions; 2) recruitment of co-repressors to methylated regions by DNA methyl-binding proteins; 3) DNA methyltransferase-mediated chromatin

remodeling; and 4) dampened efficiency of transcriptional elongation in methylation-rich gene bodies. A detailed summary of these mechanisms has been reviewed (Klose & Bird. 2006).

It has become apparent in recent years that, in addition to DNA methylation, histone modification plays an important role in controlling gene expression in both gametes (DeJong 2006) and early embryos (Nowak-Imialek *et al*. 2008; Ooga *et al*. 2008; Thomas *et al*. 2008). Nuclear DNA is packaged in the cell as chromatin – octamers of predictably-organized histone proteins wrapped by 146 bp of DNA. The N-termini of the histone proteins are highly susceptible to post-translational modifications, including (but not limited to) lysine acetylation, lysine and arginine methylation, serine and threonine phosphorylation, sumoylation, ubiquitination and ADP-ribosylation (Kouzarides 2007). The effects of histone modifications on gene expression may not be predictable enough to consider these patterns of modifications as a "code" in the truest sense, as once proposed (Strahl & Allis 2000). Rather it seems that genomic and physiologic context play a significant role in determining the effect that individual modifications might have on chromatin function. Some generalizations can be made: histone acetylation is almost universally associated with increased transcription; methylation of lysine residues can either activate (H3K4me, H3K36me, H3K79me) or repress (H3K9, H3K27, H4K20) transcription; while histone ubiquitination and sumoylation are generally considered to be repressive to transcription. Control over histone modifications is exquisite, with an impressive array of enzymes dedicated to establishing, maintaining and/or removing histone marks (Kouzarides 2007).

In addition to these primary marks, there are a number of facilitator processes and molecules that are involved in the establishment, maintenance, and transmission of epigenetically silenced (or activated) genes. These include polycomb group (PcG) proteins, trithorax group (TrxG) proteins, chromatin remodeling complexes, and ncRNAs. Polycomb group and TrxG proteins function essentially antagonistically to repress or maintain activity of genes that are important during development. Some members of the PcG and TrxG protein families have histone methyltransferase activities, with PcG proteins methylating K27 of H3 – a generally repressive mark – and TrxG proteins catalyzing H3K4 methylation, which is permissive to transcription (Kiefer 2007). Chromatin remodeling complexes (de la Serna *et al*. 2006) such as the ISWI and various SWI/SNF complexes interact with and modulate the activity of histone arginine methyltransferases (Pal & Sif. 2007) and acetyltransferases (Brockmann *et al*. 2001) which are generally associated with increased transcription. These complexes are necessary for transcriptional regulation that occurs after fertilization (de la Serna *et al*. 2006). SMARCA4, an SNF2 chromatin remodeling ATPase, is an important determinant of early porcine embryogenesis in both *in vitro*-fertilized and somatic cell nuclear transfer (SCNT) embryos (Magnani *et al*. 2008). Finally ncRNAs result in gene silencing in a variety of scenarios important to development, such as X-chromosome inactivation (Boumil & Lee. 2001) and gene imprinting (Nagano *et al*. 2008; Pandey *et al*. 2008), in addition to their role in RNA-induced gene silencing (RNAi).

Almost by definition, cellular differentiation is an epigenetic phenomenon: phenotypic and functional differences between cell types arise without reordering the sequence of the DNA. Thus for each distinct cell population, there is an associated cadre of epigenetic modifications that determine which genes are turned on/off thus giving each cell population its phenotype. As somatic cells differentiate into germ cells, somatic-specific epigenetic marks are erased and at the end of gametogenesis there is a paternal-specific pattern of epigenetic modifications established in each sperm, and a maternal-specific pattern established in each oocyte. Upon fertilization, the genome of the embryo is demethylated and otherwise reprogrammed to replace the extensive germ cell-specific epigenetic marks with those conducive to pluripotency. De novo reestablishment of epigenetic marks in appropriate cell lineage-specific patterns occurs

in the inner cell mass (ICM) and to a lesser extent in the trophectoderm of the blastocyst (Dean *et al*. 2001; Santos *et al*. 2002). A global survey of DNA methylation during early development in the pig showed that 18% of the randomly selected genomic regions changed from the gametes to the blastocyst stage (Bonk *et al*. 2007a). Some DNA methylation in early embryos is sensitive to the culture conditions, i.e. cultured embryos can have a different pattern of DNA methylation compared to *in vivo*-collected embryos (Mann *et al*. 2003; Mann *et al*. 2004).

Expression of imprinted genes– genes that are expressed in a parent-of-origin, monoallelic pattern - show how epigenetics can impact development. Many imprinted genes regulate fetal and placental growth, development, and function. Establishment of imprinting appears to be fairly conserved across species, and one such imprinted region is the well-characterized IGF2/H19 locus (Sasaki *et al*. 2000; Engel *et al*. 2004), which in most mammalian species – including pig (Han *et al*. 2008) – is heavily methylated on the paternal allele, and relatively unmethylated on the maternal allele. In the mouse the unmethylated maternal allele can bind to an insulator (CTCF) which prevents the IGF2 promoter from interacting with an upstream enhancer, thus preventing maternal allele expression of IGF2. An inactive IGF2 allele is conducive to H19 expression and is therefore expressed highly from the maternal chromosomes. Heavy methylation on the paternal allele is repressive to H19 expression, while conducive to IGF2 expression because the CTCF insulator does not bind to methylated DNA. Another imprinted locus is IGF2R/KCNQ1, which is silenced at the paternal allele by the interaction of ncRNAs with histone methyltransferases and members of the PcG complex (Nagano *et al*. 2008; Pandey *et al*. 2008). While the sperm and oocyte chromatin are hypermethylated relative to the early embryo, the demethylation that occurs after fertilization does not result in a loss of the imprint (Nakamura *et al*. 2006; Santos *et al*. 2002). The mechanisms by which these epigenetic 'memories' are maintained are poorly understood, but additional examples of how epigenetic mechanisms impact development are abundant (Hansen *et al*. 2008). For example, increased methylation of arginine residues on histone H3 predisposes blastomeres of the early embryo to contribute to the pluripotent cells of the ICM, whereas lower levels of H3 arginine methylation direct cells to a mural trophectoderm state (Torres-Padilla *et al*. 2007). Histone methyltransferase EHMT2 is a 'master regulator' of early embryonic genes: 126 genes (including POU5F1) expressed in undifferentiated mouse embryonic stem cells were converted to heterochromatin and silenced by EHMT2 upon differentiation (Epsztejn-Litman *et al*. 2008). A common mark on these gene promoters was trimethylation on lysine 9 of histone H3. Interestingly, the promoters of these 126 silenced genes exhibited de novo DNA methylation in addition to the H3K9 trimethylation. This case also serves to exemplify the functional interplay that exists between these supposed 'separate' mechanisms of epigenetic gene control, in that EHMT2 – a *histone* methyltransferase – also appears to be able to direct de novo DNA methylation by autonomously recruiting DNA methyltransferases to the silenced loci.

Much of what we know about epigenetic control of gene activity in germ cells and early embryo development comes from studies investigating the nuclear reprogramming associated with SCNT. The inefficiency of SCNT has been correlated with aberrant patterns of DNA methylation, histone modification and, consequentially, gene expression (Santos *et al*. 2003; Wrenzycki *et al*. 2006; Bonk *et al*. 2007b). Upon transfer of a somatic nucleus into oocyte cytoplasm an epigenetic reprogramming event must take place to revert the somatic chromatin back to a pluripotent-like state. This is an inefficient process, with the transferred nucleus often retaining a proportion of the somatic epigenetic marks, resulting in mis-regulated gene expression, which in turn can cause abnormalities in the embryo or fetus (Niemann *et al*. 2002). The most severe of these abnormalities result in failed development very early. However, less severe epigenetic aberrations can allow development to proceed, but often the resulting offspring have obvious

deformities and/or are compromised physiologically after birth. The term Large Offspring Syndrome (LOS) was coined as a result of the large birth weights of cloned calves, although the characterization of this syndrome as dealing exclusively with "Large Offspring" is inappropriate since cloned offspring in some species (e.g. pigs) tend to have lower birth weights relative to their naturally-conceived counterparts (Estrada *et al*. 2007); additional manifestations of LOS include hydroallantois, hydrops fetalis, hyperplasia in various organ systems, compromised immune function, respiratory distress and other skeletal and soft tissue deformations (Carter *et al*. 2002; Wrenzycki & Niemann 2003; Carroll *et al*. 2005; Wrenzycki *et al*. 2006). Of interest is the association of similar developmental defects with offspring arising from other less invasive assisted reproductive technologies such as *in vitro*-oocyte maturation, -fertilization, -culture, and intracytoplasmic sperm injection (Farin *et al*. 2006; Fernandez-Gonzalez *et al*. 2007; Lawrence & Moley. 2008). While the etiology of these disturbances is not known it is clear that epigenetics play a role. In one recent study, bisulfite sequencing at distinct genomic loci revealed significant differences in levels of DNA methylation in comparisons made between *in vivo*-produced and parthenogenetic blastocysts (5/12 loci were differentially methylated), and between *in vitro* fertilized blastocysts (6/8 loci) and SCNT blastocysts (4/12 loci) (Bonk *et al*. 2007a). Success at increasing 'normal' developmental patterns of cloned embryos is achievable by treating donor cells and/or reconstructed embryos with inhibitors of DNA methylation and histone acetylation (Ding *et al*. 2008; Iager *et al*. 2008). It has yet to be demonstrated whether such treatments can restore 'normal' transcription profiles in cloned embryos, or reduce the occurrence or severity of LOS.

The study of the epigenetic control of embryonic and germ cell transcription is a nascent field of research. What has emerged is a picture of enormous complexity as there is significant functional overlap and cooperation between marking mechanisms. The practical application of this knowledge is now beginning to be developed. This will be an area of intense interest into the foreseeable future, with continued emphasis being placed on the epigenetic control of development, and especially pluripotency.

Post-transcriptional regulation

In addition to proper chromatin configuration, many maternally-derived RNAs and proteins must be degraded by microRNAs or the binding of regulatory proteins to the 3' untranslated region of the message (Schier 2007; Stitzel & Seydoux 2007). Degradation of the maternal proteins begins immediately after fertilization and is mediated partially via ubiquitin-proteasome mediated processes (Huo *et al*. 2004) and macroautophagy. There are at least two periods of degradation; the first is at the time of fertilization, and the second is at the time of ZGA. In drosophila the main degradation of transcripts and proteins occurs at ZGA and a third of the genes whose maternal transcripts are degraded also begin transcription at the same time (De Renzis *et al*. 2007).

Macroautophagy results from the sequestration of proteins into an autophagosome that fuses with a lysosome where the proteins are degraded (Mizushima *et al*. 2008). This system, mediated by autophagy-related 5 protein (ATG5), is up-regulated after fertilization or parthenogenetic activation in the mouse, and a deficiency in this system results in embryos that die by the 4- to 8-cell stage (Tsukamoto *et al*. 2008). In the pig, ATG5 (aka APG5L) decreases ($p = 0.080$) from a ratio of 10.2 in the GV to 3.5 at the 4-cell stage, and then decreases further to 1.3 by the blastocyst stage (Whitworth *et al*. 2005). High levels of mRNA for ATG5 show that a macroautophagy system may also function in the pig.

MicroRNA regulation of oocyte maturation and embryonic development

Specificity in cellular phenotype, as affected by differentiation and cellular lineage, is the result of a particular combination mRNA transcript abundance and controlled translation of those mRNAs to produce proteins capable of eliciting biological function. Transcription and translation are both critical for specificity in cellular phenotype and miRNAs have been shown to be potent regulators of transcript abundance and protein translation, particularly during cell lineage progression and differentiation (Neilson *et al*. 2007).

What are miRNAs?

MicroRNAs are ncRNA that are processed into a functional size of 18-24 nucleotides (Bartel 2004). The mature sequence confers significant biological impact on the cells in which they are synthesized and processed through perfect or imperfect pairing to the 3'UTR of a target mRNA. The binding of a miRNA and its target mRNA 3'UTR results in posttranscriptional gene silencing (PTGS) through the action of several mechanisms, including translation inhibition, target transcript degradation and in some cases, chromatin silencing via methylation (Jackson & Standart 2007). Since the discovery of the first miRNA (Lee *et al*. 1993; Wightman *et al*. 1993) significant advancements have been made into the understanding of miRNA prevalence, biogenesis and function in the Plantae and Animalia kingdoms, largely due to the overlapping pathways and mechanisms involved with RNA interference (RNAi). Current estimates predict that 2-3% of human genes represent miRNAs which are collectively capable of conferring PTGS on an estimated 30% of the human genome (Rajewsky 2006).

MicroRNA biogenesis

Similar to mRNA transcription, microRNA expression is RNA polymerase II dependent (Bartel 2004). The capped, polyadenylated RNA transcripts containing the primary miRNA, have been coined 'pri-miRNA' (Lee *et al*. 2002). All pri-miRNAs have the spatial sequence complementation necessary to form secondary hairpin structures that are recognized by nuclear RNASEN (aka Drosha RNase III endonuclease) (Lee *et al*. 2003). RNASEN cleavage, which requires the functional cooperation of DGCR8 (Han *et al*. 2004), results in the nuclear release of ~60-70 nt stem loops, referred to as pre-miRNA (Lee *et al*. 2002; Zeng & Cullen 2003). Following RNASEN/DGCR8-mediated cleavage, pre-miRNAs are exported from the nucleus (Lund *et al*. 2004) into the cellular cytoplasm, DICER1, also an RNase III endonuclease, typically cleaves both strands resulting in 18-24 nt dsRNA molecules possessing 3' overhangs. These molecules are referred to as mature microRNAs (Grishok *et al*. 2001; Hutvagner *et al*. 2001; Ketting *et al*. 2001). Argonaute proteins (EIF2C1) then interact with mature miRNAs as the foundational component to the formation of a RNA-induced silencing complex (RISC) capable of conferring PTGS (Liu *et al*. 2008).

MicroRNA mechanism of action

The mechanism of action of microRNAs was demonstrated when the abundance of lin-4, having sequence complementary to *lin*-14 and *lin*-28, was shown to be involved with C. elegans larval stage transition from L1 to late L2/early L3 (Lee *et al*. 1993; Wightman *et al*. 1993). During this developmental transition both LIN-14 and LIN-28 abundance was reduced 90 percent while mRNA abundance of lin-14 was unchanged compared to only a 50% reduction of *lin-28*

mRNA (Seggerson *et al*. 2002). Both transcriptional degradation and translational repression are widely involved in miRNA function. In addition, targeted deadenylation, which accelerates mRNA decay, also occurs (Zhang *et al*. 2007). MicroRNA target recognition occurs primarily through the binding of residues on the 5′ end of the miRNA to complementary sequence in the 3′UTR of mRNA target for PTGS (Brennecke *et al*. 2005).

Biological importance of miRNAs

The biological importance of miRNA function is the suggested role of conferring robustness to a given cell's mRNA and protein profile (Stark *et al*. 2005) and is accomplished by providing alternative molecular networks that can contribute to the mRNA abundance and protein production. With respect to embryogenesis, miRNA functions are strategically involved with cell fate and are required for cell lineage destinations. This is evidenced by the tissue specificity of many miRNAs and is further supported by the fact that genes associated with tissue specific Gene Ontology (GO) categories have 3′UTRs enriched for miRNA binding sites whereas genes associated with non-tissue specific GO categories lack significant miRNA complementation sites in the 3′UTR (Beuvink *et al*. 2007; Kawahara *et al*. 2007; Wang *et al*. 2007; Zhao *et al*. 2007).

Importance of miRNA function for oocyte and early embryonic development

Successful embryonic development in mammals requires broad translational arrest and mRNA clearance to deplete maternally derived mRNAs and proteins in coordination with ZGA and protein production; as shown in the pig (Schoenbeck *et al*. 1992). Maternal depletion of mRNA is in part controlled via the 3′UTR of the expressed transcripts (Brevini *et al*. 2007). So it is not surprising that differential expression of microRNAs is temporally associated with oocyte maturation and early embryonic development in a variety of species (Biemar *et al*. 2005; Watanabe *et al*. 2005; Giraldez *et al*. 2006; Tang *et al*. 2007).

The 3′UTR repertoire of the mRNAs present in porcine oocytes suggests a potential role during oocyte maturation and embryonic development. In addition the presence of DICER1 mRNA in GV oocytes at 2.3 times the abundance after ZGA suggests biological importance (Whitworth *et al*. 2005). Biological importance of DICER1 is demonstrated by the ability of long double-stranded RNAs injected into porcine zygotes to induce knockdown of corresponding target mRNA (Cabot & Prather. 2003). The required role of DICER1 function for successful development (Tang *et al*. 2007) suggests that specific mature miRNAs are responsible for PTGS necessary for embryo survival. While the average miRNA is estimated to have recognition sites for approximately 100 target mRNAs, specific individual miRNAs have the predicted ability to confer PTGS on a group of genes varying from only a few to more than 800 (Rajewsky 2006). Thus the alteration of only a few miRNA during embryonic development may contribute to large changes in transcript abundance during developmental progression or between embryo production methods (Whitworth *et al*. 2005). The loss of developmental capacity in mouse oocytes lacking functional DICER1 during oogenesis is associated with disorganized spindles, lack of chromosome alignment, as well as transcriptome and proteome alterations (Tang *et al*. 2007) . In addition to DICER1 function being required for oocyte maturation and fertility, the maternal expression of EIF2C1, an RNA binding protein essential to the formation of RNA-induced silencing complexes, is required for early embryonic development in the mouse (Lykke-Andersen *et al*. 2008). Loss of function of EIF2C1 results in the stabilization of specific maternal mRNAs and arrested development during ZGA. EIF2C1 mRNA levels in the pig GV oocyte are 10- to 13-fold higher than in a reference sample (Whitworth *et al*. 2005)

The utility of understanding transcript population changes during embryo development

At least three major benefits are derived from understanding alterations in transcript abundance in the early embryo. The first is the possibility that such information can be used to improve reproduction by explaining the ~30% loss of potential conceptuses that occurs during the first month of development. Toward that end, we have been engaged in EST projects to identify genes in reproductive tissues and early embryos of pigs (Jiang *et al*. 2001; Jiang *et al*. 2004; Whitworth *et al*. 2004). Through these efforts many tissue-specific and novel (previously uncharacterized) genes were identified. Follow-up transcriptional profiling experiments with cDNA microarrays confirmed the involvement of many of these genes in embryo development, as well as providing a better understanding of the genetic pathways that are involved in embryo development (Whitworth *et al*. 2005). Secondly, such information can provide clues to improve assisted reproductive technologies, such as altering culture conditions to improve fertilization parameters (Hao *et al*. 2006). A third benefit is the opportunity to identify gene products that are predicted to be secreted into the culture medium. If such products happen to be correlated with developmental competence, they provide a potential means to non-invasively screen *in vitro* produced embryos to better define their quality.

Identification of transcribed genes that encode secreted molecules that are higher in *in vivo* produced embryos compared to *in vitro* produced embryos (Whitworth *et al*. 2005) may permit identification of molecule(s) that could be correlated with developmental completence. Several abundant and/or differentially transcribed genes were identified that are candidate markers because many:

- possess enzymatic activity that can be detected (e.g. PAGs, cathepsin D and ADAMs),
- possess enzyme *inhibitory* activity (e.g. Bikunin) that can be quantified,
- possess a defined biological activity that can be measured (e.g. IFN-gamma),
- exhibit relatively specific binding characteristics that might be exploited (e.g. Galectin 1 and IGFBP7), and
- are known to be bound by antibodies (e.g. CD9 antigen, Relaxin, Laminin receptor) that could be used to measure marker release.

Our group is currently working to determine if the data generated from transcriptional profiling can be leveraged to identify markers, such as those above, that can serve as way to identify embryos with low or high developmental potential (e.g. Telugu *et al*. 2009).

Conclusion

Control of development through the cycle of embryonic cells to somatic and germ cells is incredibly complex. Early thought was focused on transcriptional control of development. Now we have learned that transcriptional control is just one component. The factors that regulate transcription are very complex (histone modifications, DNA methylation) and post-transcriptional controls (miRNAs) appear to be just as important as transcriptional regulation alone. Nevertheless, a more complete understanding of the global control of differentiation will provide tools to improve the efficiency of *in vivo* processes. These efforts will also lead to improvements in systems of embryo production and will likely lead to the identification of factors that can be used to predict the developmental competence of embryos and gametes.

Acknowledgements

The authors wish to acknowledge the support of the National Institutes of Health through the National Center for Research Resources (RR018877, RR013438), the USDA NRI (2006-35203-17282, 2004-35205-15549, 2008-35205-05309), and Food for the 21st Century at the University of Missouri.

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Ovarian responses to lactation management strategies

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A number of lactation management strategies can be applied to reduce negative effects of lactation on post-weaning fertility. This paper focuses on effects of lactation length, Intermittent Suckling and Split Weaning on follicle development and subsequent oestrus. It is concluded that a lactation length of less than 3 weeks still leads to suboptimal reproductive performance in our modern sows. Further, both Intermittent Suckling and Split Weaning stimulate lactational follicle development and oestrus, but the variation in response between sows still limits practical application.

Introduction

During lactation, both the suckling intensity of the piglets and the negative energy and/or protein balance of the sows normally inhibit the growth of pre-ovulatory follicles and therefore, prevents the occurrence of lactational oestrus. Gonadotrophin release and thus follicle development can be suppressed to such an extent that post-weaning follicle development and subsequent fertility (weaning-to-oestrus interval, ovulation rate, embryo survival and subsequent farrowing rate and litter size) are negatively influenced too. This is most obvious in first litter sows, but is also observed in older parity sows with extensive lactational weight loss (Thaker & Bilkei 2005). A number of lactation management strategies can be applied to reduce negative effects of lactation on post-weaning fertility: optimizing lactation length, Intermittent Suckling (i.e. temporary separation of sows and piglets during the last days of lactation) and Split Weaning (i.e. a reduction of litter size during the last days of lactation). Before reviewing these strategies, a short overview is given of the mechanisms leading to lactational suppression of follicle development.

Lactational suppression of follicle development

This paper briefly describes the most important factors that affect follicle development during lactation, as Quesnel (2009) in this volume focuses on lactational and nutritional influences on follicle development.

Antral follicle development depends on the gonadotrophins LH and FSH. During established lactation, LH-levels and pulsatile LH release are suppressed due to the suckling-induced inhibition of the GnRH-pulse generator (De Rensis *et al*. 1993). The level of LH-suppression is related to the suckling intensity, but also to the negative energy balance of the sows; in

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primiparous sows on a low feeding level, LH-levels were reduced compared to sows on a a high feeding level (e.g. (Quesnel & Prunier 1998; Van Den Brand *et al*. 2000). Effects of lactation on FSH are less consistent, and seem more dependent on ovarian negative feedback (inhibin) than on suckling (reviewed by (Prunier *et al*. 2003).

In the course of lactation, LH-pulsatility normally restores (e.g. Van Den Brand *et al*. 2000), which may be related to a decrease in suckling frequency and intensity or to the increase in pituitary LH response to GnRH (e.g. Bevers *et al*. 1981; Rojanasthien *et al*. 1987). Concomitantly with the increase in LH-pulsatility, follicle size increases in the course of lactation. Thus, with progressing lactation, the follicle pool on the ovaries achieve a greater diameter, but most sows do not develop follicles beyond 3-4 mm until after weaning (Lucy *et al*. 2001), although occasionally sows develop pre-ovulatory follicles (~8mm) and ovulate during lactation.

The inhibition of LH release during lactation influences both lactational follicle development and the resumption of ovarian activity after weaning (Shaw & Foxcroft 1985, Quesnel *et al*. 1998). Additionally, the positive feedback mechanism to oestradiol matures in the course of lactation, increasing the ability of sows to mount a pre-ovulatory LH-surge of sufficient magnitude (Sesti & Britt 1993). These mechanisms together form the basis for lactational effects on subsequent fertility parameters, such as: weaning-to-oestrus interval, ovulation rate and even embryo survival (reviewed by Prunier *et al*. 2003), eventually affecting farrowing rate and litter size.

Lactation management strategies

To counteract negative consequences of lactation on subsequent fertility, several management strategies can be applied. These strategies aim to improve the energy balance of the sows and reduce the intensity of suckling in the last part of lactation either by reducing litter size (Split Weaning) or by limiting the period of suckling (Intermittent Suckling). Since lactation length in itself is a major determinant of post-weaning ovarian activity, it is discussed first.

Lactation Length

In 1982, Varley reviewed effects of day of weaning on subsequent reproductive functioning. He concluded that the weaning-to-oestrus interval was around 7 days when sows were weaned at 3 weeks or beyond, but increased when sows were weaned at less than 3 weeks of lactation. Further, lactation length did not seem to influence ovulation rate or fertilization rate, but embryo mortality around the time of implantation seemed to increase in sows with lactation lengths shorter than 24 days. As a result, subsequent litter size was reduced substantially. The negative consequences of short lactation lengths on litter size were attributed to the compromised uterine development after weaning.

Since the review by Varley (1982), sows have changed substantially. Due to genetic selection, current sows have a higher percentage of lean muscle tissue, a lower level of backfat and improved reproductive performance as shown by the lower percentage of sows with delayed weaning-to-oestrus intervals and increased litter size. So, how does lactation length affect reproductive functioning in modern sows?

Hardly any studies have investigated immediate post-weaning reproductive physiology of sows in relation to lactation length. An interesting exception is the study by (Willis *et al*. 2003) who compared sows weaned at Day 14 with sows weaned at Day 24 of lactation. They found a lower LH-pulsatility pre- and post-weaning, consistent with an HPO that is not fully recovered at Day 14 of lactation. They also found higher FSH levels and a delayed increase in post-weaning oestrogen levels with weaning at Day 14, consistent with a suppressed follicular development at Day 14.

How does suppressed follicle development at weaning affect further reproductive functioning? A few studies have analysed farm data on sow performance in relation to lactation length. These studies show that the weaning-to-service interval is consistently short when lactation length is beyond 21 days, but weaning-to-service interval increases after shorter lactation lengths (see Fig. 1). In sows with short lactations that did not show oestrus by day 6 after weaning, Knox & Rodriguez Zas (2001) consistently found smaller follicles at weaning and at 3 days after weaning, suggesting a suppressed follicle development in these sows.

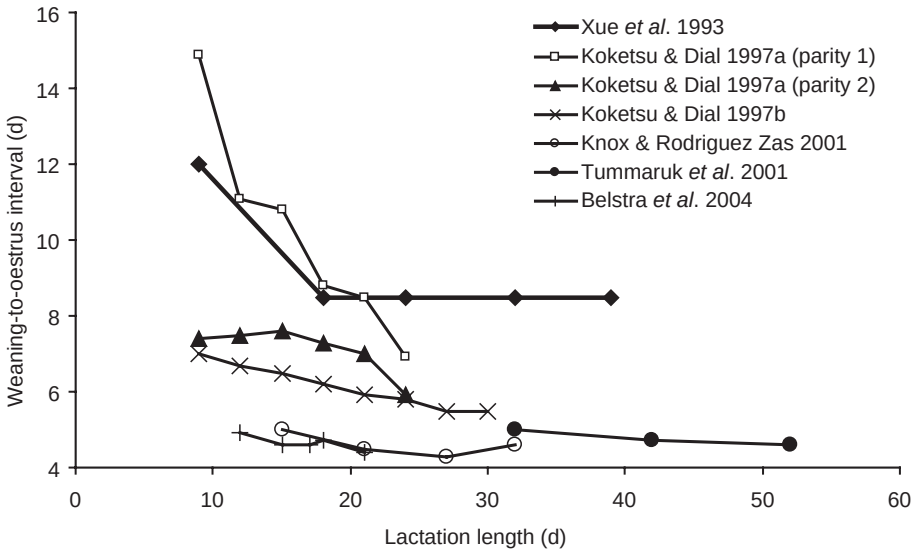


Fig. 1 Effect of lactation length on weaning-to-oestrus interval NB Knox and Rodriguez Zas (2001): only sows showing oestrus within 8 days from weaning. The percentage of sows showing oestrus within 8 days was 35%, 94%, 98% and 96%, for the shortest to longest lactation length classes; Tummaruk *et al*., (2001): sows with oestrus within 20 days [90.3%; percentages not shown per lactation length class]; Belstra *et al*., (2004): only sows showing oestrus within 10 days from weaning. The percentage of sows showing oestrus within 10 days was 92%, 92%, 90%, 94% and 98% for the shortest to longest lactation length classes.

Also when oestrus is not delayed, reproductive processes may still be affected. For example, Knox & Rodriguez Zas (2001) found that in sows with short lactation (< 16d), the percentage of sows showing oestrus was reduced (35%), and also the percentage of these sows that ovulated (78%) compared to sows with a longer lactation length (e.g. for sows with a lactation length of 25-31 d: 98% showed oestrus, of which 98% ovulated). The sows that failed to ovulate either showed a short/intermittent oestrus with only small or medium sized follicles or ovarian cysts at that time or had a long oestrus period with normal sized pre-ovulatory follicles that did not ovulate within the first 5 days of oestrus. In ovulating sows, lactation length did not affect follicle size at ovulation. Thus, even when oestrogen production of sows with short lactations is sufficient for oestrous behaviour, the immaturity of the positive feedback system may prevent/ disable the occurrence of an LH-surge (Sesti & Britt 1993), and consequently, these sows fail to ovulate. If the LH surge fails in sows with normal sized pre-ovulatory follicles, follicles may become cystic. There are no recent reports outlining the influence of lactation length on the development of cystic follicles or cystic ovaries; Svajgr *et al*. (1974) found that the number of cystic follicles doubled for sows with a lactation length of 13d compared to 24d (1.3 vs 0.6). Consistent with the earlier review by Varley (1982), Willis *et al*. (2003) did not find effects of lactation length on ovulation rate.

A possible disadvantage of longer lactations for farm reproductive parameters can be the increase in the number of sows with lactational ovulation. This may especially occur in multiparous sows from specific prolific breeds, but may occur in any sow with a low number of suckling piglets or a high feed intake during lactation. Lactational ovulation is often not noted and, such sows will thus be marked as 'delayed oestrus'.

Lactation length may also affect subsequent farrowing rate and litter size, as reviewed by Varley (1982). Also in more recent literature, short lactations (less than 3 weeks) negatively affect subsequent litter size and farrowing rate (e.g. Koketsu *et al.* 1997, Le Cozier *et al.* 1997). Further, the limited information that is available on reproductive performance for long lactations seems to indicate a positive influence of lactation lengths above 4 wks, both for farrowing rate (+3%) and litter size (+0.6 piglets) (Gaustad-Aas *et al.* 2004), although such effects are not always found (Tummaruk *et al.* 2001) and may not result in a higher number of piglets per sow per year (Xue *et al.* 1993).

Summarising, especially short lactation lengths (<3wks) have a clear negative effect on post-weaning follicular development and subsequent interval to oestrus, ovulation response and even farrowing rate and litter size. These effects are related to the level at which lactation has suppressed follicle development. Effects of short lactation lengths on weaning-to-oestrus interval seem less evident in recent literature (see Fig. 1), although the true effect is masked by the relatively short cut-off points (e.g. oestrus up to 8 days from weaning (Knox & Rodriguez Zas 2001) and oestrus up to 10 days from weaning (Belstra *et al.* 2004). Nevertheless, such reduced effects of short lactation lengths on weaning-to-oestrus interval may be related with the ongoing selection for short weaning-to-oestrus intervals. However, if this means ovulation of less developed follicles and oocytes, such sows may still have lower subsequent performance.

Intermittent Suckling

One way to reduce the suckling stimulus of the piglets and as such stimulate follicular development and subsequently lactational ovulation, is by introducing daily periods of separation of sows and piglets during the last part of lactation. This procedure is termed reduced or limited suckling, interrupted suckling, or Intermittent Suckling (IS). Recently, Langendijk *et al.* (2006) and Gerritsen *et al.* (2008b) reviewed effects of Intermittent Suckling on subsequent fertility. They showed that the enormous variation in (lactational) oestrous response of sows in the different Intermittent Suckling regimes can be attributed to the regimes used, but also to differences between sows. In short, up to 90% of sows may show lactational oestrus if the Intermittent Suckling does not start too early in lactation (preferably later than Day 18) and lasts for at least 10h per day. During separation, sows should be housed out of sight and hearing from the piglets, preferably allowing some boar contact. The genotype of the sow is of major importance and also the parity, since primiparous sows have a lower oestrous response.

Interestingly, sows that respond to the treatment with oestrus do so in a synchronous fashion at a seemingly normal interval of 4 to 5 days from start of treatment (see Gerritsen *et al.* 2008b). Extending the duration of the treatment period does not seem to influence the oestrous response. As a consequence, a treatment period of 14d compared to 7d (Soede *et al.* 2009) did not significantly increase the oestrous response. Interestingly, in that study, the sows that did not show a lactational oestrus within the period that they were subjected to IS, showed a 'normal' weaning-to-oestrus interval once they were weaned. Thus, the oestrous response of sows in an Intermittent Suckling regime seems to be an 'all or none' phenomenon; either a 'normal' duration of the follicular phase, or no response, as was also concluded by Stevenson & Davis (1984).

Even though a considerable number of sows may show lactational oestrus in an Intermittent Suckling regime, other sows show varying patterns of response in terms of follicle development. In the experiments of Gerritsen *et al*. (2009); Gerritsen *et al*. (2008a) and Langendijk *et al*. (2009), ultrasound was performed daily to check the ovarian response to the Intermittent Suckling regimes. The following follicle development patterns could be distinguished; sows showing follicular growth with follicle diameters not reaching pre-ovulatory size (<6mm) and sows developing follicles of pre-ovulatory size (>6mm). Within the second category, follicles either ovulated, regressed or became cystic (>10mm, not ovulating). Fig. 2 shows the patterns of follicle development for each of these categories. Table 1 shows the number of sows for each of these ovarian response for weaned control sows (Day 21 of lactation) and for sows in which Intermittent Suckling started at Day 14 or at Day 21 of lactation. Failure of sows to develop follicles beyond 6 mm was particularly observed when IS commenced early in lactation (13% for D14 vs 0% for d21), illustrating the recovery of gonadotropic secretory capacity during this period of lactation. Sows with follicular development beyond 6 mm, mostly ovulated and showed oestrus. However, in some sows follicles regressed without ovulation (Table 1). This phenomenon (pre-ovulatory sized follicles regressing) has, to our knowledge not been described before and has not been observed before by our research group. FSH and LH release on the first day of IS did not seem limiting for normal follicle development in these sows (Langendijk *et al*. 2009). Nevertheless, the follicles in these sows were anoestrogenic (no increase in peripheral oestradiol levels) despite an increase in follicle diameter. In sows ovulating normally, an increased concentration of oestradiol was observed from day 2 of IS. We suggest that the follicles that regressed were not fully responsive to the increased FSH and LH secretion after commencement of IS. Some sows that showed pre-ovulatory follicle development (>6mm) developed cystic follicles. Particularly sows that commenced IS at 14 d of lactation were prone to show this deviation in the follicular phase: in 13 % of all D14 sows subjected to IS (vs 0% for D21) all follicles turned cystic, or some follicles became cystic with the other follicles luteinising and forming corpora lutea. The sows with all follicles turning cystic had a normal follicular phase oestrogen rise, but had a very low or no LH surge and (consequently) no signs of luteinisation (no drop in oestradiol, no rise in progesterone), see Gerritsen *et al*. (2008b).

Table 1. Follicle development[1], ovulation and oestrus response [between brackets] in Intermittent Suckling regimes with 12h daily separation starting at day 14 (IS14) or day 21 of lactation (IS21)[2]

	Treatment		
	Control[3]	IS14	IS21
	N = 36	N = 79	N = 23
Follicular development remained <6mm	0% [0%]	13% [0%]	0% [0%]
Follicular development reached >6mm			
Ovulation within 8d after start IS/wean	94% [a] [100%]	66% [b] [90%]	87% [b] [95%]
No ovulation			
Regression of follicles	0 [0%]	9% [0%]	13% [100%]
Cystic follicles	6% [100%)	13% [80%]	0

[1]Daily ultrasound from onset of IS or from weaning onwards
[2] Results are based on (Gerritsen et al. 2009; Gerritsen et al. 2008a; Langendijk et al. 2009);
[3] Control sows weaned at Day 21 of lactation
[ab] $P < 0.05$

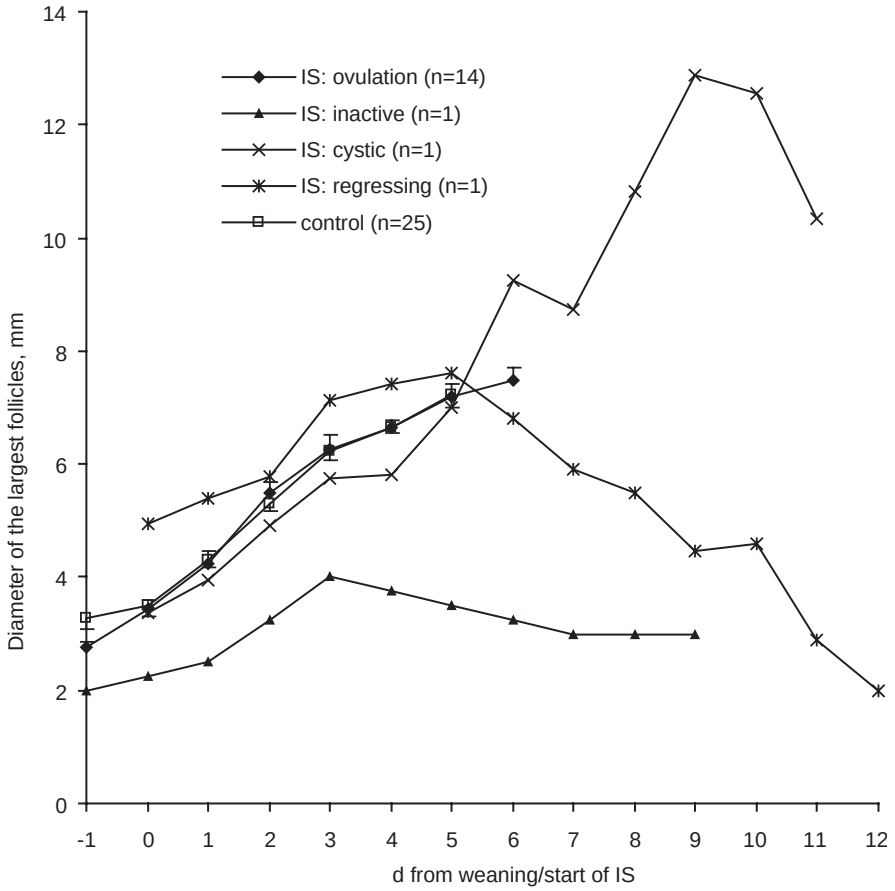


Fig. 2 Different patterns of follicle development in sows during the last day of full lactation (full lact) and after subsequent weaning (Control) or onset of Intermittent Suckling (IS). In IS, the majority of sows the follicles reached ovulatory size (>6mm) and subsequently ovulated (IS:ovulation), but in others follicles never reached ovulatory size (IS:inactive, one example sow shown) or did reach ovulatory size and either developed cystic ovaries (IS:cystic, one example shown) or follicles regressed (IS: regressing, one example shown). [Langendijk, unpublished results].

This again illustrates that during early lactation, the postive feedback of oestradiol on the LH surge centre is still developing or there is insufficient LH to induce an adequate LH surge.

In the early studies on Intermittent Suckling, the majority of sows ovulated after weaning. Thus, from those studies it remains unclear if and how Intermittent Suckling and/or lactational inseminations affect pregnancy. Recent Intermittent Suckling studies (reviewed by Gerritsen *et al.* 2008b), suggest that pregnancy rate, embryo survival rate and embryo development are negatively affected if IS-induced ovulation takes places around Day 19-21 after farrowing (IS started at Day14) and IS continues after ovulation, which may be related with lower progesterone levels in these sows. When sows are inseminated more than 3 weeks after farrowing, neither litter size, nor farrowing rate are negatively affected by lactational inseminations in an IS-regime (Soede *et al.* 2009) or during 'normal' lactation (Gaustad-Aas *et al.* 2004).

Summarizing, recent studies with Intermittent Suckling show that there is a large variation in oestrous response, in which specifically the onset of treatment, the genotype of the sows and parity of the sows is important. The oestrous response is an 'all or none' phenomenon, which means that sows either show oestrus after a follicular phase of normal duration or do not respond at all and show a normal return to oestrus after weaning. The majority of sows that show oestrus, normally ovulate with a normal ovulation rate, even though in some sows follicles may become cystic. When sows ovulate during an intermittent regime, they have a similar chance of becoming pregnant and remaining pregnant and a similar litter size compared to sows that are inseminated post weaning, but the negative effects of short farrow-to-insemination intervals also hold for Intermittent Suckling.

Split weaning

With Split Weaning, the suckling stimulus of the piglets is reduced and the energy balance of the sows is improved by the permanent removal of a part of the litter a few days before complete weaning. In the different studies reported, there was a considerable variation in the Split Weaning regimes; Split Weaning lasted for 3 to 7 days before complete weaning, leaving 2 to 6 piglets for the remaining lactation which lasted 17 to 35 d. Matte *et al*. (1992) reviewed the reproductive performance of split wean sows and concluded that the reduction in the interval from weaning to oestrus was mostly affected by the number of piglets that remained with the sow during the last days of lactation. The largest reduction in weaning-to-oestrus interval was seen when only 3 piglets remained with the sow for 4.7 days. Interestingly, Grant (in: Varley & Foxcroft 1990) showed that effects of Split Weaning (leaving 5 piglets with the sow) on both LH levels and follicle development after 7 days of treatment were larger when the 6 anterior teats had been covered compared to when only Split Weaning was applied. Since piglets had a similar growth rate in both treatments, and thus the metabolic demands on the sows were similar, it appears that effects on LH and follicle development are primarily mediated by the neural stimulation of suckling. Only few experiments have since evaluated effects of Split Weaning on reproductive physiology or performance of sows and these later experiments have all focused on the lower parity sows (Mahan 1993; Vesseur *et al*. 1997; Zak *et al*. 2008; Foxcroft et al. unpublished results). These studies also consistently show that the weaning to oestrus/insemination interval is reduced when Split Weaning is applied. Two recent studies have evaluated the role of the gonadotrophins in this effect. Degenstein *et al*. (2006) showed that during the whole period of Split Weaning (day 16-19), prolactin levels were reduced and FSH levels were increased. Further, Zak *et al*. (2008) found an acute increase in LH levels and LH pulse frequency in the 10h following Split Weaning at day 18. At the day of weaning (3 d later), average LH levels were similar to the control animals, both before and after final weaning. The day after weaning, split wean sows had more follicles with a diameter larger than 3 mm. Thus, the increased LH levels, the higher FSH levels during the split-wean regime and possibly the lower prolactin levels together stimulate follicle development and shorten weaning-to-oestrus intervals.

As far as we know, hardly any information is available on the quality of ovulation in split weaned sows. However, the reproductive characteristics that have been measured show a slightly increased ovulation rate (17.7 compared to 15.5 (Zak *et al*. 2008)), a similar embryo survival rate at day 28 (64% versus 60% (Zak *et al*. 2008)); similar subsequent litter size (Vesseur *et al*. 1997), and increased farrowing rate in parity 2 sows (97% versus 86% (Vesseur *et al*. 1997)).

Thus, removing the majority of the litter for the last 3-5 days of lactation consistently shortens the weaning-to-oestrus intervals through a stimulation of follicular development, primarily by a reduction in suckling stimuli. The improvement in weaning-to-oestrus intervals is only seen in groups of sows with an otherwise extended weaning-to-oestrus interval. Further, the few papers that have investigated Split Weaning effects beyond oestrus are not very conclusive. Therefore effects on subsequent fertility remain unclear.

Table 2. Oestrus response in recent Split Weaning studies

Split wean (d before weaning)	Litter size before split-wean	Remaining piglets	Lactation length (d)	Parity	WOI[1] (d) (compared to Controls)	Source
7	9.8	4	30	1+3	3.1 (C: 6.6)[2]	(Mahan 1993)
7	10.3	6	28	1	5.6 (C: 5.9)[3]	(Vesseur *et al.* 1997)
7	10.5	6	28	2	4.6* (C: 5.4)[4]	(Vesseur *et al.* 1997)
3	9.6	4 (lightest)	21	1	4.6* (C: 5.7)	(Zak *et al.* 2008)
3	NM	5 (lightest)	19	1+2	4.9 (C: 5.2)	Foxcroft *et al.* (unpublished)

* $P<0.05$, NM not mentioned
[1] WOI = Weaning-to-Oestrus interval
[2] Control sows were weaned at Day 23
[3] of sows that showed oestrus within 15d after weaning (52% and 58%)
[4] of sows that showed oestrus within 15d after weaning (62% and 63%)

Concluding remarks

As shown above, lactation management strategies such as Intermittent Suckling and Split Weaning may stimulate lactational follicle development and thereby postweaning oestrus onset by the combined effects of a reduced suckling intensity (most important) and improved energy balance of the sow. However, the benefits seem rather marginal in our modern sows that have been selected for short weaning-to-oestrus intervals and still not all sows will respond to the treatment. Concerning effects of lactation length, also for our modern sows, a 3-week lactation still seems to be required for optimal post-wean performance.

Besides applying optimal lactation management strategies, negative consequences of lactation on post-weaning fertility can also partly be counterbalanced by optimizing post-wean management, e.g. by boar stimulation and nutritional management. However, since the post-weaning follicular period is normally very short, even optimal post-wean conditions may not be sufficient to optimize post-lactional fertility. Another possibility would be to allow sows to recover from lactation by postponing post-wean oestrus and insemination e.g. by short term use of a progesterone analogue.

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Studies on fixed-time ovulation induction in the pig

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A technology that allows for manipulating of oestrus and ovulation, and would then also allow for fixed-time insemination, can be of great benefit for swine farms that operate using sow batch management due, at least in part, to savings in labour and the production of large batches of evenly developed pigs. Thanks to the current knowledge on endocrine regulation of follicle development and ovulation, and the availability of numerous reproductively active substances such a technology is now available. It covers procedures for synchronising oestrus based on the use of altrenogest in gilts and of batch-wise weaning in sows, for stimulating follicle development using eCG and for inducing of ovulation using hCG or LH as well as GnRH analogues. While the procedures for oestrus synchronisation stand alone, other procedures require additional treatments. If fixed-time insemination is the goal, oestrus needs to be synchronised and follicular development and ovulation induced by the use of GnRH analogues and hCG with ovulation occurring within 36-42 hrs. It is a general recommendation to inseminate those animals twice, i.e. 24 and 40 hrs after ovulation induction. However, the aforementioned technology requires healthy animals and a solid management and cannot be used to compensate for poor management.

Introduction

More than half a century ago research efforts were made to synchronise the oestrous cycle and ovulation in pigs with the ultimate goal of fixed-time artificial insemination (AI). Tanabe *et al*. (1949) were the first to treat pigs with equine chorionic gonadotrophin (eCG) and sheep pituitary extracts in order to stimulate follicle development and ovulation. Further studies using methallibure to suppress follicular growth followed by treatment with eCG to stimulate follicle development and human chorionic gonadotrophin (hCG) for inducing ovulation (Polge & Day 1969), and those conducted by Hunter (1967,1974) on ovulation induction as well as on follicle and oocyte development were milestones in the development of procedures to manipulate the oestrus cycle in pigs. Results of these early studies facilitated Eastern-European, particularly East-German research efforts into those procedures starting in 1970 at a time when the farm inventories were growing and there was an increasing need for this kind of biotechnology for management purposes. The general approach was to manipulate female reproductive functions, such as follicular development as well as ovulation and parturition. However, the goal was to

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mimic what occurs physiologically in the female pig. The ultimate goal was to synchronise all reproductive processes with the advantage of periodic and batch-wise AI, parturition as well as weaning, thereby enabling the practise of all-in-all-out and to produce large groups of pig in the same reproductive state with the same health and immunisation status. Indicative of the success made over the years in reproductive biotechnology in 1990, controlled reproduction was used on 86 % of the 1.1 million breeding sows and gilts in East-Germany. It has been learned that research into and the practical use of biotechnological procedures requires understanding of reproductive processes and the availability of appropriate substances (i.e. hormones or hormonally active substances). While those substances are available in general to manipulate almost all key reproductive processes in the female pig, they are not equally available since their use in practice requires national approval. As compared to Europe, hormones or hormonally active substances are still rarely used in the North and South American as well as the Asian swine industry. However, during the last decade due to increased costs for labour, feed and energy resulted in new interest in using those substances that allow for manipulating reproductive processes in the female pig.

Follicle development and ovulation

Follicular development in gilts and sows has been reviewed in detail elsewhere (Prunier & Quesnel 2000, Schwartz *et al*. 2008). In gilts the follicle cohort destined to ovulate is stimulated by increased post-ovulatory FSH, but under the influence of the high luteal progesterone (P4) concentration during dioestrus follicles do not grow to ovulatory size. Only after luteolysis when P4 is low and thus does not negatively feedback on gonadotrophin synthesis, follicles finally grow from 4 mm to ovulatory size within 4-6 days. However, there is a well-defined balance between stimulatory (e.g. LH and FSH) and inhibitory (e.g. P4 and inhibin) factors that favours pre-ovulatory follicle development. As has been shown after gonadotrophin deprivation, FSH is necessary to support follicle development beyond 2-3 mm and LH beyond 4 mm (Driancourt *et al*. 1995). Once the follicles reach a larger preovulatory stage FSH declines (Guthrie & Bolt 1990) and LH pulse secretion changes from luteal (high amplitude - low frequency) to follicular patterns (low amplitude - high frequency). Final stage of follicular development is associated with decreased FSH and increased LH receptor expression along with increased production of oestradiol, as well as increased fluid accumulation within the follicular cavity. Increased pre-ovulatory oestradiol finally elicits the GnRH-mediated LH release from the pituitary, thereby initiating ovulation and the release of a mature oocyte.

Sows commonly have a lactational anoestrus. During lactation and before weaning, follicles exhibit a wave-like pattern of growth, and emerge from a cohort of 20-30 follicles of 2 mm that grow to not larger than 5 mm (Lucy *et al*. 2001). This is because suckling inhibits secretion of GnRH and subsequently LH due to a concerted action of prolactin, oxytocin and endogenous opioid peptides that prevent final growth of these follicles to reach ovulatory size before weaning (Varley & Foxcroft 1990). Once the piglets are weaned, follicles start to grow to 7-8 mm before ovulation. Initially FSH increases and then decreases at weaning. Basal LH and LH pulse frequency increases; these are key regulators of post-weaning follicle development which affect the weaning to oestrus interval (van de Brand *et al*. 2000). Furthermore, adrenal hormones and nutritional mediators such as glucose, insulin and free fatty acids, as well as endogenous opioids and leptin have been shown to have an influence on post-weaning follicle development and ovulation. In conclusion, follicle development including the final ovulatory follicle growth and maturation, as well as ovulation itself, requires fine-tuned hormonal events. Understanding these mechanisms is necessary in any attempt to manipulate the reproductive process in female swine.

Manipulation of follicle development and ovulation

Synchronisation of oestrus

In a batch farrowing system, oestrus in sows is naturally synchronised by weaning. Oestrous cycles of gilts need to be synchronised when they are introduced into such systems. For this purpose, protocols based on either suppression of follicle development and/or mimicking the luteal phase are available (reviewed by Estill 2000). Progesterone and its synthetic derivates did not prove fully effective for oestrus synchronisation. In contrast, non-steroidal substances such as methallibure (Aimax, ICI 33828, Suisynchron®) have been shown to yield good results, but were banned by US and European countries before being commercially available for the swine industry because of their teratogenic effects. In East-Germany, zinc-methallibure (Siusynchron®) was successfully used in hundreds of thousands of gilts between 1973 and 1989, but was then also banned and replaced by allyltrenbolone (Hühn *et al*. 1996). At present, allyltrenbolone (altrenogest, as Regumate® in Europe and Matrix® in North America) is the only licensed substance with progestagene type effects to be used in female pigs in Europe and in North America. If it is given orally and fed in a dose of 15 to 20 mg/day/gilt over a period of 14 to 18 days, it has been proven effective at suppressing follicle development. Recommendations on the daily dose of altrenogest and the duration of administration differ between countries. Production sites in France and also some in Germany prefer feeding 20 mg/day/gilt for 18 days (Martinat-Botte *et al*. 1990). Other sites in Germany use feeding altrenogest in doses of 16-20 mg/day/gilt over 15 days and achieved good fertility results (Hühn *et al*. 1996). For North America, the general recommendation is 15 mg/day/gilt for 14 days. Feeding of altrenogest for a shorter period is possible, if the stage of oestrous cycle is known at first administration (Kirkwood 1999). Gilts usually show oestrus within 5 to 7 days after altrenogest withdrawal (Martinat-Botte *et al*. 1990, Hühn *et al*. 1996). Other approaches have been to include progesterone or GnRH agonists in a slow release device during a defined time, in conjunction with hCG or oestrogen to extend the lifespan of the corpora lutea, and the 'breed-and-abort' procedure using $PGF_{2\alpha}$. However, all of the aforementioned procedures except those based on the use of altrenogest are not currently used in the swine industry.

Stimulation of follicular development

Though synchronisation of oestrus is possible, with weaning group of sows or treatment with altrenogest in gilts, the onsets of oestrus can however still spread over a week. This is due, at least in part, to insufficient follicle development. Gonadotrophins may then be used after weaning or after altrenogest in order to stimulate this follicle development and to achieve a better synchronisation effect.

Equine CG, which is a glycoprotein similar to equine pituitary LH, has been proven to have superior effects on follicle development in pigs. This gonadotrophin exhibits both LH- and FSH-like activities (Farmer & Papkoff 1979) with a FSH/LH activity ratio that differs between 0.14 to 0.31 as estimated by bioassays conducted in rats and mice (Bergfeld & Haring 1983). A different ratio of FSH *versus* LH activity may account for variations in the ovarian response seen in pigs after use of different eCG batches (Bergfeld & Haring 1983, Ciller *et al*. 2008), and should be considered when analysing the effectiveness of different treatment protocols that are based on eCG. As evident from numerous East-German experiments, 800 to 1,000 IU of eCG are most effective at stimulating follicle development in gilts and 600 to 1,000 IU eCG in sows. However, within- and between-farm differences occur, as indicated by variations in the ovarian response of sows that received the same eCG treatment but were located at different

farms. This observation needs to be considered when determining an optimal dose of eCG (Bergfeld *et al*. 1984). It is recommended to test different eCG dosages first and then pick the one that yielded the optimum fertility. Besides the dose, the time when treatment occurs with eCG affects follicle development. For instance, the application of eCG one day after, or the last day of methallibure feeding in gilts (Polge *et al*. 1968) or 48 *versus* 24 hrs after methallibure (Brüssow & Bergfeld 1984) was associated with an increased number of stimulated follicles and also with a lower dosages of eCG needed for stimulation. Whenever a protocol includes the use of eCG it needs to be evaluated, for example by evaluating the ovary at slaughter, laparotomy, laparoscopy and ultrasound.

Besides pure eCG, different combinations of the gonadotrophins eCG and hCG have been used for stimulation of follicle development and oestrus in gilts and sows (Estienne *et al*. 2001, Knox *et al*. 2001). However, using such combinations there is evidently the risk of inducing ovarian cysts and/or of premature luteinization of follicles. The latter is assumed due to the higher LH activity of those combinations compared to pure eCG, most likely as the result of the extra hCG (Bergfeld *et al*. 1982). Increasing eCG and hCG in a combination from 400 to 700 IU and from 200 to 350 IU, respectively, was associated with an increased number of gilts with cystic ovarian degeneration (36 % *versus* 88 %) and a decrease in the pregnancy rate (50 % *versus* 65 %). In contrast, when gilts were treated with 1,000 IU eCG, only 4 % of them developed ovarian cysts (Schlegel *et al*. 1978). Moreover, if 1,000 IU eCG was given to primi- and multiparous sows 24 hrs after weaning and compared to 400 IU eCG/200 IU hCG (Suigonan®, Intervet, Unterschleissheim, Germany), the pregnancy rates, litter sizes and piglet indices were all higher in sows that received eCG only (Barbe *et al*. 1997). Taken together the majority of the studies that were conducted in former East-Germany suggest that pure eCG stimulates follicle development with better fertility results than do combinations of eCG and hCG. This is certainly the reason why standardized, lyophilized eCG preparation (e.g. Pregmagon®; IDT Dessau, Germany) is given preference in the German pig industry for the purpose of stimulating follicle development in pigs. However, in the United States, a drug that combines 400 IU eCG and 200 IE hCG, i.e. PG 600 (Intervet Canada, Guelph, Ontario, Canada) is the preferred medication, and has been shown being effective for a multitude of reproductive purposes (Kirkwood 1999).

Synthetically produced GnRH agonists have been used to stimulate follicle development in pigs, and are considered as an alternative to eCG. Recently, a synthetic analogue of lamprey GnRH-III (Peforelin; Maprelin® XP10, Veyx-Pharma, Schwarzenborn, Germany), was shown to exhibit selective FSH releasing activity in barrows (Kauffold *et al*. 2005), has been used in primi- and multiparous sows to stimulate follicle development and oestrus, and yielded similar fertility results than eCG (Engl *et al*. 2006). However, a general recommendation for its broad use would greatly benefit from further endocrine studies and supportive field results.

Induction of ovulation

Though the onset of oestrus and to some extend also ovulation can be synchronised in both gilts and sows by using eCG, the time when ovulation occurs can be still extremely variable. If fixed-time insemination is the goal, ovulation needs to be induced, either by using gonadotrophins with predominately LH activity such as hCG or by using GnRH analogues. Human CG (Hunter 1967) or equivalent substances such as pituitary extracts (Tanabe *et al*. 1949), aiming to mimic the endogenous pre-ovulatory LH-peak, were effective in gilts at inducing ovulation which occurred at approximately 40-42 hrs after treatment. If animals are treated with eCG to stimulate follicle development and then followed by hCG treatment to induce ovulation, the

interval between both treatments is however crucial to the ovulation-inducing effect of hCG. Considering that hCG should be given as close as possible to the time when the endogenous LH peak occurs in order to induce ovulation. Indeed, when this interval has been set at 78-80 hrs after eCG and the responses compared to non-treated controls, treated animals ovulated more uniformly, with ovulations occurring 42-53 hrs after hCG, whereas no control animal ovulate during this time period (Bergfeld *et al.* 1976).

Porcine LH (pLH, Lutropin-V, Bioniche Animal Health, Belleville, Canada) has been shown to be as effective as hCG at synchronising ovulation in weaned sows (Cassar *et al.* 2005, Bennett-Steward *et al.* 2008). If eCG was given the day of weaning and pLH 80 hrs later, sows ovulated between 34-42 hrs post pLH (Cassar *et al.* 2005).

After GnRH, a decapeptide, was discovered and its structure known, the pharmaceutical industry started to synthesize GnRH and thus made it available for use in swine industry. In contrast to hCG, GnRH acts at the pituitary level and stimulates the release of endogenous LH, thereby approximating what is a more "biologically normal" event than does hCG. Once more the East-German pig industry took the initiative in the development of one of the first GnRH analogues, i.e. Gn-RH vet "Berlin-Chemie" for use in swine in the late 70s. Brüssow & Bergfeld (1979) and Bergfeld & Brüssow (1979) performed intensive studies on the effect of different doses of this GnRH analogue alone or in combination with hCG on ovulation and observed that, 900 μg of this GnRH analogue alone or different combinations of GnRH/hCG (100-300 μg GnRH/100-300 IU hCG) stimulate ovulation similar to what was achieved with only hCG.

Later, another GnRH agonist (D-Phe^6-LHRH, Gonavet®, Berlin-Chemie, Berlin, Germany) was demonstrated to be even more effective at synchronising ovulation in swine than Gn-RH vet "Berlin-Chemie". As observed by repeated laparoscopy (Brüssow *et al.* 1990), in gilts treated with 50 μg D-Phe^6-LHRH started ovulating 35.5 $\pm$ 2.7 hrs after treatment and finished ovulation on average 5.9 $\pm$ 1.7 hrs later. However, animals varied in their response to D-Phe^6-LHRH, with variations being related to the interval between the GnRH injection and the LH peak, the maximum of the LH peak and the overall time needed for ovulation (Table 1). Typical LH secretion patterns observed in gilts after GnRH (Gonavet®) treatment are shown in Fig. 1.

Table 1. Effects of 50 μg D-Phe^6-LHRH (Gonavet®) on LH and ovulation in gilts (from Brüssow *et al.* 1994)

Criteria	Early* responder (n =6)	Medium* responder (n = 6)	Late* responder (n = 4)
GnRH – LH surge (hrs)	2.2 $\pm$ 0.4^a	5.0 $\pm$ 1.0^b	9.0 $\pm$ 1.0^c
LH maximum (ng/ml)	19.6 $\pm$ 9.8^a	7.3 $\pm$ 3.2^b	3.8 $\pm$ 0.8^c
GnRH – commencement of ovulation (hrs)	35.6 $\pm$ 2.4^a	34.6 $\pm$ 2.5^a	39.6 $\pm$ 1.5^b
GnRH – completion of ovulation (hrs)	39.0 $\pm$ 1.8^a	37.2 $\pm$ 2.5^a	42.0 $\pm$ 1.5^b
LH peak - commencement of ovulation (hrs)	33.4 $\pm$ 2.5	29.4 $\pm$ 3.9	30.6 $\pm$ 0.6
LH peak – completion of ovulation (hrs)	35.8 $\pm$ 1.7^a	32.2 $\pm$ 2.7^b	35.5 $\pm$ 3.8
Duration of ovulation (hrs)	3.6 $\pm$ 2.3	2.8 $\pm$ 1.7	2.4 $\pm$ 0.2

^{a,b} $p < 0.05$,

* Animals were classified as early, medium and late responder according to their LH peak as response to GnRH.

Field trials involving a total of 2,744 gilts that were all injected with 50 μg D-Phe^6-LHRH (Gonavet®) 78-80 hrs after 1,000 IU eCG and artificially inseminated twice at fixed times, i.e. 24 and 40 hrs after GnRH, demonstrated that D-Phe^6-LHRH yields superior fertility results than if ovulation was induced with hCG. A similar observation has been made with 71,600 sows that were treated with 50 μg D-Phe^6-LHRH compared to 300 μg GnRH/ 300 IU hCG combination 55-58 hrs after eCG and artificially inseminated twice at 24 and 42 hrs after GnRH (Brüssow

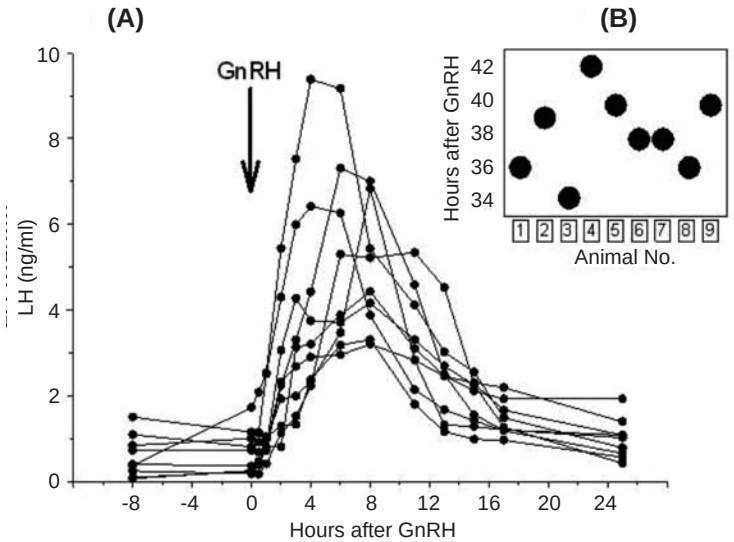


Fig. 1 LH response (A) and time of ovulation (B) in gilts (n = 9) treated with 50 μg D-Phe^6-LHRH (Gonavet®) 80 hrs after eCG

et al. 1996). Other GnRH analogues that have been tested for ovulation induction in swine are buserelin (Möller-Holtkamp *et al*. 1995), goserelin (Brüssow *et al*. 2007) and triptorelin (Taibl *et al*. 2008). All of them are effective at stimulating pre-ovulatory LH secretion in both gilts and sows. Currently however, D-Phe^6-LHRH is still the only GnRH analogue that has been licensed for use in swine in a number of European countries including Germany. The intravaginal application of GnRH containing gel (Baer & Bilkei 2004, Taibl *et al*. 2008) has been tested for the purpose of ovulation induction, but did not reach a stage beyond research. Another neuronal peptide, kisspeptin, which is a product of the KISS1 gene (Kotani *et al*. 2001) and synthesised as a preprohormone, has gained increasing interest lately since it is involved in the regulation of GnRH release, and if given to female pigs has been shown to stimulate LH release in a dose dependent manner (Lents *et al*. 2008). Though kisspeptin is thus a promising candidate to induce ovulation in pigs the question of whether or not it will ever be used in the pig industry for this purpose as part of a fixed-time insemination protocol needs further investigation.

Protocols for fixed-time ovulation and insemination in practice

The German pig industry has a long and strong history of using biotechnology in pig reproduction. Supported by continuing research efforts, the industry has many years experience with this type of technology (Hühn *et al*. 1996, Brüssow *et al*. 1996), and has thus been able to recommend different protocols that can be used for oestrus synchronisation and fixed-time insemination in pigs (Table 2). However, other approaches are also possible such as the one reported recently and performed with 3,000 sows on commercial farms in Canada (Cassar *et al*. 2005, Bennett-Steward *et al*. 2008). In these studies, sows were injected with 600 IU eCG at the day of weaning and were given 5 mg pLH 80 hrs later. They were then inseminated at fixed-times 36 and 44 hrs after pLH. Compared to untreated controls, the farrowing rate of treated sows was significantly increased (86 % *versus* 69 %).

Table 2. Treatment protocols for ovulation induction and fixed-time insemination in gilts and sows as recommended for use in practice based on experience from the East-German swine industry

Method	Gilts	Sows
Synchronisation of oestrous cycle	18 days oral application of altrenogest (20 mg/gilt/ day) (08:00 h)	Lactation until weaning
	15 days oral application of altrenogest (16 mg/gilt/day) (08:00 h)	
Stimulation of follicle development	800 – 1,000 IU eCG 24 hrs after last altrenogest (08:00 h)	*Primiparous sows* 1,000 IU eCG 24 hrs after weaning (08:00 h)
		Multiparous sows 600 – 800 IU eCG 24 hrs after weaning (08:00 h)
Induction of ovulation	GnRH* or hCG** 78-80 hrs after eCG (14:00-16:00 h)	*Lactation > 4 weeks* GnRH* or hCG** 56-58 hrs after eCG (16:00-18:00 h)
		Lactation 4 weeks GnRH* or hCG** 72 h after eCG (16:00 h)***
		Lactation 3 weeks GnRH* or hCG** 78-80 hrs after eCG (14:00-16:00 h)
1st AI	24-26 hrs after GnRH* or hCG**	24-26 hrs after GnRH* or hCG**
2nd AI	38-40 hrs after GnRH* or hCG**	40-42 hrs after GnRH* or hCG**

* e.g 50 μg Gonavet®, ** 500 IU hCG, *** weaning one day earlier in the afternoon

Conclusions

A technology is currently available that allows for manipulation of almost all key reproductive processes in the female pig including oestrus and ovulation, making fixed-time insemination possible. Batch farrowing systems may profit from using this technology due, at least in part, to savings on labour and production of large batches of uniformly developed and healthy pigs. This technology does however not allow for compensation of health problems and/or mismanagement. Understanding of reproductive processes and the availability of hormonally active drugs are essential requirements when deciding to use this technology. However, several factors related to the farms themselves, as well as to the drugs may influence the effectiveness and outcome of this technology. It is thus recommended to adapt this technology to each individual farm.

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Post-weaning Altrenogest treatment in primiparous sows; the effect of duration and dosage on follicular development and consequences for early pregnancy

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Many first litter sows suffer from a suboptimal reproductive performance in the second cycle, shown by an increased weaning-to-oestrus interval, a low farrowing rate and a small litter size (e.g. Thaker & Bilkei 2005). This so-called 'second litter syndrome' may be related to the suppressive effects of a negative energy balance on lactational follicle development, since several authors have shown that pre-follicular phase feeding levels (either during lactation or during the luteal phase of the oestrus cycle) affect the antral follicle pool and subsequent follicle development (Quesnel *et al*. 2000), oocyte development (Zak *et al*. 1997), ovulation rate (Hazeleger *et al*. 2005), embryo development (Algriany *et al*. 2004) and embryo mortality levels (Almeida *et al*. 2000). Postponing oestrus by administration of a progesterone analogue (altrenogest) from weaning onwards positively affects subsequent reproductive performance (e.g. Martinat-Botte *et al*. 1994, Patterson *et al*. 2008). Success of altrenogest treatment is probably related to the improvement of follicle development during treatment. Therefore, the objective of this study was to investigate follicle development at weaning and during and after different altrenogest treatments, and relate this to subsequent ovulation rate and early embryonic development.

For this study, 48 primiparous sows were weaned from their (9.4 ± 1.1) piglets at Day 21 ± 3 of lactation (= Day 0) and were randomly assigned to the following treatments: control (no altrenogest, n = 11), RU8-15 (15 mg of altrenogest, n = 11, Day -1 till Day 7), RU8-20 (20 mg of altrenogest, n = 12, Day -1 till Day 7) or RU15-15 (15 mg of altrenogest, n = 12, Day -1 till Day 14). From weaning onwards, trans-abdominal ultrasound was performed daily and the five largest follicles on one ovary were measured until ovulation occurred. Oestrus detection was performed twice daily and sows were inseminated 12 hours after first detected oestrus on 12 hours intervals during standing oestrus. Sows were slaughtered 5 days after ovulation to determine ovulation rate and to recover embryos. At slaughter a blood sample was collected from each sow to determine progesterone concentrations. Embryo morphology and quality was assessed after which embryos were treated to spread and count cell nuclei and number of accessory sperm cells, and to determine the number of cell cycles (^{2}log of the nuclei count) and homogeneity of the litter (expressed as the range in number of cell cycles for embryos with more than 90% of the litter average in cell cycles).

Follicle size at weaning was 2.6 ± 1.1 mm. Follicle size increased during treatment to reach a plateau of 4.6 ± 1.6 mm around Day 6, irrespective of treatment. This increase resulted in larger follicles at the onset of the follicular phase for treated animals (4.8±1.8, 4.8±1.4 and 4.9±0.9 mm for RU8-15, RU8-20 and RU15-15) compared with controls (2.9±0.8; P = 0.0002). Follicles of treated animals remained significantly larger until the fourth day of the follicular phase. Pre-ovulatory follicle size tended to be larger for treated animals (7.9 ± 2.4mm, 7.9 ± 0.7mm and 8.6 ± 1.3mm for RU8-15, RU8-20 and RU15-15, respectively) than for controls (6.9 ± 0.9mm; P = 0.07).

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The interval to onset of oestrus (from weaning for controls and from 24h after last treatment for treated animals) was shorter for treated animals (4.6 ± 1.4, 4.7 ± 0.9 and 5.2 ± 1.6 days for RU8-15, RU8-20 and RU15-15) compared with controls (6.5 ± 1.4 days; $P = 0.0001$). Duration of oestrus was not affected by treatment, neither was ovulation rate (18.9 ± 4.0 vs. 21.2 ± 5.4 vs. 18.8 ± 4.0 vs. 19.5 ± 3.1 for RU8-15, RU8-20, RU15-15 and controls, respectively ($P > 0.1$)), luteal weight, or progesterone levels at slaughter. Average fertilization rate of the recovered embryos and oocytes was 90.6%, which was not affected by treatment. The majority of the recovered embryos consisted of compact morulas (26%) and blastocysts (53%). No treatment effect was found on embryo quality (visual appraisal), number of accessory sperm cells, number of cell cycles or homogeneity of the litter. Further, none of these parameters were related with follicle size at weaning, follicle size at the start of the follicular phase or pre-ovulatory follicle size. For treated animals, the increase in follicle size during treatment was significantly related with ovulation rate ($P = 0.05$); every mm increase in size during treatments resulted in an increase in ovulation rate of 1.1.

The fact that altrenogest treatment after weaning did not affect ovulation rate and embryonic development may be related with the low lactational burden of these sows (on average 9.4 piglets and a relative weight loss of 4%). However, even under these circumstances treated animals had larger follicles than controls at the onset and first four days of the follicular phase, tended to have larger pre-ovulatory follicles, and the follicle growth during treatments was positively related with subsequent ovulation rate. Further studies need to evaluate subsequent reproductive performance of different altrenogest treatments.

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Assessment of follicle population changes in sows from day of weaning and during estrus using real-time ultrasound

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Follicle selection and maturation for determining ovulation rate in the pig appears to occur during much of the follicular phase and up to the time of ovulation. There is evidence to suggest that counts of follicles classified as ovulatory-sized at onset of estrus may not reflect the final corpora lutea counts. Follicle size heterogeneity has been reported at estrus and may be related to increased embryonic asynchrony and mortality (Hunter *et al*. 1989). It is not clear which follicles ovulate at estrus but reports indicate follicles >4 mm are LH responsive (Dufour & Mariana 1993; Lucy *et al*. 2001). Counts and size measures for ovulatory follicles may differ by the follicle size classification system and in response to whether follicles were assessed by physical or ultrasound measurement (Soede *et al*. 1998; Knox *et al*. 2002; Bracken *et al*. 2006). We hypothesized that the numbers of follicles classified as ovulatory at estrus may not reflect expected ovulation rate or litter size. We performed two experiments to characterize the changes in follicle populations from onset of estrus (Experiment 1) and from time of weaning to ovulation (Experiment 2).

In experiment 1, our objectives were to measure proportions of weaned sows having large, medium and small follicles on the two days before ovulation. A total of 21 sows that had expressed estrus and ovulated between day 2 and 3 and which had real-time ultrasound digital video recordings for both the left and right ovaries on the first (period 1) and second day (period 2) of estrus were included in this study. The images of the ovaries were obtained transrectally using an Aloka 500V ultrasound with a 7.5 MHz linear transducer. The images were digitally recorded and follicles individually counted and measured using a digital display system that was calibrated to the measures of the ultrasound. The follicles were classified as small (S, <3.5 mm), Medium 1 (M1, 3.5-4.99 mm), Medium 2 (M2, 5.0-6.49 mm), Large 1 (L1, 6.5-7.99 mm), Large 2 (L2, 8.0-9.49 mm), and Large 3 (L3, 9.5-12.0 mm). Data were analyzed using the GLM procedures of SAS for the main effects of sow and period (day 1 and 2 of estrus). The response measures included the proportions of sows having the specified size class, numbers of follicles in class, and the size of the follicles.

Period did not affect the percentage (22%), number (<3 follicles), or size of small follicles (3.1 mm). Period during estrus also did not affect the percentage of sows with M1 follicles (90%), or their size (4.4 mm), but numbers were reduced ($P<0.05$) in period 2 (6.5 vs. 4.5). Period did not affect the percentage of sows with (100%) or numbers (14.1 follicles) of M2, but size was increased ($P<0.05$) in period 2 (5.8 vs. 5.9 mm). Period did not affect the percentage of sows having L1 (100%), but numbers (7.8 vs. 9.1, $P=0.01$) and size (7.2 vs. 7.3, $P<0.05$) were both increased in period 2. Period tended ($P<0.10$) to increase the percentage of sows having L2 in period 2 compared to period 1 (37 vs. 63%) but there was no affect on numbers (2.2) or size of these follicles (8.6 mm). There was no effect of period on the proportion of sows having L3 (14%), or their number (1.7), or their size (10.8 mm). In assessing the total number of large follicles (all those > 6.49 mm), there were more ($P=0.01$) large follicles in period

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2 (10.6) compared to period 1 (7.9). Since numbers of follicles >6.49 mm were increased at period 2, it is evident that growth continues during estrus. Further, since numbers of large follicles were lower than expected ovulation rate for this population of sows (>16 CL), it appears that follicles must ovulate from the larger M2 follicle class to achieve final ovulation rate. The results of this study support the observation for heterogeneity of ovulatory follicles at estrus and could help explain how follicle size heterogeneity might contribute to variation in oocyte and embryo quality and survival.

Experiment 2 was performed using 16 sows that were scanned daily from weaning until ovulation. Data were included only from sows that expressed estrus and ovulated and displayed no ovarian cysts. All sows in this study were 3rd parity PIC C-22 sows, which displayed a weaning to estrus of 5.2 ± 0.2 days and ovulation on day 6.1 ± 0.05. All sows were mated twice at onset of estrus resulting in an 87% pregnancy rate, 80% farrowing rate, 12.9 total born, and 10.8 pigs born alive. Data were analyzed by sow and day of weaning (d 0). Sow had no impact on small or M1 follicles but did impact M2 and larger follicles. Day post weaning had a significant effect on most follicle parameters assessed. The numbers of small follicles differed by day ($P<0.001$) with more than 12 small follicles on d 1 and <5 on days 2-6. Most sows (>84%) maintained small follicles on d 1-2, but few had small follicles on days 4-6. M1 follicles were present for most sows (>84%) on all days but numbers differed by day and ranged from 3-9 follicles. The number of M2 follicles/sow ($n=6$) did not differ by day but percentages of sows with M2 follicles were 50% on day 1 and ≥85% on days 2-6. The number of L1 follicles differed by sow and day. Sows had ≤ 3 L1 follicles on d 1-3, and on d 5 and 6, increased their numbers to 5-6 L1 follicles. The percentage of sows with L1 follicles was low on d 1, and reaching 46% on d 2, and then increasing to >85% of sows on d 3-6. The L2 class was not present on d 1-2 but was present in ~50% of sows on days 3-4, and in >80% of sows on d 5-6. The L3 class was represented in only 10% of sows and estimates were < 1 follicle on average. The total numbers of follicles >6.49 mm on day 1 of estrus was 8.3 with the total number of large follicles reduced on the second day of estrus since ovulation was already in process in half of the sows. Since total numbers of large follicles was lower than CL counts (11.3) in sows that could be scanned for CL counts, and was also lower than total born pigs, we analyzed all follicles ≥5.0 mm. The result of the new count was that there were 14.6 follicles at estrus and suggests that ultrasound measures should include follicles 5.0 mm follicles at onset of estrus for assessment of ovulation rate. In summary, it would appear that for experiments 1 and 2, heterogeneity occurs in follicle sizes that ovulate, and that follicles 5.0 mm or greater at onset of estrus in weaned sows will ovulate and contribute oocytes that will effect eventual for litter size.

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Temporal candidate gene expression patterns in the sow placenta during early gestation and the effect of maternal L-arginine supplementation

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The trend towards high ovulation rates in mature commercial sows has resulted in intra-uterine crowding in the immediate post-implantation period, with negative impacts on placental development and later fetal development (Town *et al*. 2004). Factors that improve placental angiogenesis could offset the effects of intra-uterine crowding by supporting placental development at critical times in gestation. Feeding of L-arginine has been shown to have beneficial effects on placental vascularization in gilts (Hazeleger *et al*. 2007) and on litter size born in gilts (Mateo *et al*. 2007) and sows (Ramaekers *et al*. 2006). In the present study, we investigated the effects of L-arginine supplementation to commercial sows on global placental gene expression, and on temporal changes in the expression of a panel of eight candidate genes known to be involved in angiogenesis, in early pregnancy.

Multiparous sows (n = 48) were either non-supplemented Controls or were fed an L-arginine supplement (20g.d) from d 15 through 29 of gestation. A representative number of sows were euthanized on days 16 through 49 of gestation and embryonic and placental tissues were collected from two average-sized conceptuses from each uterine horn and placed in RNAlater for later analysis. To obtain temporal expression profiles for specific genes involved in placental angiogenesis, total placental RNA was extracted from all Control samples collected, reverse transcribed and real-time PCR used to determine the transcript abundance of: vascular endothelial growth factor (VEGF)-A; the two VEGF receptors, fms-related tyrosine kinase 1 (FLT1) and fetal liver kinase-1(flk-1/KDR); hypoxia-inducible factor (HIF)1A; the Angiopoietins (ANGPT) -1 and -2 and their receptor, TEK tyrosine kinase; and finally Angiogenin (ANG)-1. The delta ΔCt values were calculated using 18S as an internal control, and data were analyzed using regression analysis (SAS Institute Inc., Cary, NC). To determine the cumulative effect of L-arginine treatment, real-time PCR for these same candidate genes was also performed on the d 30 placental samples from both Control and L-arginine sows. The relative ΔCt values for d 30 samples were again calculated using 18S as an internal control and data were analyzed using MIXED models (SAS Institute Inc., Cary, NC). Effects of L-arginine on global placental gene expression (n = 4 representative sows per treatment) were also analyzed using PigOligoArray slides and placental tissues collected at d 30 of gestation. Total RNA was extracted, purified using mRNA mini kits (Invitrogen), amplified with aminoallyl mRNA amplification kit (Ambion), and labeled with Cy3 or Cy5 in a random block dye-swap design. The hybridized slide images were captured with Genepix software and an Axon Scanner set for optimized PMT for each dye. Median spot intensities underwent Loess and quantile normalization and were analyzed using linear models, all in limma (Smyth, 2004).

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There were no differences in ovulation rate, embryo survival, live embryos, and embryo or placental weight between control and L-arginine treated sows at day 30 of gestation (Table 1).

Table 1. Reproductive Characteristics of the experimental sows at day 30 of gestation.

Item	Control sows (n = 6)	L-arginine sows (n = 6)	P-value
Parity	4.0 ± 0.35	3.8 ± 0.37	0.698
Ovulation Rate	25.8 ± 1.72	23.2 ± 1.80	0.305
Live Embryos	15.9 ± 1.14	13.8 ± 1.19	0.216
Embryo Survival (%)	64.1 ± 4.6	60.6 ± 4.8	0.603
Embryo Weight at Day 30	1.98 ± 0.11	2.26 ± 0.12	0.105
Placental weight at Day 30	30.60 ± 3.66	34.94 ± 3.43	0.402

Temporal gene expression profiling in the Control samples revealed that angiogenin (ANG1) and VEGFA mRNA expression did not change ($P > 0.05$) from d 17 through 49 of gestation. However, mRNA expression increased ($P < 0.0001$) over early gestation for flk-1/KDR, a specific receptor for VEGFA, and for HIF1A, FLT1, TIE2, ANGPT1 and ANGPT2. Of these 8 genes, L-arginine supplementation only affected placental ANG1 mRNA expression ($P = 0.028$) at d 30 of gestation in treated sows compared to Control sows (11.3 ± 0.58 vs 13.6 ± 0.58). This is a promising result as ANG1 was one gene whose expression did not change throughout early gestation in Control placental tissues. We are currently analyzing the effect of L-arginine on candidate gene expression at d 45 of pregnancy to determine if effects of L-arginine are more pronounced at later stages of gestation. The expression of ANG1 protein in d 45 placenta is also being investigated. Analysis of microarray data revealed that 60 genes were differentially expressed between Control and treated sows at an F-test p-value of < 0.01. Only 5 genes showed higher expression in the Control sow placentae; whereas expression of other genes was higher in L-arginine supplemented sows (cell proliferation-inducing gene 15, RING finger protein 4, Apolipoprotein C-II, Retinol binding protein, and Progestin Receptor alpha).

Given the limited effects of L-arginine feeding on reproductive characteristics, these results suggest that there are other factors influencing placental development and uterine crowding which confound the possible beneficial effects of L-arginine supplementation. Multiparous sows can vary greatly in their ovulation rates, and may have much lower embryo survival rates by d 30 of gestation compared to lower parity sows, and this variance may contribute to lack of consistent effects of treatment. Testing of L-arginine in gilts or first parity sows with similar uterine crowding in early gestation may more clearly define these effects. Nevertheless, evidence of higher expression of angiogenin 1 in L-arginine treated sows by real time PCR, and upregulation of multiple genes in the microarray analysis, confirms the potential for L-arginine to improve placental angiogenesis in early gestation.

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Estrogen and Interleukin-1β regulation of trophinin, osteopontin, cyclooxygenase-1, cyclooxygenase-2, and interleukin-1ß system in the porcine uterus

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Embryonic loss during early gestation limits litter size in swine production. Failure of the conceptus to attach properly to the uterine surface may contribute to the high rate of embryonic loss observed in swine. Attachment to the uterine surface is a highly synchronized event that requires precise communication between the expanding conceptus and endometrial tissue. Conceptus attachment to the uterine surface includes upregulation of adhesion molecules at the maternal/fetal interface for attachment as well as a pregnancy specific inflammatory response. Trophinin and osteopontin are cell adhesion molecules that may function in initial attachment between conceptus trophectoderm and uterine luminal epithelium of the pig and human. Leukocytes infiltrate the endometrium during implantation, and the pro-inflammatory cytokines Cyclooxygenase (COX)-1 and COX-2 are expressed in human and pig endometrium during pregnancy, where they are proposed to regulate conceptus implantation and uterine angiogenesis. Interleukin-1β (IL-1β) increases during implantation in the mouse, human and pig and may regulate uterine inflammatory cytokines. Estrogen also controls uterine events necessary for attachment and implantation of the mouse and pig conceptus and may act in synergy with IL-1β to prepare the uterus for the implanting embryo. Furthermore, trophinin expression was induced by IL-1β in human endometrial cells, and uterine osteopontin expression is regulated by estrogen in the pig and mouse. The objective of the current study was to evaluate the hypotheses that estrogen regulates the uterine inflammatory response induced by IL-1β during the establishment of pregnancy.

Cyclic gilts were treated with corn oil or estradiol cypionate (5 mg) on Day 11 of the estrous cycle. On Day 12, gilts were subjected to mid-ventral laparotomy and uterine horns were randomly infused with either saline or porcine IL-1β (15 μg). Uterine horns were removed at 4h and 36h post-infusion (4 gilts/trt/sampling periods) and endometrial mRNA was quantified by quantitative RT-PCR.

Estrogen did not influence ($P > 0.1$) concentrations of endometrial COX-1 and COX-2 mRNA; however, IL-1β increased ($P = 0.01$) endometrial COX-2 mRNA by 3.5 fold and tended ($P = 0.06$) to increase COX-1 mRNA by 2.5 fold 4h post infusion. Cyclooxygenase-1 and COX-2 regulate uterine prostaglandin secretion, which is essential to normal implantation and pregnancy in pigs (Kraeling *et al*. 1985). Cyclooxygenase-2 null mice are infertile and fail to implant; however, implantation is not impeded in the Cox-1 null mouse (Lim *et al*. 1997). Although the conceptus induces uterine COX-2 expression at implantation sites, estrogen did not increase COX-2 mRNA in ovariectomized mice which is true in our pig study (Chakraborty *et al*. 1996). Furthermore, IL-1β regulates ovulation in mice through COX-2 and prostaglandin production (Davis *et al*. 1999). We hypothesize that conceptus IL-1β regulates uterine prostaglandin secretion by increasing endometrial COX-2. Prostaglandins regulate angiogenesis

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and the conceptus may increase uterine vascularity through IL-1β induced COX-2.

The elongating porcine conceptus secretes IL-1β on Day 12 of pregnancy during conceptus attachment and maternal recognition of pregnancy, and this correlates with endometrial Interleukin-1 receptor type 1 and interleukin receptor accessory protein gene expression suggesting conceptus interleukin upregulates its own endometrial receptors (Ross *et al*. 2003). In the current study, IL-1β increased ($P = 0.05$) its own expression by 3.5 fold and tended ($P = .08$) to increase Interleukin-1 receptor type 1 by 2.5 fold; however, estrogen not IL-1β increased ($P < 0.05$) interleukin receptor accessory protein (2.5 fold increase). Similarly, IL-1β increased Interleukin-1 receptor type 1 in human endometrial stromal and glandular cells (Soloff *et al*. 2004). Interleukin-1β and estrogen differentially regulated uterine interleukin receptors; therefore their combined effects may alter the endometrial response to IL-1β during implantation.

Endometrial osteopontin and trophinin were increased ($P < 0.05$) at 36 h but not 4h after estrogen treatment. Estrogen treated gilts had 11.3 fold greater OPN and 3.6 fold greater trophinin endometrial mRNA than gilts treated with corn oil. Trophinin mRNA and protein are located at the maternal/fetal interface during implantation of pigs, mice, and humans (Suzuki *et al*. 2000, Nakano *et al*. 2003, Sugihara *et al*. 2008). Uterine trophinin is increased by estrogen in mice but IL-1β in human endometrial cells (Sugihara *et al*. 2008), suggesting expression is pigs and mice are controlled by similar mechanism which differs from humans. The fact that OPN and trophinin receptors are located on conceptus trophectoderm suggests a role in attachment and communication at the maternal/fetal interface. We hypothesize that IL-1β and estrogen secretion by the pig conceptus differentially modulates uterine expression of COX-1, COX-2, trophinin, osteopontin, and the interleukin-1 β system providing an inflammatory environment that is essential to establish pregnancy.

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Proteomic study to identify factors in follicular fluid and/or serum involved in *in vitro* cumulus expansion of porcine oocytes

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Follicular fluid, as a transudate of serum, constitutes the micro environment of the maturing oocyte. In a previous study, we have shown that follicular fluid (pFF) is superior to autologous serum in promoting cumulus expansion during *in vitro* maturation (IVM) of porcine oocytes (Bijttebier *et al*. 2008). After ultrafiltration of both fluids, the fraction containing molecules >30kDa includes the factor(s) responsible for the observed differences in cumulus expansion (J Bijttebier, unpublished observations). This suggests the factor is likely to be a protein. The present study aimed to identify those proteins responsible for the observed differences in cumulus expansion after IVM in 10% serum (>30 kDa) versus 10% pFF (>30 kDa) obtained from sows in the preovulatory stage of the estrous cycle.

Shotgun proteomics analysis of the pFF and serum fractions >30kDa from 3 sows was performed by application of the 'isobaric Tag for Relative and Absolute Quantitation'(iTRAQ) technology (Applied Biosystems) followed by 2D-LC ESI-Q-TOF MSMS. Of each sample, 100μg protein material was loaded and runs were performed in duplicate. The processed data, obtained from Mascot Daemon, was searched against the pig EST database for protein identification (http://pigest.ku.dk). Protein ratios resulting from duplicate runs were averaged, log-transformed and analyzed by Student's t-test. In addition, 600 prepubertal gilt oocytes were matured *in vitro* for 26h in NCSU23 supplemented with 10% pFF (>30kDa) or 10% serum (>30kDa) of each of the 3 sows. After IVM, the expanded cumulus matrices were collected and subjected to proteomic analysis. Proteins in the matrix extracts were separated using 2D-PAGE. Two spots that were absent in matrices matured in pFF were excised and submitted to mass spectrometric analysis using ESI-Q-TOF MSMS. The processed data, obtained from Mascot Daemon, was searched against the pig EST database for protein identification.

First of all, serum and pFF were not depleted for high abundant proteins like albumin, because the depleted sample did not show the same biological effect on the IVM of porcine oocytes. Therefore an exclusion list was used based on the first run to exclude abundant peptides derived from albumin. Proteomic analysis of serum and pFF revealed 63 unique proteins present in both fluids of which 13 showed significantly ($P<0.05$) different expression levels (10 proteins levels on $P<0.01$). Seven of these proteins were more abundant in serum whereas 6 of them were more abundant in the pFF fractions. Alpha2-macroglobulin (A2M) and ch4 and secrete domains of swine IgM, which were both down regulated in pFF, were also identified as the protein spots that were absent in the cumulus matrices after IVM in 10% pFF (>30 kDa) compared to IVM in 10% serum (>30 kDa).

In conclusion, 2 proteins that are upregulated in autologous serum were also solely retrieved in the cumulus matrices of oocytes matured in 10% serum (>30 kDa). One of them, A2M,

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might be involved in the observed differences in cumulus expansion. Alpha2- macroglobulin, as a major proteinase inhibitor, might degrade ADAMTS-1, which has been shown to be essential for gonadotropin-regulated cumulus expansion of porcine cumulus-oocyte-complexes (Shimada *et al*. 2004). However, further experiments should be performed to elucidate the mechanisms responsible for the possible inhibiting effects of A2M on cumulus expansion.

Table 1: Differentially expressed proteins ($p < 0.05$) in autologous serum and pff of three sows in the preovulatory phase of the estrous cycle. Protein ratio values in italics correspond to proteins that are more abundant in pff compared to serum

Protein	Protein ratio pFF/serum	P-value protein ID
α2-macroglobulin	0.274	0.000
apolipoprotein CIII	0.375	0.000
angiopoetin-like protein 2	*11.783*	0.011
ch4 and secrete domains of swine IgM	0.201	0.000
clusterin	*1.334*	0.050
complement component C4	0.893	0.000
fibrinogen β chain	*1.607*	0.014
IgG α chain C region	0.804	0.008
IgG E ε chain	0.916	0.002
inter-α -globulin inhibitor H1	*1.175*	0.001
antithrombin III	*1.465*	0.000
angiotensinogen	1.117	0.08
IgG VDJ chain	0.846	0.001

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Impact of selection for uterine capacity on the placental transcriptome

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Direct single trait selection for 11 generations resulted in a 1.6 pig advantage for uterine capacity (UC) while average birth and placental weights at term remained unchanged. Uterine capacity was defined as the total number of fully-formed pigs produced to term when ovulation rate was not limiting, using a unilateral hysterectomy-ovariectomy model. A serial slaughter experiment conducted throughout gestation determined the critical time period for the line difference in litter size was already established between d 25 and 45 of gestation and generated direct evidence of differential relative growth rates for placental tissues at these times. Timing of line differences in fetal survival as well as anecdotal evidence of tissue structural differences pointed to the developing placental tissue as a target of particular interest. Our objective was to gain insight into placental transcriptional changes during this critical stage of gestation and identify genetic loci impacted by quantitative selection for uterine capacity.

Thirty gilts each from the UC and control (CO) lines were subjected to unilateral hysterectomy-ovariectomy at approximately 160 d of age and mated within line at approximately 280 d. Gilts were slaughtered at d 25, 30, or 40 of gestation. Fetal and placental tissues were obtained from each live embryo. Fetal liver samples were used to extract DNA and determine sex of each fetus by PCR. Two male and two female embryos closest to the litter mean for placental weight were chosen to represent each litter sampled (n = 3 litters per line and time point combination). Placental tissues were pooled within litter and total RNA was extracted. Samples were labeled and hybridized to Affymetrix porcine array chips (n = 18) using the manufacturers suggested protocols. Signal intensities were normalized using GC content Robust Multi-array Average (GCRMA) on the probe level data. Filtering was based on perfect match intensities as implemented for Affymetrix Human arrays. Two-way ANOVA (two lines and three stages) was performed. Threshold values were set at a minimum of 1.5 fold difference and the false discovery rate was set to $P < 0.05$ (Benjamini and Hochberg algorithm). Less stringent two-way comparisons (t-tests) were also conducted between lines within each gestation stage. GeneSifter® software web tools were utilized to conduct the analyses and generate the gene lists.

An additional analysis was conducted to examine the potential for a bioinformatics method of identifying single feature polymorphism (SNP) targets between the two lines associated with the behavior of the expression data. A gene by gene linear mixed model of probe intensity variation from 11 probes per gene target was investigated to identify line x probe interactions. Log2-transformed perfect-match intensities for all observations were fit to a linear mixed model that broadly corrected for effects of breed and array. A gene-specific mixed model was fit to the normalized intensities (residuals from first model) accounting for fixed breed, probe, and breed-by-probe interaction effects, and a random array effect. Probes that produced statistical evidence (qvalue ≤ 0.05 to account for multiple testing and $|\text{fold change}| \geq 2$) for the breed-by-probe interaction were identified as candidates containing SNP. The interaction was declared

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significant when Prob t < 0.0064 and would indicate a putative coding region polymorphism associated with line within that probe location. Validation of this approach was conducted in a previous experiment comparing Meishan and occidental breed placental tissues with an 87% success rate using the same threshold values of q < 0.05 for false discovery rate and with greater than 2-fold difference in estimated corrected probe-by-breed interaction effects. The great majority of these single feature polymorphisms are discovered in the middle portions of the 25-mer probe targets.

A total of 4171 targets on the array exceeded P value and threshold limits for the main effect of stage (2230 up-regulated and 1839 down-regulated from d 25 to d 40). Two targets, LIM domain and actin-binding protein 1 (LIMA1) and a hypothetical protein LOC51524 (transmembrane protein 138) approached highly stringent thresholds for significance of the main effect of line (P < 0.08) in the two-way ANOVA with both targets up-regulated in the UC line and increasing during these stages of gestation. Expression data for both loci were validated as differentially expressed by QPCR (P < 0.05) and also increased with gestation age. The function of LIMA1 is to bind to actin monomers and filaments, increase the number and size of actin stress fibers and inhibit membrane ruffling. It inhibits actin filament depolymerization, promotes bundling of actin filaments, delays filament nucleation and reduces formation of branched filaments. Both genes could be predicted to play roles in cell migration, suggesting participation in placental folded bilayer formation.

Less stringent analyses of two-way comparisons within each time point indicated increased expression occurred more frequently than decreased expression in the UC line. The number of up-regulated targets relative to the CO line identified totaled 219, 407, and 234 for gestation stages d 25, 30, and 40, respectively. In contrast, the number of down-regulated targets relative to the CO line identified totaled 36, 71, and 47 for gestation stages d 25, 30, and 40, respectively. This overall expression profile is consistent with observations reached previously, that these lines differed in relative growth patterns of fetal and placental tissues. Genes of interest identified in these lists revealed the importance of pathways such as integrin signaling pathway (calpains, collagen, fibronectin) and lipid metabolism (adiponectin receptors).

Analysis of probe x line data yielded a large number (5617) of putative transcribed SNP between the lines. Merging these data with the expression information indicated an overlap of 762 targets where day effects were also significantly different in the two-way ANOVA, and an overlap of 179 targets from the pair-wise contrasts within each stage. Therefore this overlapping list of targets offers evidence of both expression differences in the placenta as well as genetic variation between the lines that could be contributing to genetic variation for uterine capacity. Both lines originated from the same four-breed composite genetic base prior to the 11 generations of selection applied. Due to the small sampling of data generated from the microarrays, it is also likely a large number of these putative line-specific polymorphisms are associated with effects of genetic drift. However, these targets offer an intriguing start to directly untangling the impact of genetic selection for uterine capacity. It is clear that a more thorough investigation by cluster and pathway or network analysis will also require better annotation of targets on the array. The experimental and analytical approaches in this study identified both expression and polymorphism differences to pursue for identification of genetic markers contributing to genetic variation in uterine capacity from a unique germplasm resource.

Enrichment of porcine spermatogonia by differential culture

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Undifferentiated spermatogonia are a potential source of pluripotent cells and could be used for targeted genetic alteration in pigs. Our understanding of mechanisms maintaining porcine spermatogonial stem cells (SSCs) in vivo and of conditions to propagate SSCs in vitro remains limited. This is largely due to the small number of SSCs present in the testis and the lack of specific morphological and cell-surface markers to isolate a purified population. The goal of this study was to establish a modified differential culture system to effectively enrich SSCs from prepubertal porcine testes for subsequent culture.

Germ cell enrichment was quantified by immunocytochemistry and RT-PCR analysis of proteins and genes known to be specifically expressed in spermatogonia (PGP 9.5, VASA and DBA). Testes were collected from 10 week-old pigs and washed with PBS and transported on ice within 24 h to the laboratory in PBS that was supplemented with antibiotics. Testes were pooled for the isolation of germ cells (2-4 testes per trial). Cells were isolated by a two step enzymatic digestion (Honaramooz et al, 2002). The cells were incubated in DMEM containing 0.1 mM 1-mercaptoethanol, 0.1mM MEM non-essential amino-acids, 200mM L- Glutamine and 5% FCS supplemented with 100 IU penicillin streptomycin, in tissue culture dishes coated with 0.01% gelatin 12 h prior to use, at a concentration of $50x10^6$ cells per dish (60x15 mm) for 1 h at 37 °C in 5% CO_2 in air. By counting cells in the supernatant it was determined that 50% of the total cells attached to the culture plates after 1 h, most of which were somatic cells. Germ cells largely remained in suspension and were transferred to new culture dishes. After an additional 14 h of incubation, unattached cells were collected, concentrated by centrifugation for 5 min and counted before use for long term culture. Enrichment of germ cells at each time point (0, 14 h) was determined by immunocytochemistry for alkaline phosphatase activity, and expression of DBA, PGP 9.5 and VASA. Counterstaining for vimentin was employed to identify somatic cells. mRNA was isolated for RT-PCR analysis to confirm expression of PGP 9.5 and VASA. Isolated cells were seeded at a density of 5×10^5 cells in 6-well plates in DMEM medium as above, supplemented with glial cell-derived neurotrophic factor (GDNF, 20 ng/ml), epidermal growth factor (EGF; 200 ng/ml), and basic fibroblast growth factor (bFGF; 200 ng/ml). Comparison between groups was by Student's t-test.

The two-step differential culture increased the concentration of germ cells from $5.4 \pm 3\%$ in the initial cell suspension to $46.6 \pm 22\%$ in the non-adherent population at 14 h of culture (Table 1). Enriched germ cells formed more (21 ± 7 versus 10 ± 3.4; $P < 0.01$) and larger colonies at day 7-10 post culture than those arising from control cells not subjected to differential culture (Table 2).

The cultured cells grew vigorously in medium that was supplemented with growth factors as mentioned above. Cultured cells formed colonies by 5-6 days, which became compacted

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and formed three dimensional structures at day 8-10. When these colonies were removed physically and transferred to a new culture dish without feeder cells they differentiated to cells with fibroblast like morphology. These cells did not form three dimensional colonies and most cells stained negative for PGP 9.5 and VASA.

Table 1: Germ cell enrichment by differential culture

Testis cells (0h)	Germ cells	Enriched testis cells (14h)	Germ cells
Cell no. (x 10^6)	(%)	Cell no. (x 10^6)	(%)
290	8.2	12	74.0
300	5.3	13	41.3
300	4.1	8	62.5
300	5.3	6	35.0
200	4.0	6	17.5
278 ± 44	5.4 ± 1.7	5.4 ± 3.3	46 ± 22

Table 2: Colony formation after 7-10 days in vitro

Replicates per experiment (0h)	Colony No. per dish (0h)	Replicates per experiment (14h)	Colony No. per dish (14h)
2	6	2	11
2	12	2	30
2	15	2	25
2	12	2	19
2	9	2	22
	10 ± 3.4		21 ± 7.0

0h: Cells plated without enrichment
14 h: Cells plated after enrichment
Student's T-test $P < 0.01$

These results indicate that the two-step differential culture protocol results in a 10-fold enrichment of germ cells from prepubertal porcine testis. Propagation of germ cell colonies in cells maintained in vitro for 7-10 days requires the presence of feeder cells. Enriched germ cell populations can now be used more efficiently for further expansion and genetic modification of porcine germ cells in vitro.

This study was supported by grant # 2-R01RR017359-06 from the NIH/NCRR

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Cloning and expression of pluripotent factors around the time of gastrulation in the porcine conceptus

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The expression of the transcription factors Nanog, Sox-2 and Oct-4 is required for maintaining the inner cell mass and ensuing epiblast of the developing mouse embryo as well as pluripotency of embryonic stem cells in culture. Nanog and Oct-4 are down regulated about the time of gastrulation (Rosner *et al*. 1990, Chambers *et al*. 2003) whereas Sox-2 expression is observed in other tissues including the developing nervous system (Avilion *et al*. 2003). In embryonic stem cells, these factors suppress differentiation and promote self-renewal by forming an autoregulatory and feedforward network. The expression pattern of these markers in farm animal species is not well characterized and may differ from that of the mouse (Degrelle *et al*. 2005). Therefore, we have partially cloned the porcine Oct-4, Nanog and Sox-2 transcripts and characterized their expression in day-10, -12, -15, and -17 embryonic and extraembryonic tissues as well as endometrium, myometrium, placenta and fetal liver at day 40 of pregnancy (day 0 is the onset of estrus).

Embryos were flushed from sows 10, 12, 15, and 17 days post-insemination. Day-10 and -12 embryos were processed as whole conceptuses. Day-15 and -17 embryonic tissue (embryonic disk) was separated by closely trimming the adjacent extraembryonic tissue (proximal extraembryonic) with a scalpel using a stereo-microscope (5 to 50X). Additional extraembryonic tissue (distal extraembryonic) was collected after removal of the embryonic disks. Total RNA was isolated using RNeasy Mini or RNeasy Micro Kits (Qiagen; Valencia, CA) according to manufacture's instructions. Sequence for each transcription factor was obtained by full-length RNA ligase-mediated rapid amplification with either the RLM-Race (Ambion; Austin TX) or GeneRacer (Invitrogen; Carlsbad CA) kits according to manufacture's instructions. Total RNA was reverse transcribed and real-time PCR was peformed using TaqMan probe-based assays (Applied Biosystems; Foster City CA). Threshold values were normalized using 18s ribosomal RNA as the endogenous control. Using the adjusted threshold values, tissue means were compared by the GLM procedure of SAS (SAS Institute Inc.; Cary NC) and pair-wise comparisons were made between tissues. For each gene, the tissue with the lowest adjusted threshold value was designated as the reference tissue. Relative expression differences were calculated by taking the difference in threshold values with the reference tissue and raising it by 2^n.

The coding sequence for porcine Nanog (Genbank: DQ447201) including 452 base pairs of the Nanog promoter, and partial coding sequences of Oct-4 and Sox-2 were obtained. The homeodomain and c-terminal tryptophan repeats are highly conserved in porcine Nanog compared to the mouse, human and bovine. In the promoter, the highly conserved Octamer and Sox binding sequences are also present.

Oct-4 and Sox-2 expression (see Table 1) was lowest in day-40 tissues except for fetal liver which was 20 and 71 fold, respectively, higher than endometrium. The pattern of Nanog RNA expression differed from Oct-4 and Sox-2. Day-40 tissues demonstrated the highest expression of Nanog, including endometrium (7 fold), fetal liver (27 fold), placenta (40 fold) and myometrium (72 fold) when compared to day-15 distal extraembryonic tissue. Expression of these transcription factors in fetal liver may indicate the presence of a stem cell population

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in the developing liver. This could coincide with hematopoiesis in the developing fetus. Expression in the endometrium, placenta and myometrium was unexpected. Expression of Nanog in adult mouse tissues has been reported (Hart *et al*. 2004) and the relatively high expression in these gravid tissues may be associated with rapid growth or other physiological responses in pregnancy.

Oct-4 expression levels were similar for day-10, -12 and -15 conceptuses and disk but dropped 3 fold in day-17 disk. On the other hand, Sox-2 was up regulated 1000 fold in the day-15 disk and 2000 fold in the day-17 disk when compared to the day-12 conceptus. The up regulation of Sox-2 occurs when the initial neural structures are appearing in the disk.

Overall, the expression of Nanog, Oct-4 and Sox-2 in pig pregnancies reveals similarities to that in the mouse but expression in the early fetal period may indicate functions beyond early embryogenesis. Further study of the regulatory circuits at these stages is needed.

Table 1. Relative Expression of Nanog, Oct-4 and Sox-2 in the Conceptus and Gravid Uterus

Tissue	n	Nanog	Oct-4	Sox-2
D10 Conceptus	3	7.8^{c}	61.3^{d}	29.2^{de}
D12 Conceptus	4	6.9^{c}	52.8^{d}	21.9^{de}
D15 Embryonic Disk	5	12.3^{cd}	65.7^{d}	1134.6^{f}
D15 Proximal Extraembryonic	5	3.9^{bc}	51.3^{d}	7.4^{bcd}
D15 Distal Extraembryonic	5	1.0^{a}	8.5^{b}	1.4^{ab}
D17 Embryonic Disk	3	11.2^{cd}	17.7^{bcd}	2022.6^{f}
D17 Proximal Extraembryonic	3	3.2^{abc}	34.9^{bcd}	1.4^{ab}
D17 Distal Extraembryonic	3	1.2^{ab}	8.9^{b}	3.9^{abc}
D40 Endometrium	3	7.0^{c}	1.3^{a}	1.0^{a}
D40 Myometrium	3	72.6^{e}	1.0^{a}	1.8^{ab}
D40 Liver	3	27.4^{de}	1.5^{a}	71.5^{e}
D40 Placenta	3	39.9^{de}	26.7^{bcd}	5.2^{abcd}

^{abcdef}Expression values having different superscripts within column are different ($P < 0.05$)

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Prenatal programming of postnatal development in the pig

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Studies of low birth weight offspring have a long history in pig science. These pigs have reduced growth potential and poor carcass quality compared to their higher birth weight littermates. In contemporary commercial sows with between 10 and 15 total pigs born/litter, between-litter differences in average birth weight appear to make the largest contribution to variation in postnatal growth performance, independent of numbers born. Low birth weight is a characteristic of a subpopulation of these sows, likely as a consequence of an imbalance between ovulation rate and uterine capacity due to ongoing selection for litter size. Based on experimental studies, we hypothesize that increased crowding at day 30 of gestation primarily affects placental development and persistent negative impacts on placental growth then affect fetal development. However, embryonic myogenic gene expression is already affected at day 30. Latent effects of metabolic state on oocyte quality and early embryonic development have also been reported. In contrast to effects of uterine crowding, the embryo is primarily affected by previous catabolism. The large body of literature on gene imprinting, and the interactions between metabolism, nutrition, and methylation state, suggest that classic imprinting mechanisms may be involved. However, the potential use of genomics, epigenomics, nutrigenomics, and proteomics to investigate these mechanisms brings new demands on experimental design and data management that present a considerable challenge to the effectiveness of future research on prenatal programming in the pig.

Introduction

Studies of extremely low birth weight or runt pigs have a long history in pig science. Runt pigs (< 0.8 kg of birth weight, bw) can be identified as lying outside developmental norms for gestational age at a very early stage of pregnancy. They have a low chance of survival to weaning and of generating viable economic returns to producers. In a review of studies of within-litter variation in prenatal development (Adams 1971, Widdowson 1971, Hegarty & Allen 1978, Flecknell *et al*. 1981), Wootton *et al*. (1983) suggested that the extremes of intrauterine growth restriction (IUGR) or "runting" were identified within a discrete subset of

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fetuses. Subsequently, van der Lende & de Jager (1991) considered the relationship between within-litter differences in prenatal development and postnatal performance and concluded that the lower pre-weaning growth of the runt pigs in the litter was not completely explained by their lower birth weight. They suggested that IUGR, or runting, had a more complex effect on developmental potential and that the phenomenon of IUGR within a litter was associated with specific patterns of embryonic survival (van der Lende *et al*. 1990). The largest litters *in utero* were also most likely to include runt fetuses. Furthermore, these data were consistent with the conclusion that within-litter variation in development was already established at the early fetal stage (day 27 to 35) of gestation. More recently, Finch *et al*. (2002) used statistical criteria to classify pigs within a litter as either IUGR (clearly low birth weight pigs that were also outliers in the fitted distribution of litter weights), or simply small for gestational age (SGA; more than two standard deviations below the litter mean weight but not statistical outliers). In contrast to data from mature sows discussed later in this review, in these Large White x Landrace gilt litters, no relationship was established between litter size and mean litter weight at day 30 or 100 of gestation, or at birth. Another important conclusion was that in these gilt litters, the authors detected both IUGR and SGA littermates at day 30 of gestation when the effects of crowding would be considered as having minimal impacts on embryo size. These data further support the conclusion that variations in within-litter birth weight are established by day 35 of gestation; however, the mechanisms by which this occurs, if not explained by uterine crowding, are not completely understood.

In general, growth performance in the pre-weaning, nursery, and grow-finish stages of production are impaired in low birth weight pigs (Quiniou *et al*. 2002, Rehfeldt & Kuhn 2006). Within-litter comparisons of associations between low (~ 1.0 kg) and high (~1.9 kg) birth weight pigs and postnatal development reviewed by Foxcroft *et al*. (2007a) indicate that low birth weight littermates have reduced growth potential and poor carcass quality, linked to lower muscle fibre numbers at birth. These postnatal effects of birth weight are consistent with earlier studies of fetal muscle development in the pig and effects of nutrient restriction on myogenesis (see Rehfeldt & Kuhn 2006). Therefore, the low birth weight of the individual pigs within a litter, and within-litter variation in birth weight, are already of considerable economic interest for postnatal performance in pork production systems.

In the remainder of this review, we wish to contrast the incidence of within-litter variation in individual birth weight with even greater variation in average birth weight of pigs between litters. Changing interactions among the component traits determining litter size at term (ovulation rate, embryonic survival to day 30, and uterine capacity for normal placental and fetal development) need consideration. In hyper-prolific dam-line sows these interactions characteristically result in large numbers of pigs *in utero* in late gestation, but relatively poor survival through the peri-parturient period and low birth weight in both live and stillborn pigs. In less prolific commercial dam-lines, selection for increased litter size has still produced inadvertent crowding of embryos in early gestation in some sows, and this phenotype becomes more prevalent as sows mature, resulting in increased between-litter variation in birth weight. By dissecting the component traits contributing to litter outcomes in these different sow populations, it is possible to suggest the underlying physiological mechanisms involved. Finally, litter phenotypic outcomes can be further complicated by imprinting effects of sow metabolic state on early litter development.

These trends in pig litter phenotypes will be discussed in the context of the large body of research which arose from epidemiological studies in human populations that suggested epigenetic mechanisms likely determine prenatal programming of postnatal outcomes. Experimental studies in laboratory rodents and sheep continue to define the mechanisms

linking maternal nutritional state to prenatal programming and are of particular relevance to the growing number of "nutrigenomic" studies in the gilt and sow. These mechanisms also presumably underlie the indirect effects of limited placental size due to crowding *in utero* on embryonic and fetal development. Finally, existing knowledge on prenatal programming in the pig will be discussed from two perspectives. Firstly, the possible impact of multi-generational epigenetic effects on boar and sow fertility. Secondly, the demands on experimental design and data management that need to be met in order to realize the full potential of "omic" analyses in future research on prenatal programming in the pig.

Between-litter variation in birth weight and implications for prenatal programming.

Responses to selection of hyper-prolific dam-line sows

As reviewed by Boulot *et al*. (2008), selection for truly hyper-prolific dam-line sows has been associated with litter traits that are causing problems for efficient and sustainable production. Although the total number of pigs born per litter increased from 11.9 in 1996 to 13.8 in 2006 (Gondret *et al*. 2005, IFIP 2007), total mortality also increased. Overall, 8% of all pigs were stillborn, accounting for 40% of total mortality. Also, with pigs born alive averaging 12.7 per litter, pre-weaning mortality was also high (15%) and mainly explained by crushing of weak littermates. Although the average number of pigs weaned still shows a positive trend in these hyper-prolific dam-lines, the growth potential and economic competitiveness of these offspring is likely an issue.

Selection for higher total numbers of pigs born is associated with increased within-litter variation in piglet birth and an overall decrease in birth weight (Quiniou *et al*. 2007: Table 1). The proportion of pigs weighing less than 1.0 kg increased from 3 to 15% and the proportion of pigs > 1.4 kg fell below 50% in litters of 16 pigs or more. This suggests that in the large litters born to hyper-prolific sows, growth potential of the live-born pigs that survive to weaning will have been seriously compromised by intra-uterine competition, with an increasing number of stillborn pigs and littermates that die in the immediate period after farrowing (Foxcroft *et al*. 2007a). Ongoing studies of the reproductive characteristics of a similar line of French hyper-prolific sows imported into Canada confirm these suggestions. In the higher parity sows in this population, high ovulation rates are associated with substantial crowding of viable conceptuses at day 30 of gestation (JCS Harding, personal communication) and the overall distribution of birth weights shows the same shift to a lower median birth weight, and an increase in variance in litter birth weights, discussed earlier (M Duggan, personal communication).

A better understanding of the characteristics of specific hyper-prolific dam-lines is needed. This information, and an increasing focus on maximizing net revenues per sow, in terms of saleable pork products relative to the input costs per kg of pork sold, should allow more commercially sustainable dam-lines to be developed. Ultimately, selection of sows with increased uterine capacity offers the best opportunity for increasing the number of pigs born per litter without compromising the post-natal growth performance of these pigs (Johnson et al. 1999, Quinton et al. 2006, Rosendo et al. 2007). Foxcroft et al. (2007a) suggested that selection for litter characteristics also needs to include data from multiparous sows, with litter average birth weight being used in addition to total pigs born live, as a means of identifying the most efficient lifetime production. The complex interactions among the component traits determining litter size and quality were also highlighted in the review of Distl (2007), who commented that in

Table 1. Effect of litter size on piglet birth weight (bw) variation. (From Boulot *et al.* 2008)

Litter size (class)	< 9 to 9	10 to 11	12 to 13	14 to 15	16 to > 16
Mean parity	2.6	2.3	2.5	2.6	3.5
Litter: (n =)	161	134	245	334	506
No. total born	7.2	10.6	12.6	14.5	17.6
No. born alive	7.0	10.2	11.9	13.8	16.2
No. stillborn	0.3	0.4	0.6	0.7	1.5
Mean bw, kg	1.89[a]	1.67[b]	1.57[c]	1.47[d]	1.38[e]
CVbw, %	14.9[d]	17.4[c]	20.2[b]	21.3[b]	23.7[a]
Distribution in bw classes, %					
< 1.0 kg	3[e]	5[d]	8[c]	10[b]	15[a]
1-1.4 kg	8[e]	16[d]	21[c]	29[b]	34[a]
1.4-1.8 kg	27[c]	39[b]	43[a]	43[a]	38[b]
> 1.8 kg	63[a]	40[b]	28[c]	19[d]	13[e]

[a,b,c,d,e] Within each row, means with no common superscript differ ($P < 0.05$).

genomic studies, candidate gene effects and quantitative trait loci (QTL) are unlikely to be detected unless each of the component traits contributing to litter outcomes is adequately described in specific sow populations. This provides much of the rationale for studies described in the next section, which attempted to describe these phenotypic traits in contemporary commercial sow populations.

Embryonic and placental development are affected by uterine capacity in early gestation

Characteristics of IUGR would be a predicted outcome when the number of developing conceptuses *in utero* exceeds functional uterine capacity. Earlier studies indicated that uterine capacity was generally not limiting until after day 30 of gestation, and that effects of moderate crowding of embryos before day 30 could be compensated by increased placental efficiency later in gestation (see reviews of Foxcroft *et al*. 2007a,b). The dynamic changes in placental development that occur during gestation that might contribute to such compensatory effects are reviewed elsewhere in this volume (Vallet *et al*. 2009). However, particularly in studies with higher parity contemporary commercial sows, increased placental efficiency does not appear to fully compensate poor placental development earlier in gestation. Even in individual gilts with 20 or more ovulations, and little or no embryonic loss by day 28-30 of gestation, substantial intra-uterine crowding limits placental development (Almeida *et al*. 2000). Depending on the particular sow population studied, a consistent trend for increased ovulation rates in higher parity sows (Vonnahme *et al*. 2002, Town *et al*. 2005, Patterson *et al*. 2008) may be associated with increased numbers of embryos (Vonnahme *et al*. 2002, Patterson *et al*. 2008) and negative effects on placental weight (Vonnahme *et al*. 2002) at day 30. However, the interactions among ovulation rate and embryonic survival rate on the one hand, and placental and embryonic weight at day 30, and placental and fetal weight at day 50 on the other, are complex and are affected by parity of the sow (Table 2).

Several conclusions can be drawn from these data: (1) the proportion of sows with > 25 ovulations increases with parity, (2) the higher ovulation rates in these sows are positively associated with increased numbers of embryos in the uterus at d 30, despite a negative effect on the percent of embryos surviving to day 30, (3) in turn, the higher number of embryos at day 30 has a negative impact on placental weight at day 30, which persists to day 50, and (4) consistent with Town *et al*. (2004), no immediate effect of increased numbers of embryos on

embryonic weight at day 30 is present; however, reduced fetal weight at day 50 was evident, resulting in an interaction between parity, ovulation rate, and gestational age (Table 2). Thus, even within the productive lifetime of commercial sows, the changing dynamics of prenatal loss have important implications for placental and fetal development. Furthermore it is evident that the number of embryos present at day 30 of gestation in commercial sows exceeds normal uterine capacity, limiting embryonic and placental development (Foxcroft & Town 2004).

Table 2. Interactions among parity grouping, ovulation rate and either numbers of embryos, embryonic survival, and embryonic and placental weight at day 30, or numbers of fetuses, fetal survival, and fetal and placental weight at day 50, in commercial dam-line sows. (Extended data from the study of Patterson *et al.* (2008)

Parity	Gestation Day	Ovulation Rate Class (n =)	Mean Ovulation Rate	Nos. Live Embryos/ Fetuses	Embryonic or Fetal Survival (%)	Embryonic or Fetal Weight (g)	Placental Weight (g)
P2-3	30	< 25 (n = 121)	20.6 ± 0.2	14.9 ± 0.6	71.1	1.2 ± 0.8	16.0 ± 2.0
		≥ 25 (n = 25)	26.4 ± 0.5	15.7 ± 0.6	60.6	1.1 ± 1.5	14.5 ± 5.5
	50	< 25 (n = 119)	20.3 ± 0.2	12.9 ± 0.6	62.7	38.7 ± 0.8	120.8 ± 2.0
		≥ 25 (n = 26)	27.2 ± 0.5	13.2 ± 0.6	48.6	45.8 ± 1.6	113.6 ± 4.3
P4-6	30	< 25 (n = 28)	21.5 ± 0.5	15.8 ± 1.0	70.0	1.4 ± 1.7	16.7 ± 4.2
		≥ 25 (n = 21)	26.8 ± 0.5	17.3 ± 1.0	66.2	1.4 ± 1.7	15.9 ± 4.8
	50	< 25 (n = 26)	21.2 ± 0.5	10.8 ± 1.1	50.9	52.1 ± 1.8	121.8 ± 4.4
		≥ 25 (n = 22)	27.6 ± 0.5	10.6 ± 1.0	38.6	46.5 ± 1.6	104.9 ± 4.7
P7 +	30	< 25 (n = 17)	21.8 ± 0.6	12.6 ± 1.2	60.3	1.5 ± 2.5	18.9 ± 5.3
		≥ 25 (n = 18)	26.3 ± 0.6	14.8 ± 1.2	55.2	1.4 ± 2.3	15.4 ± 5.3
	50	< 25 (n = 31)	21.8 ± 0.4	12.4 ± 1.1	57.4	54.4 ± 2.0	107.1 ± 3.9
		≥ 25 (n = 19)	28.4 ± 0.6	10.7 ± 1.5	38.6	50.4 ± 3.1	102.0 ± 3.9
		Parity (P)	0.07	NS	0.07	< 0.001	NS
		Gestation (G)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
		P*G	0.06	< 0.05	0.06	< 0.001	< 0.06
P-Value		Ovulation Group (O)	< 0.001	NS	< 0.001	NS	< 0.05
		P*O	NS	NS	NS	< 0.01	NS
		G*O	NS	0.09	NS	NS	NS
		P*G*O	NS	NS	NS	< 0.01	NS

Experimental approaches to the study of prenatal programming of litter development.

An initial experiment with weaned third parity sows indicated that although an increasing number of viable embryos determined by laparotomy at day 30 did not affect average litter birth weight, characteristic impacts of IUGR were evident in neonates necropsied at term (Town 2004). In a second experiment, unilateral oviduct ligation in the weaned sow was used to effectively reduce the number of embryos and fetuses developing in the uterus compared to the relatively crowded intra-uterine environment of control sows (Town *et al*. 2004). Even though the difference in uterine crowding achieved did not replicate the extremes of intra-uterine crowding discussed above in commercial sows, the higher number of embryos at day 30 limited placental but not embryonic weight at that stage, and negatively affected both placental and fetal weight at day 90. Among the various measures of IUGR recorded, there were specific effects on the brain:muscle weight ratio, brain to muscle fibre number ratio, estimated total number of secondary muscle fibres, and associated differences in the wet weight and cross sectional area of the semitendinosus muscle (Table 3). Further analyses determined that even at day

30, down-regulation of myogenin expression was present in the relatively crowded embryos (Tse *et al*. 2008). This provided some of the first evidence that functional uterine capacity for optimal placental development at day 30 is below the threshold for embryonic survival *per se* at this stage of gestation reviewed elsewhere (Foxcroft *et al*., 2007a). The data confirm our hypothesis that increasing the number of conceptuses surviving to the post-implantation period initially limits placental development at day 30, and as a consequence, then limits placental function later in gestation and produces fetuses at day 90 that already show characteristics of IUGR. The recorded effects of inadvertent intra-uterine crowding in early gestation on expression of myogenic regulatory genes would also contribute to differences in fetal muscle fibre development, birth weight, and post-natal growth performance in affected litters.

As discussed later, the effects of prenatal programming on postnatal performance are not limited to effects on muscle development and growth. Epidemiological studies in human infants led to the "Barker hypothesis", linking pre-natal programming of the fetus to lifetime health outcomes (see Barker 1994). Harding *et al*. (2006a,b) discussed similar associations in the pig in the context of development of the immune system and early postnatal survival. In a preliminary analysis of brain sparing effects, it was demonstrated that the organs most notably affected in stillborn pigs with low relative birth weights compared to their littermates were the heart, liver, and spleen. These and other developmental complications, undoubtedly underlie the problems of managing low birth weight pigs through the lactation and nursery stages of production. In ongoing studies in mature commercial sows, the unilateral oviduct occlusion technique is being used by the same group to explore the impact of differences in intra-uterine crowding early in gestation on development of immune competence at birth.

A second approach to determine the origins of increased between-litter variation in litter average birth weight was the retrospective analysis of available litter phenotypic data from large breeding nucleus populations (Smit 2007) and the collection of individual litter data in a study of L-arginine supplementation to the gestation diet of commercial sows from the same population used by Patterson *et al*. (2008). Litter average birth weight and between-litter variance in litter average birth weight were both consistently lower in the largest litters (> 15 total born) in these populations (Fig. 1), analogous to the situation in hyper-prolific sows discussed earlier. This suggests that the birth weight of most pigs born in litters >15 is relatively low because the threshold of uterine capacity for supporting pigs within the upper percentiles of birth weight (1.6 to over 2.0 kg) is around 15 fetuses. At the other extreme, in litters of <10 pigs, low litter average birth weight should not be due to limited uterine capacity, unless high ovulation rates combined with high peri-implantation embryonic survival resulted in very serious limitations in placental development.

As is evident in Fig 1, in the population of litters with between 10 and 15 total pigs born, large variation in litter average birth weight existed, independent of litter size. Smit (2007) compared the characteristics of low and high average birth weight litters within this same subpopulation, having defined high and low average birth weights as being more or less than 0.2 kg relative to the average litter birth weight for the whole population (approximately 1.4 kg). The resulting dataset included 1,094 litter records representing the top 15% (High) and bottom 15 % (Low) of the population in terms of litter average birth weight (Table 3). In litters with a Low average birth weight, fewer pigs were born alive, more pigs were born dead, and fewer pigs survived to weaning, consistent with the hypothesis that these litters had been subjected to negative effects of IUGR. Furthermore, these litters exhibit lower within litter variation in birth weight possibly resulting from the prenatal loss of the smaller fetuses in a crowded intra-uterine environment. In contrast, in High average birth weight litters not subjected to extremes of intra-uterine crowding, pigs across a wider range of birth weights have the opportunity to survive to term.

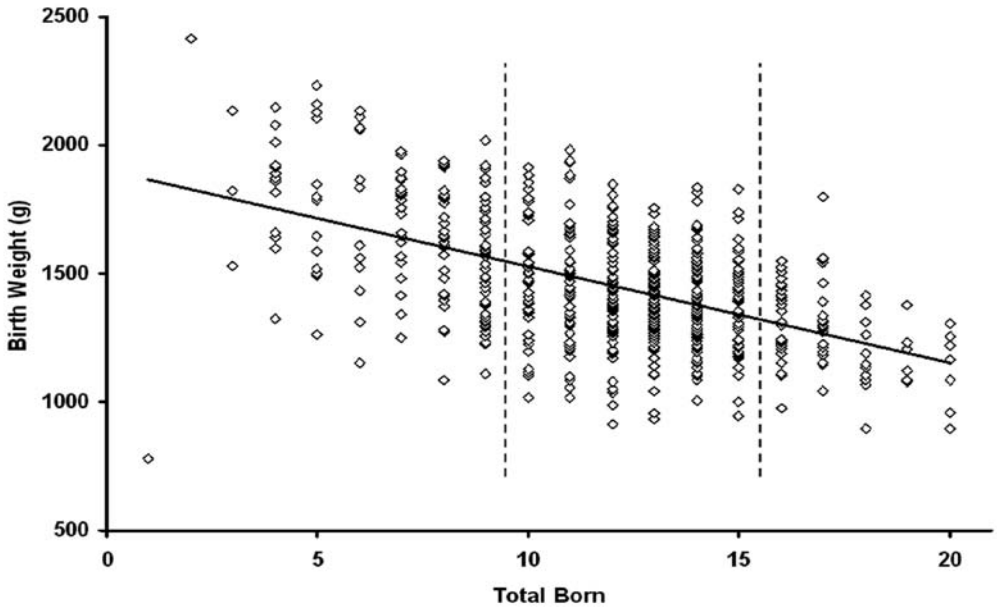


Fig. 1 Negative correlation between litter average birth weight and total born in all litters, grouped by litter sizes of less than 10, 10-15, and greater than 15, using total born as a covariate (n = 580; r^2 = -0.28; $P \leq 0.001$). (University of Alberta, unpublished data, 2007)

Table 3. Characteristics of litters classified as having a higher than average (High) and lower than average (Low) birth weight (bw), and in litter sizes of between 10 and 15 total pigs born (After Smit 2007).

	High	Low	P-Value
Average birth weight (kg)	1.8 ± 0.01	1.2 ± 0.01	< 0.001
Standard deviation in bw	0.287	0.272	0.01
Total born	12.3 ± 0.08	12.3 ± 0.07	NS
Born alive	11.7 ± 0.09	11.0 ± 0.09	< 0.001
Born dead	0.6 ± 0.07	1.2 ± 0.06	< 0.001
Weaned	10.8 ± 0.10	9.4 ± 0.10	< 0.001

The concept that low average birth weight is associated with characteristic effects of IUGR, and could be linked to effects of intra-uterine crowding on placental development in litters of between 10 and 15 pigs total born, was further explored by performing necropsies on any still-born pigs born in a proportion of the litters represented in Fig 1 and by estimating average placental wet weight immediately after farrowing in these litters (Fig 2a). Relevant to this review, necropsy was only performed if pigs were truly still-born (confirmed by lung floatation tests) and their weights fell within the mid-weight range of their respective live-born littermates. The inverse relationship between litter average birth weight and wet placental weight (Fig 2a), and between litter average birth weight and organ weights (Fig 2b), support the conclusion that low birth weight litters show characteristics of IUGR and that this may be functionally linked to limited placental development. Based on the prevalence of high ovulation rates, and an average of 15.7 and 17.3 embryos at day 30 in the parity 2-3 and 4-6 sows reported by Patterson *et*

al. (2008), these data are considered to support the principal hypothesis driving our ongoing research "that changing dynamics of pre-natal loss of embryos in contemporary commercial sow populations limits placental development and indirectly causes IUGR" and that in this situation "IUGR is a characteristic of entire low birth weight litters" (Foxcroft *et al*. 2007a).

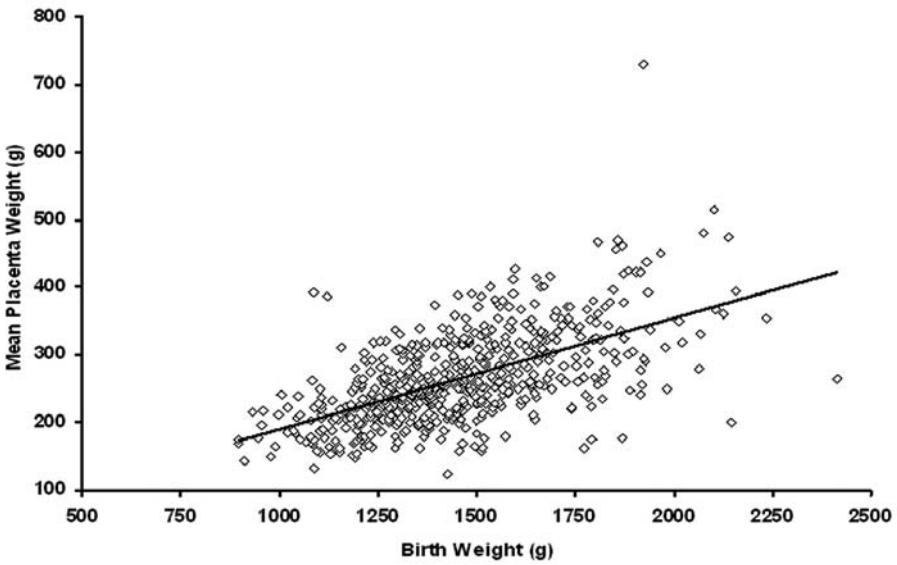


Fig. 2a Correlation between average placental wet weight and litter average birth weight (bw) for each litter (n = 586: r^2 = 0.41: $P < 0.001$)

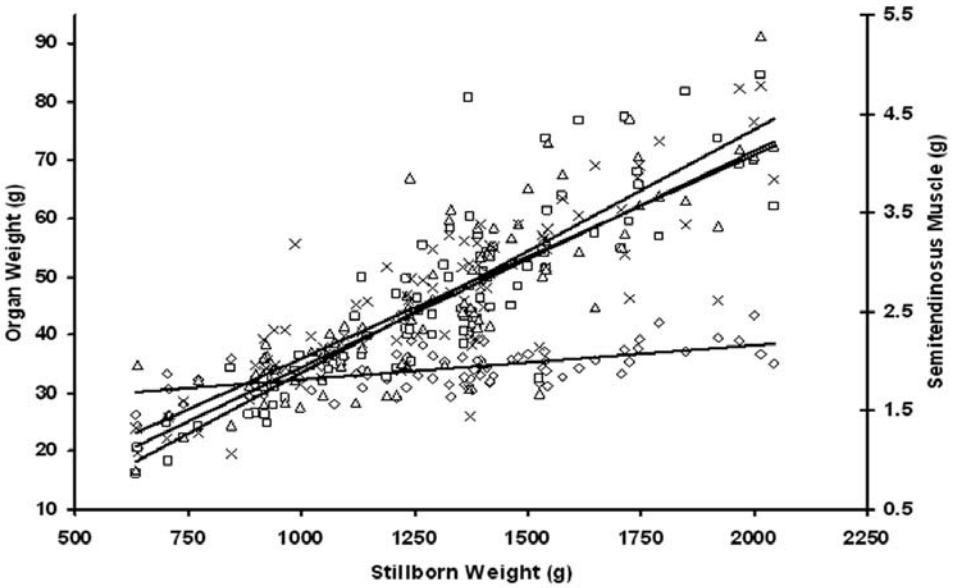


Fig. 2b Fitted linear relationships between weight of the brain (diamonds; y = 0.0059x + 26.436; r^2 = 0.35; $P < 0.001$), small intestine (squares; y = 0.0417x – 8.2041; r^2 = 0.76; $P < 0.001$), liver (triangles; y = 0.0371x – 2.6725; r^2 = 0.69; $P < 0.001$), and semitendinosus muscle (crosses; y = 0.002x – 0.0236; r^2 = 0.71; $P < 0.001$) in relation to the weight of stillborn pigs (n = 87). (University of Alberta, unpublished data, 2007)

Latent effects of metabolic state on prenatal development

The nutritional and metabolic state of the gilt or sow can exert direct and classic epigenetic effects on embryonic development, representing an alternative source of prenatal programming of whole litters due to environmental effects on the oocyte and developing embryo (see Foxcroft *et al*. 2006, 2007b). A deficit in nutrient availability at any stage of gestation may also trigger changes in litter development and Wu *et al*. (2006) reviewed evidence for the link between nutrient availability and IUGR in domestic livestock species. The extent to which nutrient restriction leads to limited, but normal, development, or results in more profound reprogramming of the developmental process (associated with characteristics of IUGR), will depend on the nature and severity of the restriction, the sensitivity of each litter to such nutrient challenges, and the window of development over which the restriction occurs. As an example, nutrient restriction due to limited placental development before day 30 may permanently reprogram myogenesis, with lasting consequences for growth potential and carcass quality (see Foxcroft *et al*. 2006). Alternatively, nutritional changes after myogenesis is complete may still affect birth weight but may not irreversibly change post-natal growth potential.

Results from an ongoing series of experiments using nutritional restriction of the sow in late lactation to understand the mechanisms mediating effects of catabolism on subsequent fertility have been reviewed previously (Foxcroft *et al*. 2007b). Selection for increased prolificacy and fertility appears to have changed the nature of the response to a catabolic state in late lactation. An extended weaning-to-estrus interval is no longer a typical response, and ovulation rate, fertilization rate, and embryonic survival to the expanded blastocyst stage are also not affected. Decreased embryonic survival to day 30 may be present but seems to affect a specific sub-population of litters (Vinsky *et al*. 2006), again indicating the variable sensitivity of particular sows to similar catabolic (environmental) challenges. It is still uncertain whether loss of embryos is associated with increased heterogeneity in early embryonic development within affected litters, but the loss of embryos may be gender specific (Vinsky *et al*. 2006, 2007b). The most consistent response to a previous catabolic state is a reduction in embryonic weight, which is usually independent of any comparable effect on placental development and is not gender-specific (Table 4).

Table 4. Least square means ± SEM for sow reproductive performance, embryonic survival and embryonic and placental weight at day 30 of gestation, in primiparous Control sows (n = 16) and sows feed-Restricted (n = 17) during the last week of a 21-day lactation (After Vinsky *et al.* 2006)

Item	Control	Restricted
Wean-to-estrous interval (days)	5.3 ± 0.3	5.4 ± 0.3
Ovulation rate (Number of CL at day 30)	18.3 ± 0.7	18.2 ± 0.6
Day of gestation at slaughter	30.3 ± 0.2	30.1 ± 0.2
Embryonic survival (%) to day 30*	**79.2 ± 4.0**	**67.9 ± 3.9**
Number of surviving male embryos	7.7 ± 0.6	7.5 ± 0.6
Number of surviving female embryos*	**6.5 ± 0.6**	**4.7 ± 0.6**
Embryo weight (g) **	**1.53 ± 0.07**	**1.38 ± 0.07**
Placental weight (g)	22.6 ± 1.9	22.8 ± 1.9

*$P < 0.05$, **$P < 0.01$ compared to Control sows

In earlier studies using oocyte *in vitro* maturation (IVM) assays, a lower proportion of oocytes recovered from previously catabolic sows reached the metaphase II stage of nuclear maturation and follicular fluid recovered from preovulatory follicles of these sows was less able to support IVM of pools of oocytes recovered from prepubertal gilts (Zak *et al*. 1997). These results

were supported by later studies linking the degree of protein catabolism in weaned sows to a decrease in the size, number and maturity of follicles at the time of weaning, and again, the ability of follicular fluid aspirated from these follicles to support IVM of standardized pools of pig oocytes (Yang *et al*. 2000, Clowes *et al*. 2003). As the timing of feed restriction coincides with the final stages of oocyte maturation and the establishment of methylation-dependent imprinting of the oocyte (Lucifero *et al*. 2002, see detailed discussion below), imprinting of oocytes may be one mechanism by which catabolism in the sow affects embryonic survival and development by day 30. Consistent with this suggestion, Vinsky *et al*. (2007a) reported that reduced embryo development and decreased female embryo survival in litters of previously feed restricted sows were associated with differences in the variance of epigenetic traits in the surviving litters at day 30. As variance in *Igf2r* expression tended to decrease ($P < 0.07$) in female embryos in these sows, whilst variance in *Xist* expression tended to decrease in male embryos ($P < 0.08$), these authors suggested that maternally inherited epigenetic defects may cause female embryonic loss and reduced growth in all surviving embryos by day 30 of gestation. However, no changes in global methylation of blastocyst DNA, and no differences in litter sex ratios or embryonic development were evident when blastocysts were studied at day 6 of gestation (Vinsky *et al*. 2007b). Collectively, these studies suggested that a subset of litters within restrict-fed sows will be most sensitive to the latent epigenetic mechanisms that ultimately trigger loss of embryos by day 30 of gestation, but that these selective mechanisms were not evident by day 6 of gestation. A number of theories linking gender-specific loss of embryos to differences in the rate of embryonic development and species-specific survival strategies were also reviewed by Foxcroft *et al*. (2007a), but evidence supporting such theories in the pig are generally lacking.

Detailed analysis of the transcriptome and epigenome of the maturing oocyte and pre-ovulatory follicle will be an important step in defining pre-ovulatory mechanisms that program subsequent embryonic development. Studies of the complex intra-follicular signalling pathways that regulate oocyte maturation in the pig are reviewed by Hunter & Paradis in this volume (Hunter and Paradis 2009). As yet there is no evidence for major changes in these regulatory pathways that would explain differences in oocyte competence in weaned, catabolic sows, compared to sows bred in an anabolic state at their second post-weaning oestrus (Paradis *et al*. 2009). However, using the same experimental paradigm as Vinsky *et al*. (2006), we have preliminary evidence suggesting that differences in embryonic development may be linked to differences in luteal weight early in gestation (Novak S, personal communication). As discussed previously (Foxcroft *et al*. 2000), this raises the possibility that steroid-dependent modifications in the oviductal and uterine environment may be yet another component of the epigenetic mechanisms controlling pre-natal development.

Epigenetic regulation of pre- and post-natal development

The large body of literature on the interactions between metabolism, nutrition, and methylation state, suggests that similar mechanisms are likely operating in the pig. A review of mechanisms determining prenatal programming of postnatal outcomes based on epidemiological studies in human populations, and experimental studies in laboratory rodents and sheep in particular, suggest avenues for further exploration in swine. The comprehensive review of Reik (2007) on regulation of mammalian development opens with the statement: "Development is, by definition, epigenetic". The complex regulatory pathways that allow somatic cells to change from a pluripotent state and follow very different pathways of differentiation as the embryo and fetus develops are elegantly described in Fig 3 (taken from Reik 2007). Within these

developmental processes, the requirements for primordial germ cell (PGC) development, and unique methylation processes involved in sperm and oocyte development, are of particular interest. The role of methylation pathways and specific DNA methyltransferases (DNMTs) in these developmental processes was comprehensively reviewed by Kelly & Trasler (2004), and the role of methylation state and other regulatory processes was further explored by Reik (2007). A process of global demethylation (erasure) in the PGC is followed by a process of *de novo* methylation (establishment) in the developing gonads. Methylation-dependent imprinting is completed in gonocytes by the peri-natal period and several DNMTs (DNMT1, DNMT3a, DNMT3b, and DNMT1l) are needed to establish and maintain methylation imprints in both the male and female germ cells (see review of Kelly & Trasler 2004 for details). Methylation-dependent imprinting of the oocyte occurs post-natally, as antral follicles emerge from the resting pool and enter the process of follicular development. Methylation is complete by the metaphase II stage of oocyte nuclear maturation, and although an oocyte-specific isomer of DNMT1, DNMT1o, is transcribed, its role seems to be more related to maintaining maternal methylation imprints during early embryonic development, than in the initial establishment of imprinting in the developing oocyte. The maintenance of the methylated state of imprinted genes in the immediate post-fertilization period in mammals seems to be a unique phenomenon and contrasts to the period of global demethylation and then *de novo* methylation of non-imprinted genes in the period between fertilization and the blastocyst stage of development shown in Fig 3. The DNA methylation mark is stable, heritable and can regulate specific monoallelic gene expression throughout life (Kelly & Trasler 2004)

The role of DNA methylation and histone modifications in the regulation of monallelic expression of imprinted genes is of particular significance to prenatal programming. For example, maternal expression of *H19* in the female is paralleled by methylation-dependent suppression (silencing) of the paternal *H19* as part of the imprinting process, whereas maternally *H19* and $IGF2_r$ are expressed but *IGF2* is silenced. The interaction of these paternally and maternally expressed imprinted genes plays a critical role in determining the pattern of early embryonic development by regulating the biologically active pool of IGFs that regulate early embryonic and placental growth. Although much of the existing information on methylation pathways and gene imprinting has been obtained from studies with laboratory rodents, comparable information on methylation-dependent imprinting of the *IGF2-H19* complex in the pig was recently reported by Park *et al*. (2009).

Because the methylation imprint in the female is established at the time of oocyte maturation, the metabolic state of the gilt or sow during the period of follicular growth preceding ovulation can exert important effects on this methylation process. Subsequently, nutrition and metabolic state can affect methylation processes and hence the global *de novo* methylation in the early embryonic stages and methylation maintenance in later gestation. The link between nutritional state and methyl donor pathways has been clearly described in the case of folate metabolism as shown in Fig. 4, taken from the review of Kelly & Trasler (2004). In turn, the mechanistic link between these pathways and extensive literature on folic acid and other nutritional deficiencies in the sow, and adverse effects on embryonic survival and embryonic growth in the pig, are self-evident (see Foxcroft 1997).

Experimental evidence for the importance of specific nutrients as intermediaries in critical methylation pathways comes from studies in protein-restricted rats in which supplementation of the restrict diet with glycine or folic acid reversed the effects of protein restriction on *DNMT1* expression and prevented the abnormal phenotype from developing (for detailed references see Burdge *et al*. 2007). Working from the opposite perspective, in the study of Sinclair *et al*. (2007) in sheep, restricting the supply of B group vitamins (B_{12} and folate) and methionine in

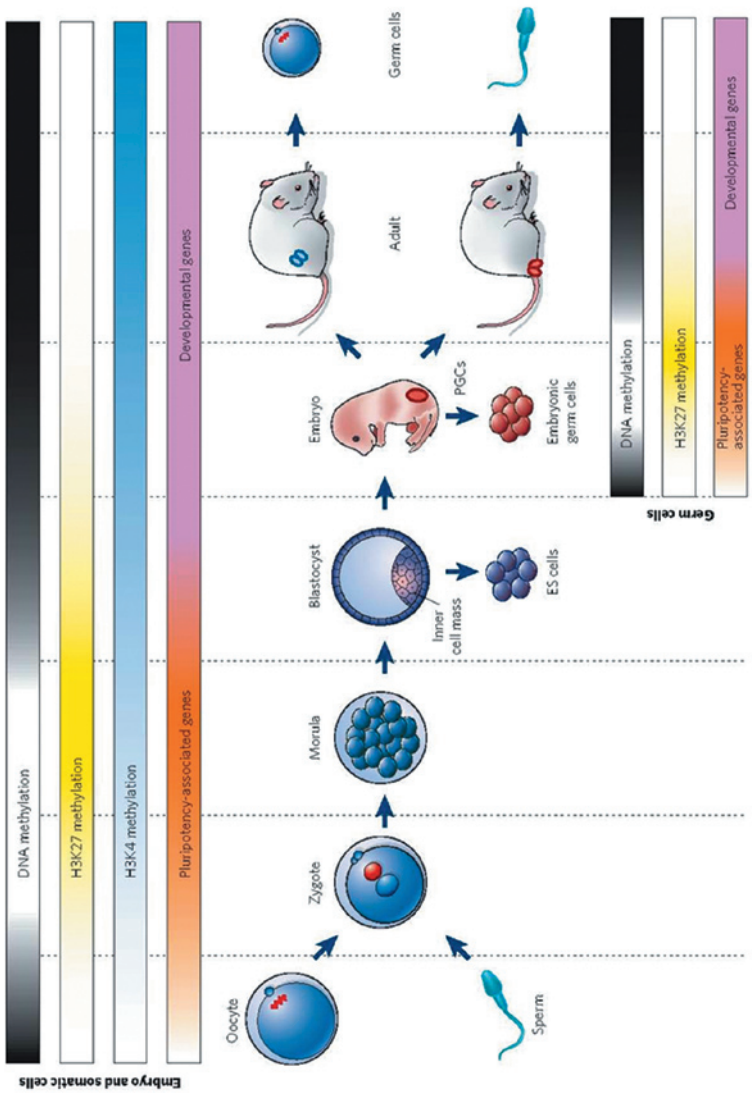


Fig. 3 Epigenetic gene regulation during mammalian development. Key developmental events are shown together with global epigenetic modifications and gene-expression patterns. Very early in development, DNA methylation is erased. In addition, pluripotency-associated genes begin to be expressed, and developmental genes are repressed by the PcG protein system and H3K27 methylation. During the differentiation of pluripotent cells such as ES cells, pluripotency-associated genes are repressed, potentially permanently, as a result of DNA methylation. At the same time, developmental genes begin to be expressed, and there is an increase in H3K4 methylation. During the early development of PGCs, DNA methylation and repressive histone modifications (such as H3K9 methylation) are also erased. Pluripotency-associated genes are re-expressed during a time window that allows embryonic germ cells to be derived in culture. Imprinted genes are demethylated during this period, and developmental genes are expressed afterwards. Flexible histone marks such as H3K27 methylation enable developmental genes to be silenced for a short time in pluripotent cells. By contrast, DNA methylation enables the stable silencing of imprinted genes, transposons and some pluripotency-associated genes. (from Reik, 2007 with permission)

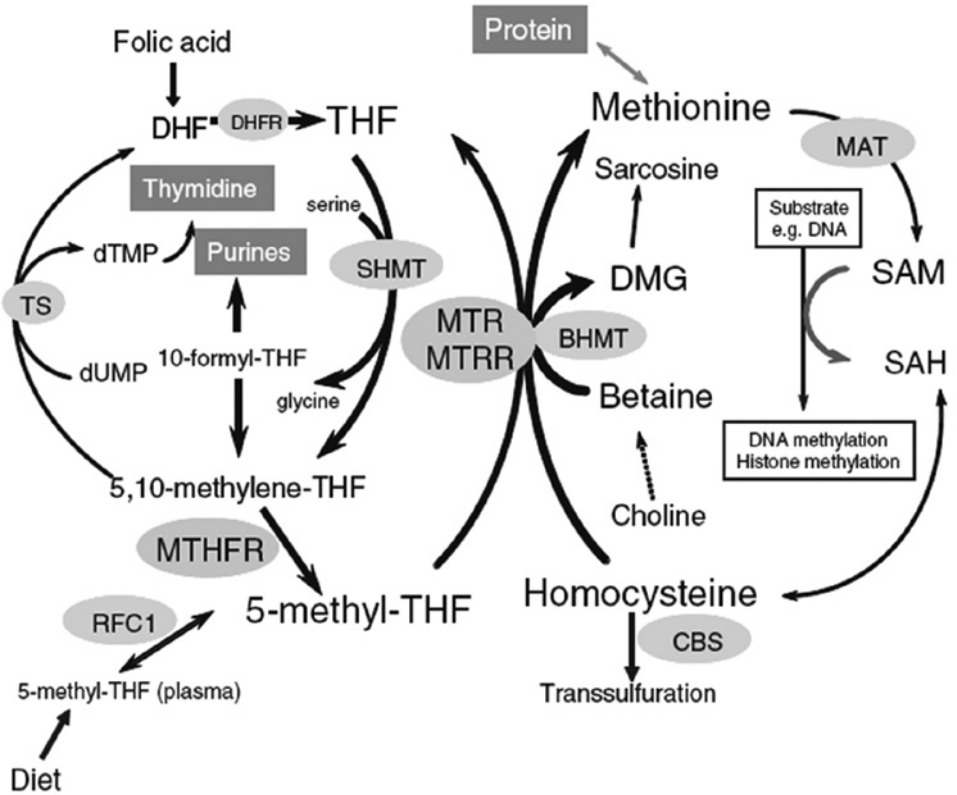


Fig. 4 The folate pathway depicting many of the enzymes involved. This pathway is intrinsic to methylation of both DNA and histones. Disruptions in this cycle can alter production of S-adenosylmethionine (SAM), which is required for methyl donation. BHMT, betaine homocysteine methyltransferase; CBS, cystathionine ß-synthase; DHF, dihydrofolate; DHFR, dihydrofolate reductase; DMG, dimethylglycine; MAT, methionine adenosyltransferase; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; MTRR, methionine synthase reductase; RFC1, reduced folate carrier 1; SAH, S-adenosylhomocysteine; SHMT, serine hydroxy methyltransferase; THF, tetrahydrofolate; TS, thymidylate synthase (from Kelly & Trasler 2004 with permission)

the diet of ewes until shortly after conception led to altered phenotypes in the offspring born to embryos coming from the nutritionally deficient dams and then transferred to normal surrogate mothers as day 6 blastocysts. The authors also reported direct evidence of altered methylation state in adult liver cells of these offspring. These studies clearly indicate the potential to study the role of similar nutrients as mediators of methylation-dependent pathways controlling phenotypic outcomes in the pig. Such nutrients might be used to counteract nutrient deficiencies resulting from limitations in voluntary feed intake in late lactation, when many younger sows enter a catabolic state because the nutritional demands of high milk production are met by mobilization of the sow's fat and protein reserves. Equally, "nutrigenomic" approaches to alleviating problems of nutrient supply to the early embryo created by intra-uterine crowding and limited placental development may be possible. The recent results of Mateo *et al*. (2007) in the gilt provided evidence of beneficial responses to L-arginine supplementation during later gestation on the number of live born pigs. Other preliminary results presented in this volume also indicate that L-arginine supplementation earlier in gestation may affect placental gene expression in mature commercial sows and could

therefore be beneficial for prenatal development. Gabler *et al*. (2007) have reported beneficial effects of feeding (n-3) poly-unsaturated fatty acids during gestation and lactation in the pig on intestinal glucose absorption measured in the offspring at weaning and it will be interesting to explore the mechanisms *in utero* that mediate these effects.

In a more general sense, nutritional manipulations, and particularly protein restriction, have been used to model the outcomes reported in classic epidemiological studies in human populations (Barker 1994). Burdge *et al*. (2007) emphasize that the concept that "the response of the fetus to nutritional cues from the mother potentially gives rise to a large number of phenotypes from one genotype" as part of a successful survival strategy. Another important concept is that whatever the mechanisms involved, these phenotypes are stable and will produce lifetime changes in the morphology and metabolic activity of the offspring. In many published studies in rodents, the link between protein restriction of the dam in gestation and altered metabolism after birth (insulin resistance, hypertension, etc) involves increased glucocorticoid receptor expression in somatic tissues, decreased expression of genes controlling corticosteroid metabolism, and indirectly an increase in hepatic enzyme activity that promotes gluconeogenesis as a response to predicted protein deficiency in the post-natal environment (see Burdge *et al*. 2007). The pathway by which protein restriction was thought to interact with glucocorticoid status to induce methylation-dependent changes in gene regulation in differentiating tissues is shown in Fig 5. In this model, a failure to maintain methylation at several levels of gene transcription ultimately leads to expression of genes (in this case in the liver) that would not normally be developmentally expressed at this stage of development if nutrition of the dam was normal.

A case for a more universal role for glucocorticoids as being "most likely to cause tissue programming *in utero*" was presented in the review of Fowden & Forhead (2004) in the context of more diverse origins of IUGR, including placental insufficiency. The diagrammatic representation of the cascade of hormonal events resulting from a change in nutritional state, and by inference changes that would also be triggered by limited placental development presented in the present review, is shown in Fig 6.

Implications for further research in the pig

The extensive literature briefly summarized above clearly links varying nutritional state of the dam, and placental competence to deliver nutrients to the developing embryo and fetus, to a wide range of possible birth phenotypes. There is also convincing evidence that the methylation state of imprinted genes that direct prenatal development are sensitive to the nutritional state of the dam and to the manipulation of specific metabolic pathways involved in maintaining methylation-dependent suppression of imprinted genes. Although the epigenetic impact of the gilt or sow "environment" on the birth phenotype of the litter is considered over a relatively short post-natal period in commercial finishing pigs, impacts on muscle development, gut development and nutrient absorption, immune status, and disease susceptibility, will have a major impact on the economic performance of the multiple phenotypes presently produced. A better understanding of the origins of these phenotypes and the extent to which they can be effectively managed will be of benefit to the pig industry. In the longer term, an understanding of the mechanisms that underlie the gene x environmental interactions that result in very different birth phenotypes from dam-line sows with essentially the same genetic merit, will allow better selection strategies to be developed for difficult to measure polygenic traits. Better management of the component traits that determine litter size, and very clearly litter quality, is an excellent example.

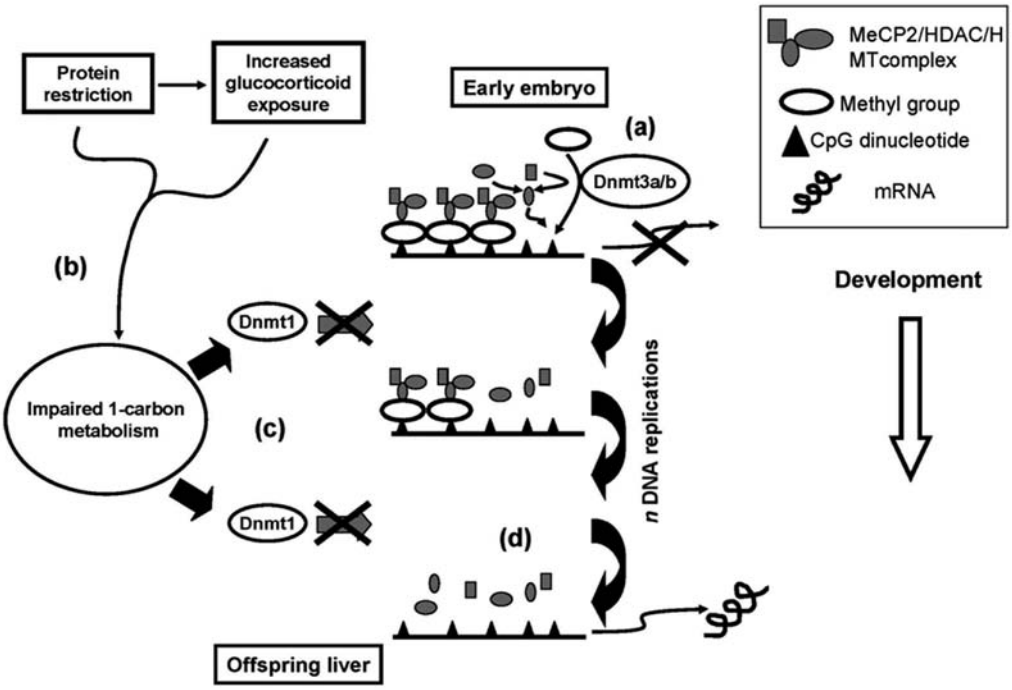


Fig. 5 A pathway for induction of altered epigenetic regulation of the expression of specific genes in the offspring of rats fed a protein-restricted (PR) diet during pregnancy. (a) Gene expression is silenced in the early embryo by the activities of DNA methyltransferases (Dnmt) 3a and 3b. (b) In the offspring of rats fed a PR diet, 1-carbon metabolism is impaired either as a direct consequence of the restricted diet or by increased glucocorticoid exposure. This down-regulates Dnmt1 expression (c). Lower Dnmt1 expression results in impaired capacity to methylate hemimethylated DNA during mitosis (d). After sequential mitotic cycles the methylation status of the promoter is reduced and expression is induced in cells which do not express the gene in control animals. Increased gene expression is facilitated by lower binding and expression of methyl CpG binding protein (MeCP)-2 and reduced recruitment of the histone deacetylase (HDAC) / histone methyltransferase (HMT) complex, resulting in higher levels of histone modifications which permit transcription. (from Burdge *et al*. 2007 with permission)

Multi-generational effects on fertility

Evidence for multi-generational carryover effects of induced phenotypes has implications for commercial pig production at the level of genetic multiplication. In theory, prenatal programming of undesirable litter phenotypes in the litters derived from gilts or sows at the multiplication level will impact the lifetime performance of the boars and gilts selected as breeding herd replacements. Such "environmental" effects of the litter of origin are very obvious in studies on the reproductive physiology of the gilt and sow, and the "litter effect" is usually controlled in better designed studies. At production level, the most rigorous selection programs for replacement gilts take account of the possible negative impact of low birth weight on lifetime performance. In many ways, this selection against low birth weight may seem counter-intuitive, because low birth weight offspring may come from the largest litters in terms of number born, and selection for increased prolificacy has been a major part of dam-line selection programs

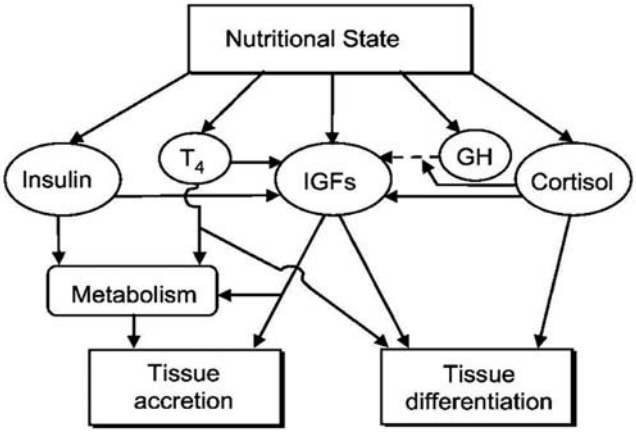


Fig. 6 Diagram illustrating the relationships between nutritional state, hormone concentrations, metabolism and tissue accretion and differentiation in the fetus. (from Fowden & Forhead, 2004 with permission)

for many years. On the positive side, the phenotypic data collected from existing commercial dam-lines, as shown in Fig. 1, shows the potential to control the gene x environment interaction and produce good sized litters that have a birth weight in the 1.5 to 2.0 kg range.

In terms of boar selection for placement in artificial insemination studs, similar effects of birth litter may have an important influence on lifetime sperm production. As in other organ systems, brain sparing effects in low birth weight male littermates should limit their testis development. Preliminary data supporting this suggestion is presented in Table 5 (FCRL Almeida, personal communication). In that particular study, new-born male littermates born to parity 4 to 6 sows, and in litters of 10 to 15 total pig born, were identified as falling into high (HW: range 1,800 to 2,200 g) and low (LW: range 800 to 1,200 g) birth weight groups relative to the average birth weight of their litter. A subset of 20 boars from each birth weight group was necropsied at birth and evidence of IUGR was confirmed by establishing that all brain:organ weight ratios were negatively impacted in LW versus HW offspring. Differences in testicular weight were associated with differences in the number of gonocytes and Sertoli cells per testicular cord.

Table 5. Mean birth weight, weight at castration (day 7), and gonadal data for male pigs born in litters of 10 to 15 and classified as high and low birth weight (bw) relative to the average birth weight of their litters. (FCRL Almeida, personal communication)

Parameter	Low bw	High bw	SEM	P
Birth weight (kg)	1.17	2.02	0.014	< 0.05
Weight at day 7 (kg)	2.03	3.30	0.029	< 0.05
Right testis weight (g)	0.42	0.95	0.012	< 0.05
Left testis weight (g)	0.46	0.98	0.018	< 0.05
Mean testicular weight, (g)	0.44	0.97	0.014	< 0.05
Right epididimal weight (g)	0.20	0.31	0.009	< 0.05
Left epididimal weight (g)	0.20	0.27	0.007	< 0.05
Gonocytes / testicular cord	0.87	1.58	0.090	< 0.05
Sertoli cells/ testicular cord	19.22	22.36	0.419	< 0.05

This above mentioned study provides some of the first evidence for significant impacts of birth weight on the population of spermatogonial stem cells (gonocytes) in the neonatal testis. Assuming that these results will be confirmed at the multiplication level in sire-line selection programs, the implications of birth weight for lifetime sperm production seem real. This suggests that prenatal programming of testis development will already predetermine the reported relationship between adult testis size and lifetime semen production.

Demands on experimental designs and genomic-epigenomic analyses

The review of Distl (2007) considered future trends in genomic selection that might address: (1) the current trends for hyperprolificacy to be invariably associated with low birth weight, and (2) the implications of overcrowding in the uterus in early gestation for later fetal and postnatal development. One of the conclusions in this paper was that: "Understanding of this "*polygenic paradox*" requires expanding the study design from studying single genes or proteins to use whole genome or protein approaches and to evaluate their multiple epistatic effects".

As we start to apply such multi-faceted analyses to understand the link between component traits for litter quality (ovulation rate, embryonic and fetal survival, several component traits for uterine capacity) and the genomic and epigenomic control of these phenotypic traits, the complexity of the experimental approach needed becomes equally complex. This seems to be a particularly difficult problem when deciding which tissues and from which litters, and even from which gender of littermate within litters, to submit to microarray analysis. For example, if the question relates to gene regulation in the placenta or embryo *in utero* in response to nutrient manipulations, the confounding combination of "noise" due to variable ovulation rate, embryo survival, uterine capacity, relative embryonic and placental development, and gender-specific effects needs to be accounted for in meaningful interpretation of microarray data. Our initial conclusion is that the complexity and elegance of the experimental designs needed to meet these challenges will a key element in the success applying "omic"- level analyses to the complex problems of prenatal programming.

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Cellular and molecular events in early and mid gestation porcine implantation sites: a review

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Commercial, North American pork breeds (*Sus scrofa*) experience significant loss of genetically-normal conceptuses during the peri-implantation (attachment) period and at mid-gestation (day 50 to 90 of the 114 day porcine gestation interval). Although exact causes for these losses are not defined, asynchronous *in-utero* development and deficits in vascularization of the endometrium and placenta appear to be involved. Understanding of normal maternal-fetal dialogue is critical to develop breeding or therapeutic strategies that improve fetal health and overall litter size in commercial pigs. The non-invasive, epitheliochorial porcine placenta permits investigation of maternal or fetal compartments without cross contaminating cells. We developed and use protocols to capture single, homogenous populations of porcine cells (endometrial lymphocytes, dendritic or endothelial cells) from histological sections using laser capture microdissection (LCM), a powerful tool for study of gene expression that reflects the *in vivo* environment. These data are compared with gene expression in biopsies of endometrium and of trophoblast from the same, attachment sites. Here we review justifications for selection of the genes we have studied and our published and in progress work. These data provide new insights into the roles of the endometrial immune environment in the regulation of the success and failure of porcine conceptuses.

Introduction

Prenatal mortality is a major economic concern for commercial swine producers in North America. In gilts, approximately, 20 to 30% of conceptuses die between gestation days (gd)12-30 and another 10-15% are lost by midgestation (114 day pregnancy, Pope *et al*. 1986, Pope 1988, Pope 1994). The ovulation rate in these animals is 16 to 18 with > 95% fertilization. This should result in 14 to 17 embryos at the beginning of pregnancy. By farrowing however, litters are reduced to ~10 (Pope 1994). The pig uterus is estimated to have the capacity to carry 12 to 14 fetuses to term (Freking *et al*. 2007).

Conceptus-derived growth factors supplemented by histiotrophic nutrition derived from the maternal uterine glands (Spencer & Bazer, 2004) support pre-elongation conceptuses. Growth and development of post-attachment (>gd15) conceptuses require endometrial-placental interactions that greatly expand local, through attachment site endometrial angiogenesis. Most

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porcine gestational losses occur as elongating conceptuses start secreting estrogen to promote endometrial attachment (Geisert *et al*. 1982). It is difficult to quantify losses of elongating conceptuses between gd13 to 20 due to their extreme fragility (Pope 1994). Trophoblast-derived estrogen is considered to signal maternal recognition of pregnancy and alters the endometrium to support attachment (Geisert & Yelich 1997). The estrogen secreted by the earliest elongating blastocysts creates a hostile endometrial *milieu* for less advanced conceptuses. Asynchronous development is a major factor influencing the first peri-attachment wave of conceptus loss (Pope 1994). A mixture of interferons (IFN) unique to porcine trophoblast is produced by early, peri-attachment embryos. Immune IFNG and IFND reach peak levels at gd15 (La Bonnardière *et al*. 1991, Cencic & La Bonnardière 2002). Porcine trophoblastic IFNs, unlike IFNT in cattle and sheep, do not act on corpora lutea but alter polarity of the uterine epithelium through the gain of basal tight junctions during conceptus attachment (Cencic *et al*. 2003).

In other species, roles have been identified for the immune system in promotion of implantation and conceptus survival as well as in conceptus demise (Raghupathy 2001, Croy *et al*. *2006,* Seavey & Mosmann 2008). In humans, pregnancy success is associated with a switch from a type 1 dominant, pro-inflammatory cytokine profile (the normal non pregnant state) to a type 2 dominant, anti-inflammatory profile in blood and in endometrium between mid to late pregnancy (Raghupathy 2001, Borzychowski *et al*. 2005, Aris *et al*. 2008).). Type 1 cytokine dominance in later gestation is considered to compromise pregnancy success (Raghupathy & Kalinka 2008). As in humans and mice, early porcine pregnancy is dominated by pro-inflammatory type I cytokines such as IFN-γ and TNF-α (Croy *et al*. 2006, Tayade *et al*. 2007, Curry *et al*. 2008). In addition to creating the endometrial cytokine *milieu*, the endometrial immune systems of humans and mice are strongly angiogenic in early gestation. The studies reviewed and reported here establish that immune cells of early porcine gestational endometrium make important, exquisitely localized contributions to conceptus attachment sites (CAS) that regulate conceptus fates.

Transcripts for genes regulating angiogenesis in porcine endometrium and CAS

VEGF and VEGFR-mediated angiogenesis at porcine implantation sites

Angiogenesis is the process of generation of new blood vessels from existing vasculature. It requires endothelial cell activation, tip cell differentiation, stalk cell proliferation and lumen formation. Maturation of new blood vessel is accompanied by recruitment of pericytes, smooth muscle cells and circulating bone-marrow derived cells (Takakura 2006, Grunewald *et al*. 2006). This is coupled with formation of an underlying basement membrane (Holderfield & Hughes 2008). Extensive, localized endometrial angiogenesis occurs during pregnancy to support each developing conceptus and its placental exchange system.

Angiogenesis is regulated by variety of growth factors but VEGF and the VEGF receptor (R) system appear to be the most important and have been targeted clinically in humans (Loges *et al*. 2009). VEGFs are a family of heparin binding growth factors. In humans, at least eight *VEGF* isoforms (*VEGF121, VEGF145, VEGF148, VEGF165, VEGF165b, VEGF183, VEGF189, and VEGF206*) are generated by alternative splicing of a single *VEGF* mRNA (Breen 2007). Similarly, eight *VEGF* splice variants have been identified in pigs. Of these, splice variants, *VEGF*120, 144, 164, 164b, 188 and 205 are exactly one amino acid shorter than the human variants while *VEGF* 147 and 182 are unique porcine isoforms (Ribeiro *et al*. 2007). VEGF signals through VEGFRI, VEGFRII and VEGFR III and binds additionally to soluble VEGFRI (sVEGFRI), a splice variant of VEGFRI and to neuropilin-1 (NRP1). VEGFRI acts primarily as a regulator of VEGF availability (Holderfield & Hughes 2008, Zygmunt *et al*. 2003). Soluble VEGFRI, which is elevated in human and mouse pregnancy complications (Levine *et al*. 2004),

prevents VEGF signaling by sequestering VEGF (Zygmunt *et al*. 2003, Breen 2007). VEGF promotes migration of vascular smooth muscle cells through its binding to VEGFRI and to NPR1 (Banerjee *et al*. 2008). It induces endothelial cell migration and proliferation primarily through binding to VEGFRII (Holderfield & Hughes 2008). VEGFRII is the dominant receptor promoting endothelial cell permeability. Placenta growth factor (PGF) is a high affinity ligand for VEGFR1 (Carmeliet 2001).

To understand the dynamic features of angiogenic gene expression at the porcine maternal-fetal interface, mRNA comparisons for *VEGF, PGF, VEGFRI* and *VEGFRII* were carried out in various endometrial tissue microdomains and in endometrial biopsies and trophoblasts at gd20 and 50 from healthy conceptus attachment sites (H-CAS) and arresting conceptus attachment sites (A-CAS). Details of sample processing and techniques are summarized in Fig. 1 and are previously published (Tayade *et al*. 2006, 2007, Linton *et al*. 2008). Angiogenic gene profiles were studied by quantitative real time PCR analyses relative to housekeeping gene, *ACTB* (β-*actin)*. Transcripts for *VEGF, PGF, VEGFRI* and *VEGFRII* were detected in virgin uterus but their abundance differed between mesometrial and anti-mesometrial sides. In pregnancy, A-CAS at both gd20 and 50 had severely reduced numbers of *VEGF* transcripts in endometrial biopsies compared with time-matched endometrium from H-CAS. *VEGF* transcripts in trophoblast were relatively stable between H-CAS and A-CAS. These differences suggested the maternal compartment had sensed "danger" signals emitted from individual conceptuses and was adjusting to promote demise of that conceptus while the conceptus maintained *VEGF* transcription in its trophoblast for survival. Transcripts for *VEGFRI and II* were variable between H-CAS and A-CAS during both gd20 and 50 time points (Tayade *et al*. 2007).

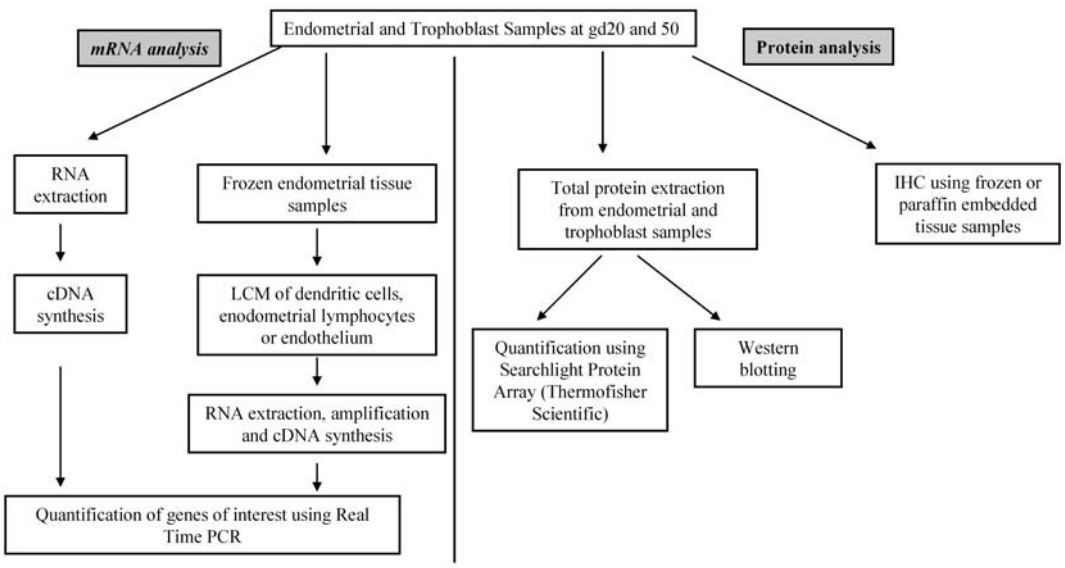


Fig. 1 Diagrammatic representation of sample utilization in various downstream applications. Each sample is processed either for mRNA or protein analysis as described in Tayade *et al*. 2006, 2007. All samples were collected from specific pathogen-free Yorkshire gilts housed at Arkell Swine Research Station, University of Guelph. Littermate H-CAS and A-CAS were identified based on gross fetal membrane vasculature at gd20 and length/weight parameters at gd50 (Tayade *et al*. 2006, 2007). The data were analyzed (unless otherwise stated) by PROC MIXED procedure of SAS using a mixed linear model that included the effects of gestation date, tissue, and health, as well as their interactions.

The numerous VEGF isoforms bind endothelial cell receptors that include the VEGFRI and VEGFRII signaling tyrosine kinase receptors and the non-signaling co-receptor NRP1. NRP1 and NRP2 were identified in 1995 in the nervous system as receptors for class 3 semaphorins (SEMA) which repel axonal outgrowth (Satoda *et al*. 1995, He & Tessier-Lavingne 1997, Kolodkin *et al*. 1997). They were subsequently found to act in endothelial cells as receptors for several pro-angiogenic factors, including $VEGF_{165}$ (Soker *et al*. 1998), PGF (Soker *et al*.1998) hepatocyte growth factor (Sulpice *et al*. 2008), and galectin1 (Hsieh *et al*. 2008). Interestingly, NPR1 as well as VEGF and VEGFRII are up-regulated on endothelial cells in hypoxic conditions (Ottino *et al*. 2004).

While NPR1 and NPR2 are only 45% homologous, they are structurally similar with five extracellular domains, each of which has specific binding partners and a short cytoplasmic tail incapable of signal transduction. The a1 and a2 domains bind SEMAs. The b1 and b2 domains bind VEGF isoforms and heparin and are implicated in cell-cell adhesion. The c domain is responsible for dimerization. As shown in Table I, NPRs also differ in their VEGF and SEMA binding partners. NPRs themselves are non-signaling co-receptors which require complexing with receptors with signaling capability to effect downstream functions. NPR1, which is expressed on endothelial cells, is associated with angiogenesis and arterial differentiation while NPR2, expressed in veins and lymphatic vessels, is involved in lymphangiogenesis (Karkkainen & Alitalo 2002, Yuan *et al*. 2002).

Table I. Neuropilin (NRP) Interactions

Gene	Pro-angiogenic Ligands	Pro-angiogenic Complex Partners	Anti-angiogenic Complex Partners	Anti-angiogenic Ligands
NRP1	VEGF165, VEGF-B, VEGF-E, PGF, HGF, PDGFBB, FGF2, TGF-β, galectin1	VEGFRI, VEGFRII,	PLXNA1, PLXNA2, PLXNA3, PLXNA4, PLXND1, integrin β1, LICAM	SEMA3A SEMA3B SEMA3C SEMA3D
NRP2	VEGF145, VEGF-C, PGF, HGF	VEGFRI, VEGFRII, VEGFRIII,	PLXNA1, PLXNA2, PLXNA3, PLXNA4, PLXND1, NRCAM	SEMA3C, SEMA3D SEMA3F, SEMA3G

HGF: hepatocyte growth factor; LICAM: neural cell adhesion molecule L1 (LICAM); NRCAM: neuronal cell adhesion molecule.

NPRs bind Class 3 SEMA, which are a large and diverse family with pivotal roles in axon guidance, organogenesis, neoplastic transformation, immune responses, vascularization and angiogenesis (Soker *et al*. 1998). SEMA functions through two receptor families; the NRPs and the plexins (PLXN). SEMA3s, a seven member family, are the only secreted semaphorins. Six SEMA3s bind to NRP1, NRP2 or both (see Table I).The seventh, SEMA3E, binds to the receptor plexin-D1, which in turn complexes with NRPs (Kolodkin *et al*. 1997). SEMA3A and SEMA3F bound by NRP and complexed with a plexin (PLXN) family member are anti-angiogenic because the complex suppresses endothelial cell migration (Guttmann-Raviv *et al*. 2007). Similarly, complexing NRPs and PLXN with SEMA3B and SEMA3F inhibited adhesion, migration and proliferation of lung cancer-derived tumor cells but the mechanism of these interactions is unknown. SEMA3A, SEMA3D, SEMA3E, and SEMA3G also have anti-tumorigenic properties and independently reduce the density of blood vessels in tumors (Neufeld & Kessler 2008).

The roles of NRPs in blood vessel development during embryogenesis and tumour progression are well-studied. However, their roles in the only normal angiogenesis found in adults, the cycling female reproductive tract, have not been thoroughly addressed. Pavelock *et al*. found that physiological variations in different sex hormones, particularly progesterone, increased

NPR1 mRNA in rat uterus (Pavelock *et al*. 2001). These data gave us the impetus to quantify expression of NPRs and their binding and complexing partners in the virgin and pregnant porcine uterus. Transcripts of *NPR1* and *NPR2*, *SEMA*-A3 *C3, E3, F3* and *G3* as well as *PLXN A1, A2, A3, A4* and *D1* were detected in the virgin pig uterus as well as in endometrial and trophoblast samples at gd20 and 50. Quantification of these genes is in progress (M van den Heuvel, unpublished observations).

Lymphocytes and dendritic cells in the promotion of endometrial angiogenesis during pregnancy

We found porcine endometrium of early pregnancy contained mildly elevated (~ 3x) numbers of uterine natural killer (uNK) cells, a unique lymphocyte subset, between gd15 to 28. During pregnancy, these cells were found scattered throughout the stroma, beneath luminal epithelium, around blood vessels and uterine glands (Engelhardt *et al*. 2002). This interval coincides with onset of angiogenesis at CAS. In contrast to the recruitment of uNK cells in humans and mice that is driven by decidual cells, porcine uNK cells were not recruited in pseudopregnancy but required conceptus-derived signals that are not yet identified (Engelhardt *et al*. 2002). Porcine uNK cells are agranular and their identification required dual antibody staining against CD16 and CD8 surface receptors to distinguish them from T cells. Recently, Dimova *et al*. (2007) examined changes in endometrial T cells (CD4 and CD8) during early porcine pregnancy. T-cells formed clusters in areas of conceptus attachment but no significant differences were found in their numbers at or between CAS. T lymphocytes increased in the sub-epithelium from 15% of leukocytes in the pre-attachment phase to 54% right after attachment at gd15. Maximal T lymphocyte numbers reached 85% at gd30 after the formation of placenta (Dimova *et al*. 2007). In our studies, we noticed scattered lymphocytes in the luminal epithelium but due to lack of commercially available porcine specific monoclonal antibodies, phenotypic characterization of the intra-epithelial lymphocytes could not be carried out. Thus, our studies of gene expression in endometrial lymphocytes have not yet been refined to address lineage subsets. We employ frozen endometrial tissue sections rapidly stained with haematoxylin and eosin and laser capture microdissection (LCM) to collect pure populations of either lymphocytes or dendritic cells (DC; Tayade *et al*. 2006, Linton *et al*. 2008) to address our hypothesis that immune cells contribute to and regulate endometrial angiogenesis during porcine pregnancy.

Endometrial lymphocytes obtained from healthy and arresting conceptus attachment sites at gd20 and gd50 were screened for the expression of *VEGF, PGF, VEGFRI and VEGFRII* by quantitative real time PCR. Endometrial lymphocytes had a much greater abundance of *VEGF* transcripts than endometrial endothelial cells or trophoblasts. Attachment sites associated with arresting conceptuses had a severe reduction in *VEGF* and gain in *PGF* transcripts in lymphocytes. Lymphocytes preferentially expressed transcripts for *VEGFRI* whereas trophoblasts had abundant *VEGFRII* transcripts showing there are differences in the mechanisms that regulate angiogenesis in the maternal and fetal compartments (Tayade *et al*. 2007).

In humans, a population of immature dendritic cells was identified in decidua via their surface expression of DC-SIGN (Dendritic cell specific ICAM grabbing non-integrin, DC-SIGN) (Kammerer *et al*. 2003, 2008). These antigen presenting cells were in contact with uNK cells in the vicinity of spiral arteries, an interaction requiring DC-SIGN on the DCs and ICAM-3 on the uNK cells (Kammerer *et al*. 2003). Because uNK cells secrete abundant VEGF but do not express VEGFRI or II (Li *et al*. 2001), we hypothesize that endometrial DC-SIGN$^+$ DCs and uNK cells may additionally communicate via VEGF and its receptors. This would also predict perivascular co-localization of these cell types to sites of active endothelial cell proliferation (Grunewald *et al*. 2006). Using a cross-reactive anti-human DC-SIGN antibody, we identified DC-SIGN + DCs

in porcine endometrium. In contrast to human endometrium where the cells are only present in pregnancy, both virgin and gestational porcine endometrium contained DC-SIGN+ cells (Linton *et al*. 2008). In pregnancy, DC-SIGN+ cells were frequently associated with lymphocytes near blood vessels. The numbers of DC-SIGN+ cells appeared to be stable between early and mid pregnancy. Using a modified immunohistochemistry protocol for rapid staining, we immunostained DC-SIGN+ DCs in frozen porcine endometrial sections, isolated them using LCM and recovered RNA for analysis. We report for the first time that porcine DC-SIGN+ DCs transcribe angiogenic factors (*VEGF, VEGFRI and II*) and thus contribute to the regulation of angiogenesis at CAS (Linton *et al*. 2008 and N F Linton, unpublished observations).

Roles of chemokines and chemokine decoy receptors at the porcine maternal fetal interface

Chemokines are families of small cytokines characterized by the presence of four conserved cysteine molecules. They range from 8 to 11 kDa and are active over a 1 to 100 ng/ml range in concentration. The major function of chemokines is to direct immune cell movement towards chemotactic stimuli (Charo & Ransohoff 2006). Chemokines are classified as CC, CXC, CX3C or XC (Charo & Ransohoff 2006). Almost all somatic cell types synthesize pro-inflammatory as well as homeostatic chemokines. Chemokines can be induced by variety of stimuli including viruses, bacteria, pro-inflammatory cytokines, anaphylatoxin C5a, leukotriene B_4, and IFNs (Drake *et al*. 2002, Huang *et al*. 2006). Although their main function is chemo-attraction, they also participate in angiogenesis, haematopoiesis, and regulate activation, proliferation, differentiation, and apoptosis in the cells they attract (Drake *et al*. 2002, Hannan *et al*. 2006).

Chemokine decoy receptors are non-signaling, membrane-bound receptors. They are responsible for regulation of cell trafficking, inflammatory responses, immune reactions, angiogenesis, and apoptosis by internalizing and degrading chemokines (Mantovani *et al*. 2001, Mantovani *et al*. 2003, Borroni *et al*. 2008). In humans and mice, three chemokine decoy receptors have been characterized. D6 and DARC (duffy antigen receptor for chemokines) bind inflammatory chemokines while CCX CKR binds homeostatic chemokines (Gosling *et al*. 2000, Townson & Nibbs 2002). In humans and mice, D6 is expressed by invasive trophoblast cells (Montavani *et al*. 2003, Martinez de la Torre *et al*. 2007). D6 knockout mice are fertile but have compromised pregnancy outcome if inoculated with lipopolysaccharide or auto-immune human anti-phospholipid antibodies during pregnancy (Martinez de la Torre *et al*. 2007). DARC and CCX CKR are also found in human and mouse placentas (Girard *et al*. 1999, Townson & Nibbs 2002, Martinez de la Torre *et al*. 2007). However, roles for decoy receptors and their binding chemokines have not been characterized in spontaneous fetal loss or in pregnancies with non invasive trophoblast. We have investigated selected members of these molecular families that are expected in porcine pregnancy.

Transcripts for chemokines that expected to bind to porcine D6 (*CCL2, CCL3L1, CCL4, CCL5* and *CCL11*), DARC (*CCL2, CCL5, CCL11* and *CXCL2*) or CCX CKR (*CCL21*) were assessed in paired gd20 and 50 endometrial and trophoblast samples from H-CAS and A-CAS. Transcripts were found for all of the genes in gd20 and gd50 endometrium and trophoblast. No significant differences were found in the expression of these chemokines between healthy and arresting conceptus attachment sites (Wessesl *et al*. 2007 and J M Wessels, unpublished observations).

Endometrial transcripts for *D6* were more abundant than those in trophoblast at gd20 and 50. Transcript numbers were stable at healthy versus arresting sites. In contrast, transcripts for the homeostatic decoy receptor *CCX CKR* were elevated in gd50 trophoblasts and endometrium

from A-CAS (J M Wessels, unpublished observations). Both molecules were localized by immunohistochemistry to uterine epithelium at the CAS. Thus, the importance of inflammation in gestational failure in pigs appears to be much less than in mice while dysregulation of porcine homeostasis may have a greater role in both maternal and fetal tissues. The role of homeostatic chemokines decoy receptors in pregnancy failure or success in other species has not been studied yet. Thus, the relevance of these findings for species with non epitheliochorial placentation is currently unknown and needs to be defined.

Role of pro-inflammatory cytokines at the porcine maternal fetal interface

The adverse effects of elevated pro-inflammatory cytokines during human and mouse pregnancy are extensively studied (Polgar & Hill 2002, Dent 2002, Patrick & Smith 2002). Several reports linked TNFA, combined with IFNG and IL-1B in promoting pathology and pregnancy failure. In women with recurrent spontaneous abortions, synergistic effects of TNFA and IFNG lead to endothelial cell injury, reduced blood supply and subsequent embryonic death (Stemmer 2000). In addition to the deficits in endometrial angiogenesis described above at porcine A-CAS, we have documented elevations in proinflammatory cytokine gene transcripts (*IFNG*, *TNFA* and *IL-1B*). Endometrial lymphocytes appeared to switch transcription abruptly from angiogenic to pro-inflammatory cytokine genes.

Based on these findings we proposed paradigm shifting functions for endometrial lymphocytes (Leonard *et al*. 2006). We feel the elevated pro-inflammatory cytokines attack maternal endothelial cells ultimately restricting blood supply to an already stressed conceptus rather than envisioning trophoblast as the target of immune attack. This raises the possibility that elevations in pro-inflammatory cytokines are an aftermath of impending conceptus death. In pigs, this immune signaling is unlikely to be an immune scavenging signal because we did not observe signs of inflammation (infiltration of neutrophils/lymphocytes) at A-CAS at gd20 or 50. Definition of this cause and effect relationship will require further study. We found significantly elevated expression of *IFNG* in endometrial lymphocytes and trophoblasts collected from gd20 A-CAS but not in gd50 A-CAS. Rather, *TNFA* was elevated in lymphocytes obtained from gd50 A-CAS. The dominance of *IFNG* during early loss (gd20) and *TNFA* during mid gestational loss (gd50) suggests distinct immune mechanisms effect conceptus health at different stages of gestation (Tayade *et al*. 2007).

Insulin like growth factors 1 and 2 in porcine fetal loss

Insulin like growth factors (IGF-1 and IGF-2) are small polypeptides of approximately 7kDa implicated in regulation of fetal and placental growth. They promote cellular differentiation, proliferation and migration and inhibit apoptosis. These processes are involved in the extensive remodeling that occurs during development of the placenta and its endometrial attachment site. The IGFs bind with high affinity to IGF receptors, namely IGF1R and IGF2R. IGF1R is a member of the tyrosine kinase family and is structurally related to the insulin receptor (Jones & Clemmons 1995, Butler & LeRoith 2001). IGF1R binds with equal affinity to both IGF-1 and IGF-2, where as IGF2R binds only IGF-2 with high affinity (Pollak 2008). The bio-availability and biological actions of IGFs are regulated by at least six insulin-like growth factor binding proteins (IGFBP1 to 6, Clemmons 1997). IGF ligation to binding proteins may augment or inhibit IGF action. Several proteases cleave IGFBPs, reducing or eliminating their ability to bind IGFs. From a series of knockout studies, it is clear that IGF2 rather than IGF1 plays important roles in mouse placental and fetal development (Baker *et al*. 1993).

The IGF system has been extensively studied during the porcine estrous cycle and in early pregnancy (Simmen *et al*. 1992, Ashworth *et al*. 2005). *IGF-1* mRNA decreased while *IGF-2* mRNA increased as pregnancy advanced. Highest levels were found in the placenta followed by endometrium and myometrium (Simmen *et al*. 1992). There is a spatiotemporal relationship between increased uterine IGF-1 and IGF-2 and estrogen synthesis by the elongating conceptuses at gd10 to 13 (Letcher *et al*. 1989, Geisert *et al*. 2001). Increased uterine IGF is associated with aromatase production for trophoblast estrogen synthesis (Green *et al*. 1995). Ashworth *et al*. (2005), documented premature loss of IGFs during the period of conceptus elongation by early exposure of pregnant gilts to estrogen. This loss of IGFs was attributed to proteolysis of IGFBPs due to endocrine disruption caused by exogenous administration of estrogen at the time of conceptus elongation (Ashworth *et al*. 2005).

We addressed whether IGF-1 and 2 are directly linked with porcine conceptus arrest at gd20 and 50. Transcripts for *IGF-1* and *IGF-2* were quantified in trophoblast and endometrial biopsies at gd20 and gd50 by real time PCR and expressed as a ratio of target gene (*IGF*) to *ACTB* (Fig. 2A and B). At gd20, trophoblast from A-CAS had fewer *IGF-1* transcripts than trophoblast from H-CAS. At gd50, trophoblasts had more transcripts than gd50 endometrial samples ($P < 0.05$). In gd20 endometrial samples (Fig. 2A), *IGF-1* was more abundant than in gd50 endometrium ($P < 0.05$). These data are consistent with other reports that *IGF-1* declines as pregnancy advances (Simmen *et al*. 1992, Ashworth *et al*. 2005). *IGF-2* transcripts were 10-fold higher in both endometrial and trophoblast samples than *IGF-1* transcripts. No significant differences were found in either tissue at gd20 or gd50 related to conceptus health status. Our results are in agreement with previous reports that *IGF-2* is predominantly expressed over *IGF-1* during porcine pregnancy (Simmen *et al*. 1992). Our studies did not provide any evidence that IGFs are directly linked with porcine fetal arrest. More comprehensive studies that includes IGF receptors and IGFBPs are in progress.

Final remarks

Our molecular interrogation of endometrial attachment sites of live fetal littermates whose gestational fates differ, has identified clear roles for immune cells, both lymphoid and dendritic, in promotion of endometrial angiogenesis within CAS. Coincident with conceptus arrest, changes occur in the immune system that could have primary and/or secondary roles in the death of specific fetuses. The immune system appears to respond to fetal distress by removing vascular support for that CAS and locally elevating pro-inflammatory cytokines. These changes may be induced in resident cells because no significant alterations were found in chemokines ligands binding to pro-inflammatory decoy receptors or in the decoy receptor D6 between H-CAS and A-CAS. Alterations in expression of the homeostatic decoy receptor CCX-CKR between H-CAS and A-CAS suggest a new regulatory pathway that may contribute to porcine pregnancy success.

Acknowledgements

This research was supported by Ontario Pork, OMAFRA, NSERC, Agriculture and Agrifood Canada and the Canada Research Chairs Program.

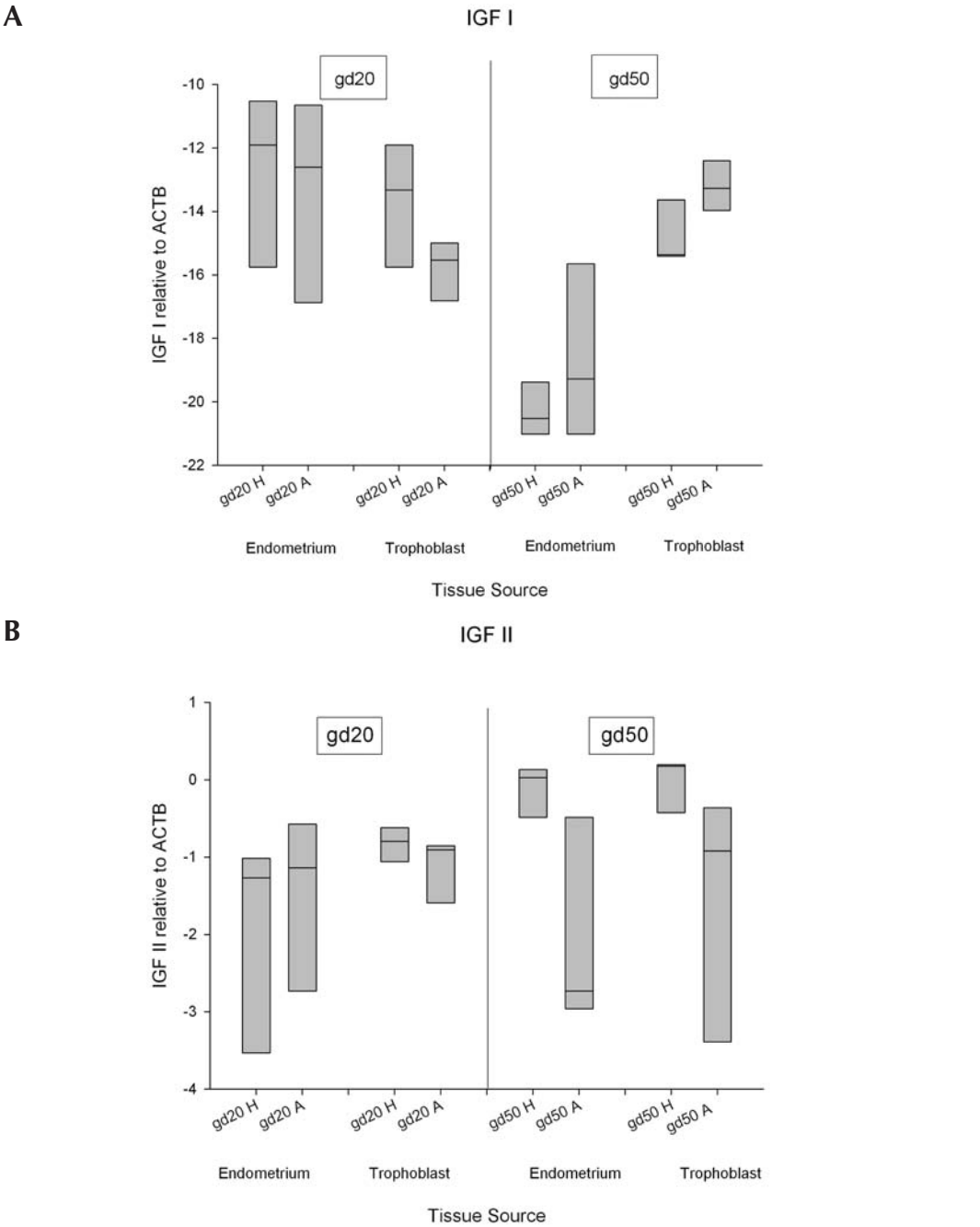


Fig. 2 Expression of *IGF1* (A) and *IGF2* (B) in porcine endomerium and trophoblasts at gd20 and 50. Expression was quantified by real time PCR using RelQuant software (Roche) and is relative to the housekeeping gene, *ACTB*. The LightCycler program was denaturation at 94 °C, 15 min; PCR amplification and quantification (95 °C, 10 s; 58 °C, 5 s; 72 °C, 20 s) with the fluorescence measurement at specific acquisition temperatures for 5 s, repeated for 45 cycles. Statistical analysis was performed using one way ANOVA and pair-wise multiple comparison procedure as per Holm-Sidak method. The bar in each box represents the data median with 6 samples per group. $P < 0.05$ was considered significant. gd: gestation days, H: healthy, A: arresting.

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Functional genomic approaches for the study of fetal/placental development in swine with special emphasis on imprinted genes

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This chapter describes the application of functional genomic approaches to the study of imprinted genes in swine. While there are varied definitions of "functional genomics", in general they focus on the application of DNA microarrays, single nucleotide polymorphism (SNP) arrays, and other high coverage genomic analyses, and their combination with downstream methods of gene modification such as silencing RNA (siRNA) and viral and non-viral transfection. Between the initial data acquisition and the actual genetic manipulation of the system lies bioinformatics, where massive amounts of data are analyzed to extract meaningful information. This area is in constant flux with an increased emphasis on detection of affected pathways and processes rather than generation of simple affected gene lists. We will expand on each of these points and describe how we have used these technologies for the study of imprinted genes in swine. First we will introduce the biological question to provide context for the discussion of the functional genomic approaches and the types of information they generate.

Part I. The biological question

While over 99% of genes in mammalian species are transcribed from both maternal and paternal alleles (bi-allelic expression), a small subset are transcribed from only one allele (mono-allelic expression). In some cases it is the maternal allele that is transcribed and in others the paternal allele. The choice of which allele is transcribed is dependent on markings placed in the chromosome during gametogenesis (Hajkova *et al*. 2002 , Reik & Walter 2001). To date less than 100 imprinted genes have been identified, yet they have profound phenotypic effects, particularly in placental and fetal development and function (Angiolini *et al*. 2006). Yet, their role is not limited to fetal and placental development but can also affect other aspects of reproduction such as rearing behavior and lactation as will be described later. Our interest in these genes came about through the reports of abnormal placentation and fetal overgrowth of somatic-cell-nuclear-transfer-derived calves (Hill *et al*. 1999). The combined syndrome has been referred to as abnormal offspring syndrome (AOS) as well as large offspring syndrome

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(LOS) (Farin *et al.* 2004 , Farin *et al.* 2006). Multiple laboratories working in this area reported epigenetic abnormalities in cloned cattle and mice, including disregulation of imprinted genes (Dindot *et al.* 2004). It was through these original observations that we became interested in this complex and fascinating group of genes, and at the same time dismayed by the almost total lack of information of their function in swine.

Evolution of imprinted genes

Imprinted genes, which are defined as genes that display parent-of-origin, mono-allelic, expression, have only been found in placental mammals (Hore *et al.* 2007) and flowering plants (Huh *et al.* 2008) while non imprinted homologues have been found in reptiles, amphibians, fishes and the egg-laying monotremes (Edwards *et al.* 2007a). Yet, even if a small rudimentary placenta is present, such as that seen in marsupials, evidence for imprinting can be found. Thus, the placenta and imprinted genes appear to have co-evolved. This underlies the relevance of these genes to the formation and function of the placenta and in fetal development.

The parental-conflict hypothesis has emerged to explain the appearance of imprinting as a result of different evolutionary pressures influencing each parent in placental mammals. The hypothesis states that imprinting evolved to control energy flow between the mother and the developing fetus (Moore & Haig 1991) . The conflicting evolutionary outcomes are that the mother (and consequently her genome) is more successful by restricting nutrient flow to the fetus/offspring so that she does not commit too much of her energy resources to each fetus, leaving her more able to reproduce in large numbers. In contrast, the father (and his genome), is represented only in the fetus, and improves his success by extracting as much energy as possible from the mother to benefit each fetus/offspring. It is here were the "conflict" lies, and a careful balance between the two contrasting forces leads to a normal fetus. Unbalancing of these forces can lead to either a smaller than normal (small for gestational age or intrauterine growth restriction) or a larger fetus (large for gestation age or large offspring syndrome; Fig. 1).

The characteristics of uniparental conceptuses support components of the parental conflict hypothesis. Androgenotes (conceptuses derived from only the male) and gynogenotes (conceptuses derived from only the female) can be produced from either two male pronuclei or two female pronuclei (McGrath & Solter 1984). Parthenotes, which are a form of gynogenote, can be easily generated by activation of oocytes and inhibition of polar body extrusion by using cycloheximide (Tsai *et al.* 2006b), resulting in a diploid embryo carrying only maternally derived chromosomes. Although neither androgenotes nor gynogenotes can produce viable offspring, their characteristics are suggestive of the role of imprinted genes in energy distribution and placental development. Gynogenotes, with a double dose of maternally expressed genes, develop into small fetuses with small placentas, as would be expected from a reduction in energy delivery to the fetus. In contrast, androgenotes develop a very large placenta also supportive of the placental conflict hypothesis. However, they also lack a fetus suggesting that maternal imprints are an absolute requirement for fetal development. In addition, as will be discussed later, there is ample direct experimental evidence supporting both the parental conflict hypothesis and the role imprinted genes play in placental and fetal development, as well as in behaviors related to control of energy flow such as nurturing behavior and milk let down.

Can placentas exist without imprinting?

While the evidence from placental mammals and flowering plants strongly supports imprinting as competition for the flow of energy between the developing embryo and the energy source,

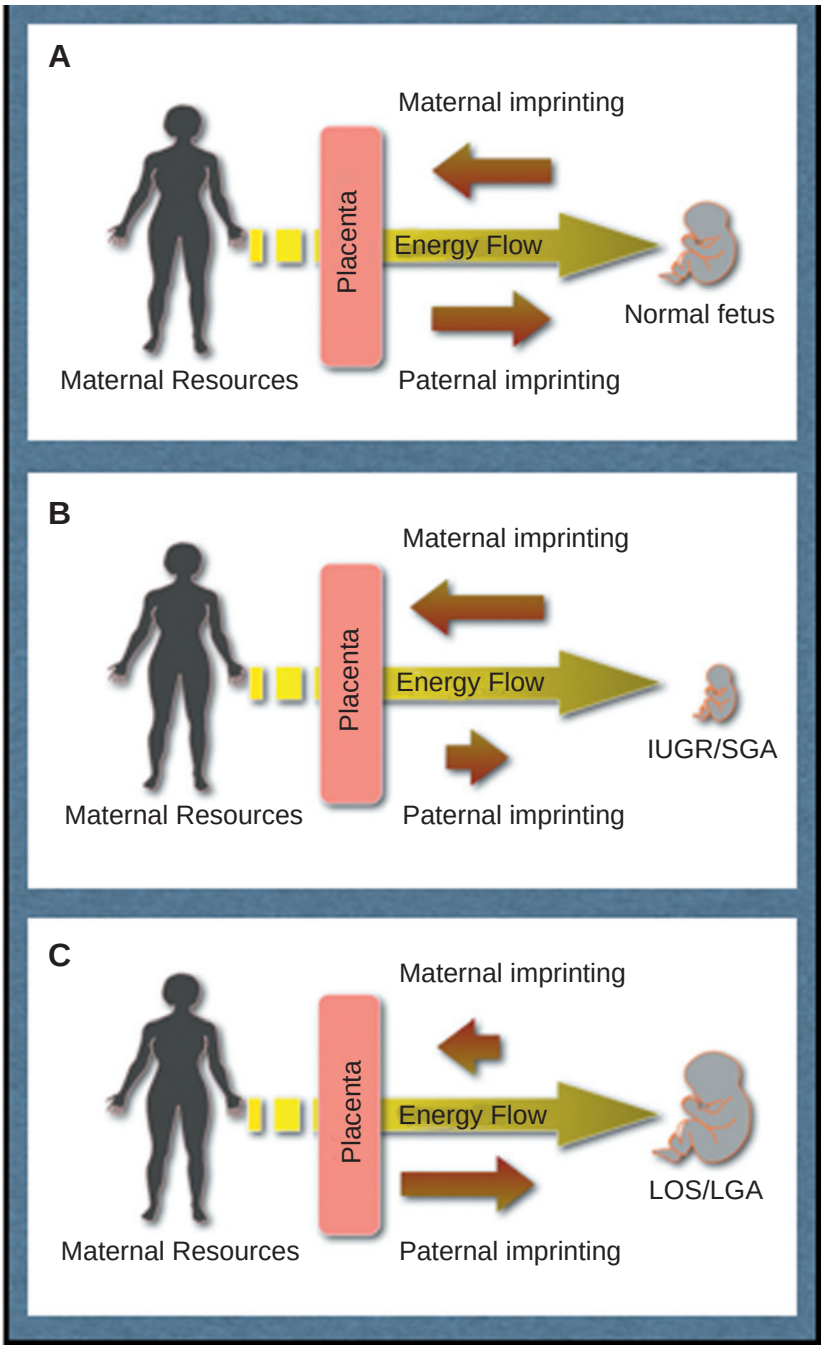


Fig. 1 Diagrammatic representation of the parental conflict hypothesis. A. A normal situation where the control of nutrients from the mother to the fetus is balanced leading to normal fetal growth. Notice maternal imprinting shifting the balance towards the mother while paternal imprinting shifts the flow towards the fetus. B. A case where the balance between maternal and paternal imprints is shifted towards the mother resulting n less nutrients reaching the fetus leading to intrauterine growth restriction/Small for gestational age (IUGR/SGA). C. The opposite case whereby nutrient flow is shifter from the mother to the fetus resulting in large offspring syndrome/large for gestational age (LOS/LGA).

the existence of placental fishes provides a paradox for the requirement of imprinted genes for placental development and function. At present, there has only been one report of analysis of imprinted genes in viviparous fishes and that single report indicates that the IGF2 gene is not imprinted in this species (Lawton *et al.* 2005). While that in itself is not sufficient evidence to classify these placental animals as a paradox, the recent observation that hammerhead sharks can reproduce by parthenogenesis is (Chapman *et al.* 2007). As mentioned above, uniparental offspring are embryonic lethal in all placental mammals species tested to date (Walsh *et al.* 1994, Hagemann *et al.* 1998 , Zhu *et al.* 2003) and it is widely accepted that this lethality is due to the presence of imprinting. The observation of successful parthenogenesis in sharks, thus, suggest that imprinting may be absent in this species. If that is proven to be the case, how did this species evolve a placenta? Or is it that the fish placenta is functional and morphologically distinct from that of placental mammals?

Fetal nutrition within the fish species ranges from wholly dependent on deposited yolk during oogenesis (vitellogenesis), through an intermediate form of nutrition dependent on histotroph secretion from the uterus/oviduct, to nutrition dependent on yolk-sac based placentation (Hamlett 1989). In the scant literature in this area it is evident that the fish placenta is quite distinct from any known mammalian placenta in multiple aspects including the continued presence of an egg envelope throughout gestation (Heiden *et al.* 2005), the small area of actual attachment to the uterus/oviduct in relation to fetal size, and the reliance on the yolk sac as the major organ of nutrient exchange (Jones & Hamlett 2004 , Reznick *et al.* 2007). This suggests that some aspects of placental development and function are independent of imprinting while others are more dosage sensitive and require the imprinting of one allele.

This is partially supported by observations in marsupials, with a rudimentary placenta, where imprinting has been observed in some genes such as IGF2 and PEG10 (Ager *et al.* 2007, Ager *et al.* 2008b) but not in others such as SNRPN, UBE3A, DIO3 (Rapkins *et al.* 2006), CDKN1C (Ager *et al.* 2008a) and DLK1 (Edwards & Ferguson-Smith 2007). A detailed analysis of marsupial placentation has been presented by Renfree *et al.* (2008) and elegantly describes how within the marsupial family different forms of placentation exist and that the more complex the placenta the greater the number of imprinted genes (Renfree *et al.* 2008). In summary, while species such as placental sharks suggest that in the absence of imprinting, a rudimentary form of placentation can exist, the preponderance of the evidence indicates that the emergence of complex placentation is associated with the presence of imprinted genes.

Experimental approaches to the study of imprinting in mammals.

The experimental evidence for the role of imprinted genes in placental and fetal development is derived from two general approaches, the analysis of uniparental animals and the direct observation of the effects of modification of imprinted genes by transgenesis. The uniparental models described above, in particular, have been very useful for broad and comprehensive analysis of imprinted genes between different mammalian species, as well as uncovering new imprinted genes (Barton *et al.* 1984 , McGrath & Solter 1984, Surani *et al.* 1984 , Cattanach & Kirk 1985 , Dean *et al.* 2001 , Zhu *et al.* 2003). A more focused approach is the analysis of the effects of transgenic manipulation of imprinted genes, usually by gene inactivation via homologous recombination. A few examples of placental defects resulting from manipulation of imprinted genes include larger placentas resulting from inactivation of the maternally expressed genes Ascl2 (Guillemot *et al.* 1995), Grb10 (Charalambous *et al.* 2003), IGF2R (Wang *et al.* 1994), PhldA2 (Frank *et al.* 2002), and p57 (Kip2) (Takahashi *et al.* 2000). In contrast, inactivation of paternally expressed genes such as Peg10 (Ono *et al.* 2006), IGF2 (Sibley *et al.* 2004), Peg3 (Li *et al.* 1999), and Mest (Lefebvre *et al.* 1998) result in smaller placentas.

The effect of Paternally Expressed Gene 3 (Peg3) deficiency is particularly intriguing as the phenotype illustrates the many roles imprinted genes can play with respect to energy utilization. The work of Curley *et al*. 2004 indicated that Peg3 deficiency influences the placenta (mentioned above), fetus and mother. At the fetal level, pups deficient in Peg3 have abnormal thermoregulation and suckling defects. Peg3 deficient mothers, in turn, have impaired maternal care, reduced feed intake during pregnancy and reduced milk-letdown. Peg3, therefore, can control energy flow at many levels, from food intake by the mother, to how much milk to provide the offspring, to how much the pup is able to extract from her during suckling (Curley *et al*. 2004). While the exact mechanism of action of Peg3 is unknown, the protein is expressed at high levels in the trophectoderm layer of the mouse placenta and in the hypothalamus. As Peg3 is known to be involved in the control of apoptosis, it has been postulated that abnormal apoptosis leads to altered hypothalamic function affecting thermoregulation, maternal behavior and milk letdown, while defects in the placenta result in reduced fetal growth.

Combined, these observations indicate how complex the function of imprinted genes can be. Yet, in most cases the phenotype supports the parental conflict hypothesis with inactivation of maternally expressed genes leading to larger placentas, and inactivation of paternally-expressed genes leading to smaller placentas. While these results are important and support the role of imprinted genes in placental development they are limited to one species, a small fraction of the known imprinted genes, and perhaps with the exception of Igf2, Igf2r, and Mash2/Ascl2 (Tanaka *et al*. 1997), the role of these genes in placental development is not known. Thus, it is the combination of the fascinating aspects of these genes, the extremely limited information of the function of imprinted genes in placenta of mice, and the absolute absence of information on their function in swine reproduction that encouraged us to embark in a comprehensive study of these genes in swine. To accomplish this goal, we used genomic approaches. In the next section we will describe the techniques we used to accomplish this goal and our positive and negative experiences with them.

Part II. Gene expression profiling methods

Gene expression profiling methods

The completion of the draft human genome sequence (Lander *et al*. 2001) demonstrated the feasibility of sequencing entire complex mammalian genomes, and marked the beginning of whole genome sequencing projects for many mammalian species. Prior to the availability of all of this genomic sequence, the classic approach in molecular genetics was the forward genetic screen. The goal of this approach was to find the genes responsible for a phenotype of interest, and indeed many interesting genes have been mapped in this way. However, the availability of whole genome and transcriptome sequences brings us to the unusual position of possessing information on the sequence of nearly all the genes in the genome, but understanding the function of a far smaller fraction. Simply put, we know where most genes *are*, but not what they *do*. So, how can we use the wealth of newly available genomic information to better understand gene function?

Microarray technology

Microarray technology provides a method of rapidly profiling gene expression genome-wide. There are two major microarray platforms currently available for gene expression profiling in swine: a commercial Affymetrix GeneChip Porcine Genome short oligonucleotide microarray

and a U.S. Pig Genome Coordination Program glass spotted long oligonucleotide microarray. The primary difference between these two platforms is that the Affymetrix platform is based on eleven 25-mer probes synthesized *in situ* on a solid support with a photolithographic mask, whereas the U.S. Pig Genome Coordination Program platform (Zhao *et al.* 2005) is based on traditionally synthesized oligonucleotides subsequently spotted onto a glass slide. In initial validation experiments, we directly compared the technical reproducibility and sensitivity of the two platforms, comparing the gene expression profiles of biparental and parthenogenetic fibroblast cell lines. From the same starting pool of total RNA, we found that the reproducibility of hybridization with the Affymetrix short oligonucleotide microarray was much higher than the U.S. Pig Genome Coordination Program microarray (Fig. 2). In probes shared across both platforms, we detected a greater number of differentially expressed genes using the Affymetrix platform. For the time being, the Affymetrix Porcine Genome Array is the most sensitive and reproducible platform for conducting gene expression profiling experiments with microarrays in swine (Tsai *et al.* 2006b). While the first generation of U.S. Pig Genome Coordination Program arrays suffered from printing defects, lower technical reproducibility, and lower gene coverage; the second generation of these arrays significantly improved coverage. However, due to their lower technical reproducibility, more than 2-3X the number of arrays are required to achieve equivalent statistical power to detect differential expression in contrast to Affymetrix Porcine GeneChip Arrays, so a cost/benefit analysis would still favor the Affymetrix platform for swine gene expression profiling.

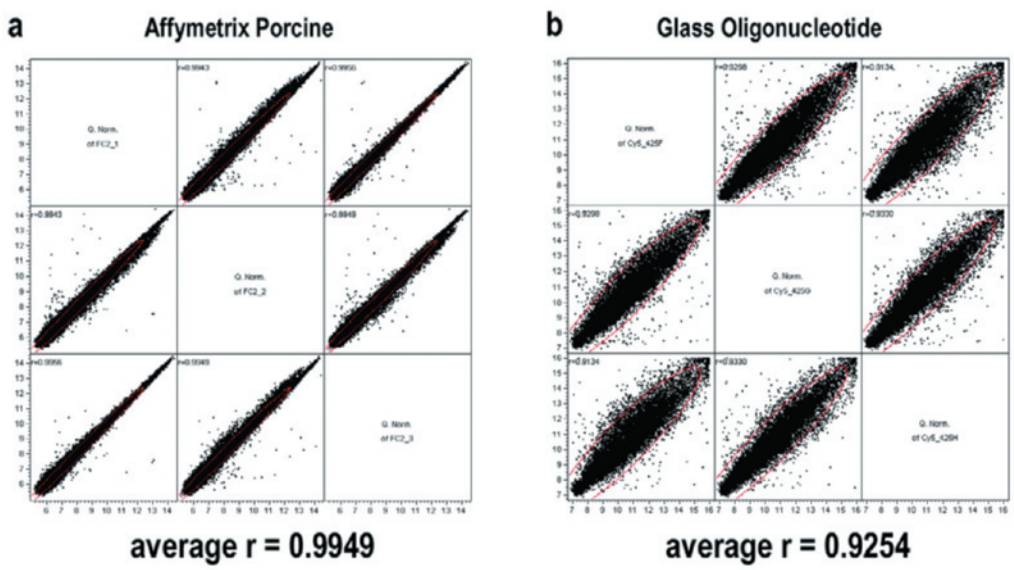


Fig. 2 Reproducibility of technical replicates in the Affymetrix and the glass array platforms. Pairwise scatterplots of control technical replicates of porcine fibroblast cell lines profiled on (a) Affymetrix Porcine and (b) U.S. Pig Genome Coordination Program long oligonucleotide glass microarrays. The lower reproducibility of the glass arrays reduces the ability of the arrays to detect statistically significant differences between experimental samples. This reduced accuracy can only be overcome by increasing the number of replicates in the glass array in comparison with the Affymetrix arrays.

There have been reports of using cross-species hybridization for the purposes of performing gene expression profiling in swine, primarily before the release of porcine specific arrays (Zhao *et al*. 2005). We compared cross-species hybridization of the same RNA described above onto Affymetrix Human U133 + 2.0 GeneChip Arrays. We found that this approach had the lowest power to detect differential expression, because of a high number of non-hybridizing probes. On average, 1-3 probes out of 11 in a probe set hybridized efficiently. Even after implementing various filtering algorithms, the sensitivity of detection was still significantly lower than using porcine specific microarrays.

Deep sequencing (RNASeq)

Microarrays provided a powerful tool for asking descriptive questions about gene expression genome wide. It is increasingly evident, however, that with rapidly evolving deep sequencing technologies microarrays will eventually be supplanted by direct sequencing of mammalian transcriptomes. In this approach, the complete transcriptome can be sequenced and matching transcripts counted rather than indirect quantitation based on hybridization intensities (Wang *et al*. 2009; (Wang *et al*. 2009; Wold & Myers 2008). This has several advantages in that: 1) there is a greater dynamic range in comparison to hybridization based technologies, single transcripts can be positively identified, 2) the technology does not rely on *a priori* knowledge of gene sequence, and 3) background from cross-hybridization is eliminated (Wang *et al*. 2009). Multiplex strategies have been developed to uniquely tag RNA samples with an unique error-correcting molecular barcode, so that the capacity of each sequencing run can be most efficiently utilized (Craig *et al*. 2008, Hamady *et al*. 2008). These strategies are based on the simple addition of 4-6 bp of sequence to the adapters that are used to create the libraries for resequencing; these unique identifiers allow the downstream determination of individual samples from a pool. The freedom from having to define which sequences to interrogate enables the possibility of novel transcript discovery. The sequence information provided allows the unambiguous identification of single transcripts, whereas detection by hybridization technologies is inevitably limited by background. Finally, the need for the complex nonlinear normalization strategies often employed in typical microarray experiments is lifted, as sequenced transcripts are simply mapped to a reference genome and counted. This may be useful in cases where one of the fundamental assumptions of most microarray normalization procedures, that the empirical distribution of transcripts is the same across samples, are on shaky ground, such as when comparing gene expression profiles across tissues or species. One limitation of RNAseq is that mapping the data produced is required by the existence of a reference genome; in swine ongoing sequencing efforts will increase the utility of RNAseq data in the coming years.

Annotation of microarrays

A determination of differential expression for a probe on a microarray or a sequence cluster from an RNAseq experiment is of limited utility without knowing what genes or transcriptional units they represent. Because of the limited annotation, with only about 20% of the probe sets present in the initial annotation of the Affymetrix Porcine Genome Array, we reannotated the probe sets against human cDNA and genomic DNA sequence (Tsai *et al*. 2006a). The approach we took was to extend the target sequence using sequence information available from The Institute for Genome Research (TIGR) swine gene index (currently Dana Farber gene index, website), and matching the extended sequence against other Ensembl human cDNA and genomic DNA sequences. This approach was successful in raising the percentage of annotated genes from

20 to 80% because the majority of the probes for this generation of microarrays are designed against the 3′ untranslated region (UTR). In many cases, our annotation strategy extended the sequences beyond the 3′ UTR. Our annotation provided a bit score (a measure of the likelihood of a correct sequence match) so that individual investigators can set their own threshold for acceptable annotation confidence. We have recently updated our annotation of the Affymetrix Porcine Genome microarray against bovine, mouse, and human. One additional unique feature of the annotation is our matching against the Affymetrix Human probe set IDs. This matching allows data generated from Affymetrix Porcine GeneChip arrays to be used with many of the pathway analysis solutions that are available for human gene expression profiling data.

Single nucleotide polymorphism (SNP) and single feature polymorphism (SFP) discovery

Affymetrix short oligonucleotide gene expression data can indicate single feature polymorphisms (SFP), as single nucleotide polymorphisms (SNP) that are close to the center of a 25-mer oligonucleotide probe can almost completely disrupt hybridization (Winzeler *et al*. 1998, Borevitz *et al*. 2003). The SFP are identified by disparate hybridizations among individual animals to one or more of the 11 targets for each mRNA on the array (Fig. 3) The exact SNP can then be identified by sequencing, however, SFP genotypes have also been directly used to generate high density haplotype maps for expression quantitative trait loci (eQTL) studies (West *et al*. 2006). Deep sequencing of the transcriptome can also contain not only information on the expression level of a transcript, but also allelic variations in the sequences obtained. The U.S. Pig Genome Coordination Program glass spotted long oligonucleotide microarrays cannot, however, be used for this purpose as a single SNP is insufficient to significantly disrupt the hybridization kinetics of a 70-mer probe.

We have demonstrated the feasibility of detecting SFP using Affymetrix short oligonucleotide arrays in swine (Bischoff *et al*. 2008). The basic idea behind the approach is to look for probes which have a substantially greater probe effect than expected, corresponding to the scenario where a SNP disrupts probe hybridization. Using this approach we detected 857 SFP between Chinese Meishan and European white composite breeds of swine, with a sensitivity of 0.65, specificity of 0.94, and a false discovery rate of 0.3. We have streamlined the method we used to determine the presence of an SFP into an easy to use downloadable procedure, *Click-'N-SNP,* which will generate a list of putative SFP given raw Affymetrix data (.CEL files) and a simple experimental design (Bischoff *et al*. 2008). Similarly, given sufficient oversampling, it is possible to detect SNPs and indels in RNASeq data. Care, however, must be taken to use the appropriate statistical models to distinguish between true SNPs and sequencing error, given that the error rate in short-read sequencing platforms is relatively high.

One motivation behind obtaining genotype information from gene expression data, whether from microarrays or RNASeq, has been a method dubbed "genetic genomics" (Jansen & Nap 2001). The principle is that there is a heritable aspect to gene expression that may ultimately contribute to phenotypes of traits of interest. By merging information on allelic differences and gene expression, as well as the gene expression contribution to function, it may be possible to gain a better picture of the genes involved and their mode of regulation. Extracting this information from gene expression data, is a "free" source of this additional genetic information, qualified by the fact that SFP obtained from microarrays only localize the polymorphism to a 25 bp window, and therefore do not fully define the sequence variation. For higher density SNP genotyping applications, a 50k porcine SNP chip has been developed.

Finally, massively parallel targeted resequencing was recently demonstrated by coupling deep sequencing with solution hybrid selection with long oligonucleotides (Gnirke *et al*. 2009),

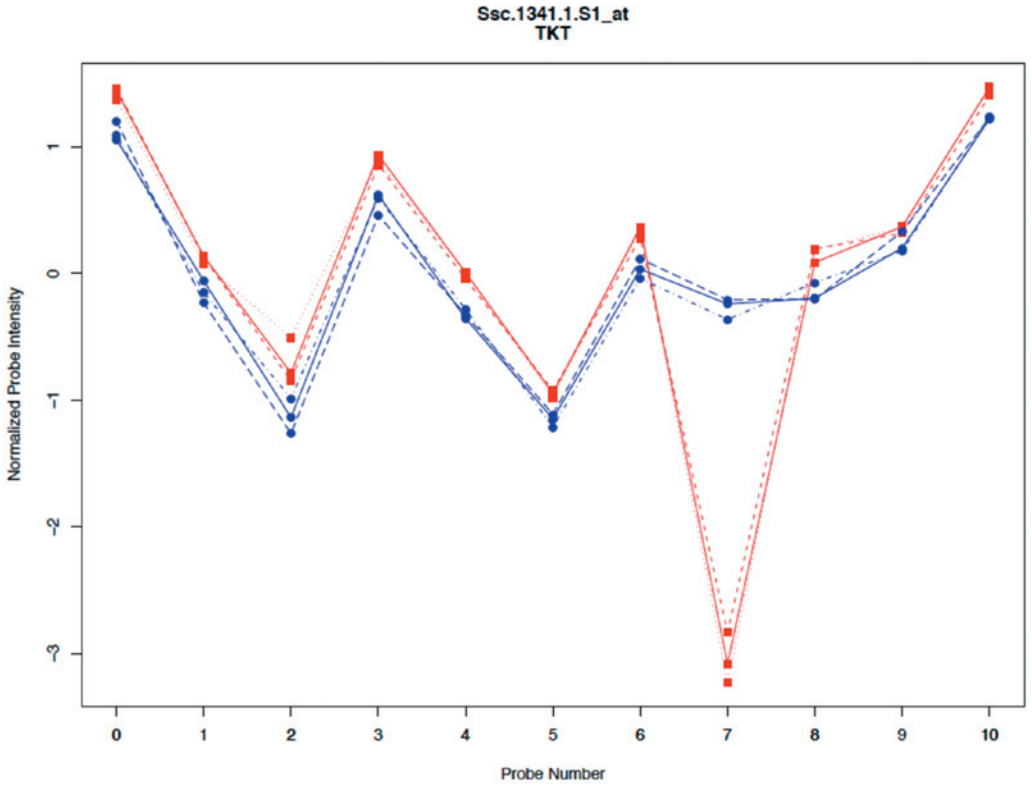


Fig. 3 Example of Single Feature Polymorphism (SFP) in swine detected between Chinese Meishan and European white composite breeds. Notice Probe 7 which exhibits hybridization intensities near background, while the remaining probes are 4-fold or more higher. This reduced hybridization was due to a SNP within that particular probe. Thus a probe-by-probe analysis can rapidly uncover a large number of potential SNPs within the population being used for the microarrays experiments.

should also be possible in swine. The principle of this approach is to synthesize a number of tiled probes against genomic region(s) of interest. These probes are around 200 bp in length, contain universal primer sequences for amplification, a T7 promoter for *in vitro transcription,* and are synthesized *in situ* on an Agilent custom microarray. After cleaving these probes off the microarray, the pool of probes is subjected to an *in vitro* transcription reaction containing biotinylated nucleotides to create a pond of biotinylated cRNA baits. This pond of biotinylated cRNAs hybridizes efficiently to sheared genomic DNA sequence. The captured, complementary genomic DNA sequence is then used as the input for resequencing library preparation. With the growing availability and near completion of a draft porcine genome sequence, an increasing proportion of the data generated via this approach will be mappable.

Part III. Moving beyond gene lists. Finding functional interactions from microarray data to study epigenetic asymmetry in porcine fetal tissues

In the following section we will enumerate a list of tools that facilitate analysis of gene expression datasets. Building on these descriptions, we draw on datasets generated by our laboratory

and apply the tools to systematically clarify functional relationships among expressed genes. The data are freely available at Gene Expression Omnibus (GEO) under accession number GSE10443.

Pattern discovery by clustering analysis

Cluster analysis software requires the ability to handle large input datasets (greater than 100,000 rows/columns) and should contain versatile microarray analysis features. Although a number exist, our laboratory has experience with the licensed package JMP Genomics (SAS, Cary, NC) and the freeware package R Bioconductor (http://www.bioconductor.org).

For pattern discovery a number of unsupervised methods exist to partition data into visual subsets by a common group of parameters or clusters (Allison *et al*. 2006 ; Kerr *et al*. 2008) which include hierarchical clustering, heat maps, k-means clustering and principal components analysis (PCA). For a concise summary of clustering methods useful to expression datasets, see D'Haeseleer (2005). Hierarchial clustering partitions data into groups of genes iteratively with each successive finer grouping being more similar. A central component of the method is depicting similarity by distance. Shorter branches represent more closely related items. In transcriptomic datasets, the distance metric is calculated from gene expression values. The output is often shown diagrammatically in a dendrogram, or branched-tree graph. An example of such output for imprinted gene expression in the placenta between day 30 control conceptuses and swine parthenote conceptuses is shown in Fig. 4).

DNA microarrays can also be depicted by two-dimensional graphical representations of gene expression values called heat maps, where color denotes expression intensity (i.e. red = low intensity, green = high intensity). A heat map can quickly show the level of expression of a gene across samples, time or treatment (Fig. 4) and is a rapid and simple way of looking at a large amount of data in a single figure. K-means analysis, a more complex method, can shuffle genes based on their geometric mean into a predicted number of clusters as defined by the hypothesis. The method is rapid, but requires a priori knowledge of how many clusters are expected, and is therefore biased.

Principal component analysis (PCA), another clustering method, reduces the complexity of a dataset by decomposing the variance into a limited number of dimensions or components using the mathematical tools of eigenvalues and covariance matrices. In this manner, the gene expression measurements can be visualized in a linear fashion to clarify how each array behaves in context with the other arrays in an experiment. The abscissa and ordinate axes represent the first and second principal components, respectively. The closer an expression array groups together indicates its similarity. Principal component analysis is a robust methodology for rapidly clustering data, and can provide a framework for quality control. An example of the use of PCA for this purpose is described in Fig. 5. The first three principal components were used as they explained 86%, 5%, and 5% of the total variation, respectively. Initial examination identified two discrepant arrays (LG2 and BG3). As 95% of the variation is contained within the concentration ellipse, both arrays were excluded from downstream analysis for technical reasons associated with RNA quality. Close examination of the array data then showed that the hybridization levels of both of these arrays were below that required to obtain a reliable signal. The arrays were then re-normalized excluding these two arrays to increase the accuracy of the data. It is interesting to note, that brain (BC, BG; blue) and placental (PC, PG; red) tissues grouped more closely than liver (LC, LG; green) or fibroblast (FC, FG; orange) day 30 swine fetal tissues and suggests brain and placental transcriptomes are more similar than fibroblast or liver transcriptomes. The main point of this example is that PCA can rapidly identify a hybridization/

technical issue in one or more of the arrays and prevent investigators from performing complex statistical analysis with data that are of low quality.

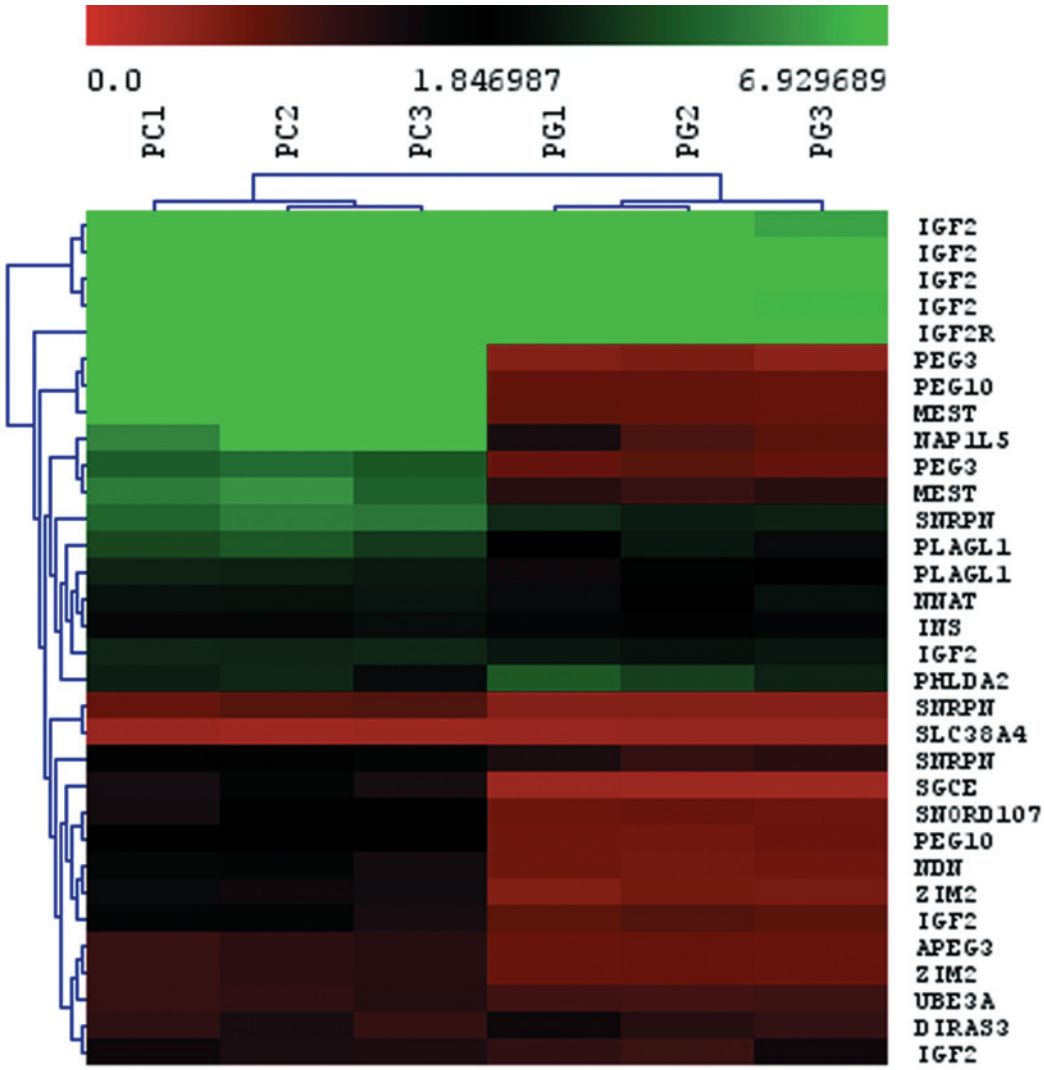


Fig. 4 Hierarchical Clustering and Heat Map of imprinted gene family in Day 30 fetuses. Microarrays containing placental RNA from biparental (PC) or parthenote (PG) D30 swine gestations were submitted to hierarchical clustering. Detransformed normalized values were used. Microarrays containing placental RNA from biparental controls or parthenote D30 swine gestations were profiled by one-color DNA short-oligonucleotide microarrays (Porcine GeneChip, Affmetrix). A gradient of red-black-green was used to denote expression intensity, where red denotes low expression, black shows median intensity, and green denotes high expression. Columns from left to right list samples of placental controls 1-3, placental parthenotes 1-3. Rows represent probe set intensities, and several genes contained multiple probesets which bind to the messenger RNA in different regions or alternatively spliced exons. Heat maps were genereated with TM4 software using version MeV v4.3.02 (Saeed, Sharov et al. 2003) http://www.tm4.org/mev.html).

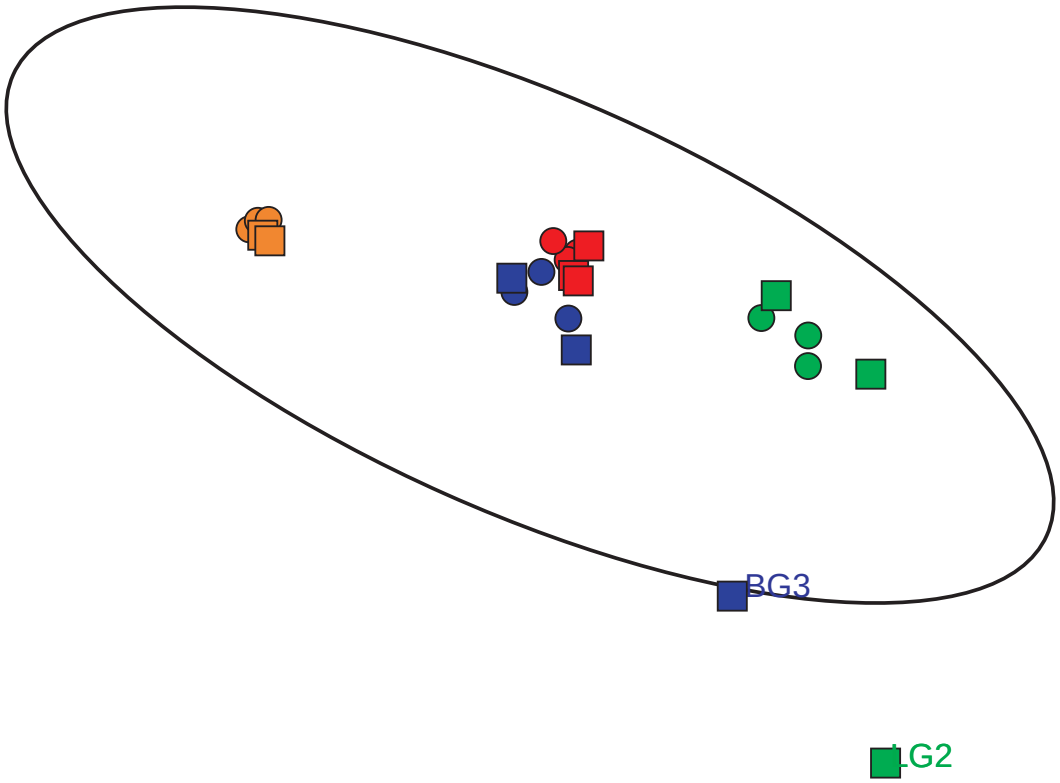


Fig. 5 Principal Component Analysis (PCA) identifies two discrepant arrays. Parthenogentic and biparental fetal tissues were hybridized to short-oligonucleotide arrays (PorcineGene Chip, Affymetrix, CA). The encircling ellipse explains 95% of the variation among samples. Circles denote parthenotes, squares represent controls. Brain = blue, fibroblast = orange, Liver = green, Placenta = red. Two arrays fell outside the concentration ellipse, BG3 and LG2, and were omitted from downstream analysis.

Enrichment analysis: functional annotation and pathway analysis

Classifying genes based on criteria such as biochemical function, genetic interaction and pathway, motif searching, and gene ontology is what broadly describes enrichment analysis. The overarching goal is to move from the daunting candidate gene lists and distill the dataset into meaningful biological processes that can be tested experimentally in the laboratory. A suite of over 68 enrichment analysis tools are now available and have recently been reviewed in Huang da *et al*. (2009). A full description of each is beyond the scope of this chapter, so we will focus on tools we have used and provide a summary of key findings. We refer the reader to Huang da *et al*. (2009) for a comparison of advantages, pitfalls, and operational classification of the current tools (e.g. statistical testing methods).

Gene ontology

Gene ontology (GO) provides a unique vocabulary that describes or annotates genes by molecular function, biological process and cellular distribution. Because the number and sophistication of GO-related programs has increased dramatically, a searching tool SerbGO (Mosquera & Sanchez-Pla 2008) is available on the web to identify which GO software application may be best for the

end-user's dataset. Alternatively, a summary of each application is available on the web at the Gene Ontology Consortium website: http://www.geneontology.org/GO.tools.microarray.shtml**.** Our group used SerbGO to identify significance analysis of function and expression (SAFE) (Barry *et al*. 2005, Gatti *et al*. 2009), Database for Annotation, Visualization and Integrated Discovery (DAVID) (Dennis *et al*. 2003), and Gene Set Enrichment Analysis (GSEA) (Subramanian *et al*. 2005) as appropriate tools for our purpose.

The porcine parthenogenetic conceptus develops as a small fetus and placenta which eventually dies at approximately day 32 of gestation (Fig. 6). To gain biological insight into pathways that differ during uniparental embryonic development compared to normal development, we used gene ontology (GO) descriptors to analyze our datasets. Two additional categories indicating parent of origin expression for each imprinted gene, were added to clarify paternal/maternal imprinting contributions. We used a permutation-based ranking method to identify functional categories differentially expressed in parthenogenetic fetuses. This approach is similar to SAFE (Barry *et al*. 2005) (significance analysis of function and expression) using SAS streamlined with JMP Genomics (SAS, Cary, NC) in lieu of Bioconductor **(**http://www.bioconductor.org/**).** Probe sets were assigned to GO categories based on the Affymetrix Porcine annotation by Tsai *et al*. (2006a). Initially, custom PHP code (http://en.wikipedia.org/wiki/PHP) reads in two files: one with the gene ID followed by any number of columns containing ranks for statistical tests that have been performed, and one containing the GO categories and the genes they contain. The algorithm calculates the rank sum for each GO category for each rank column, and subsequently permutes the gene labels with respect to their ranks. For each permutation, the GO category now contains a random set of genes and thus a random set of ranks. The permuted rank-sums are calculated, and a running total is kept of how frequently the permuted rank is less than or equal to the original rank. Dividing this by the total number of permutations provides the p-value estimate. The advantages of this permutation approach are to limit Type I errors for individual categories.

Summarized ranks of GO categories that were significant ($p < 0.05$) between parthenogenotes and biparental conceptuses for various tissues include paternally expressed imprinted genes, phosphatidylinositol binding, microtubule dynamics and lipid transporter activity. Not surprisingly, imprinted paternally expressed genes were ranked significant ($p < 0.006$) across all five datasets corresponding to each tissue indicating that parthenote profiling can reliably detect transcript dosage differences, regardless of tissue surveyed. Notably, there were marked differences in proliferation, biogenesis and biosynthesis pathways as predicted by the parent-conflict hypothesis. Consistent with our observations and others that parthenogenote conceptuses are developmentally delayed, various structural proteins ranked highly significant in most tissues. The artificial category "imprinted, maternally expressed" showed no significant difference, and may be related to a power-related problem to detect a theoretical 2:1 ratio upon comparison of maternally expressed genes between parthenotes and biparentals. As many biological pathways were affected that do not contain imprinted genes, we feel these data support conclusions that a gene network is present that extends beyond imprinted genes but is epistatically affected by them (Varrault *et al*. 2006). Thus, utilizing these methods it is possible to go from a list of genes, which in most cases is too large to properly address experimentally, to a more biologically relevant list of biological processes that are likely to be affected. This can greatly facilitate hypothesis generation as well as the design of physiological /biochemical experiments.

Pathway and interactome analysis

Additionally, to uncover new meaningful biological relationships it is often helpful to visualize gene signatures in the context of curated biochemical pathways as provided by resources such

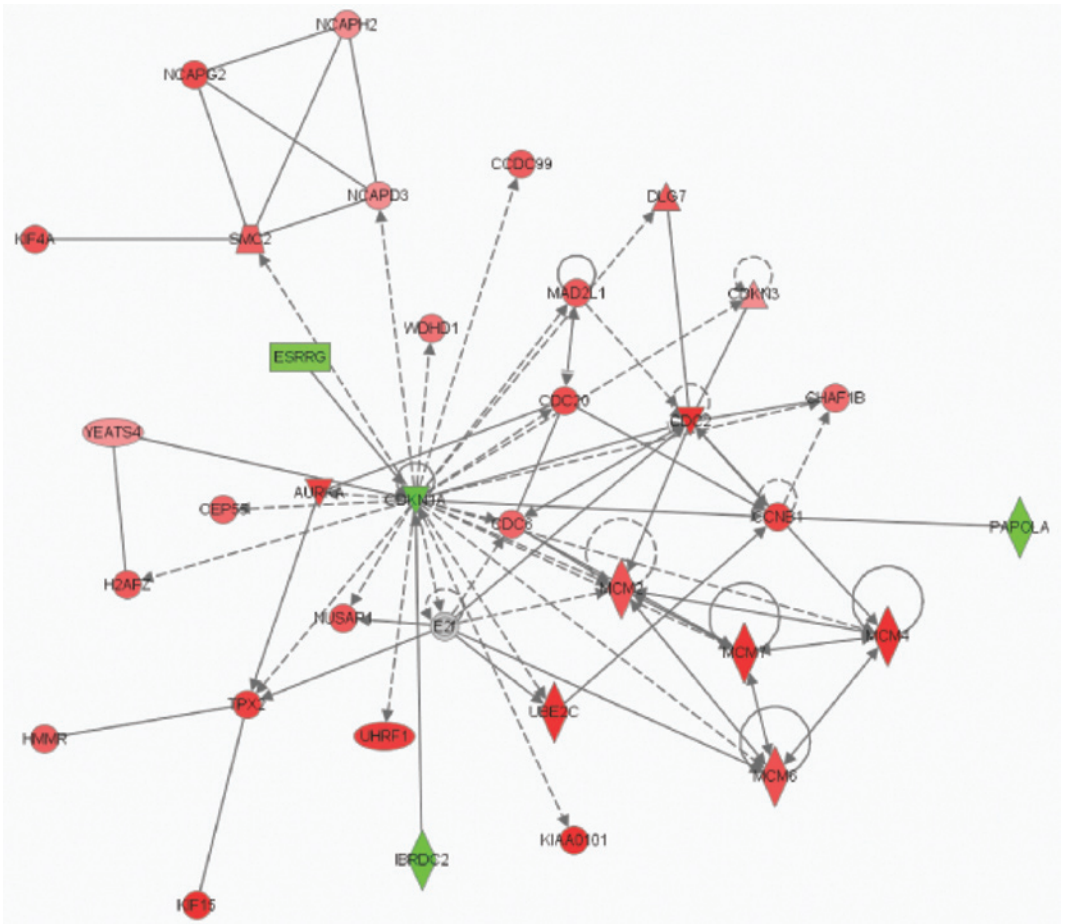


Fig. 6 Functional networks of genes dysregulated in swine parthenogenetic tissues. Ingenuity Pathway Analysis was used to map genes differentially expressed in swine parthenogenetic fetal tissues into functional pathways. Green represents up-regulation of genes with respect to the parthenote, while red represents down-regulation in the parthenote. The following panel depicts highest affected pathway in the combined analysis of all tissues. One of the central nodes or hub is CDKN1A. Up-regulation of CDKN1A results in cell cycle arrest at the G1/S checkpoint and induces apoptosis.

as KEGG (Kyoto Encyclopedia of Genes and Genomes) (Okuda *et al.* 2008), BioCarta (http://www.biocarta.com/genes/allpathways.asp), and Reactome (Matthews *et al.* 2009). For this process, open-source software such as Cytoscape (Cline *et al.* 2007 , Yeung *et al.* 2008) and MadNET (Segota *et al.* 2008) are excellent programs that aid the investigator by mapping array expression datasets on canonical biochemical pathways. A handful of commercial applications like Ingenuity Pathways Analysis (IPA; IngenuitySystems, www.ingenuity.com) are also available. In general, one should choose the application that best suits the scientific question, helpful criteria include text mining options, curated pathway plug-ins, user-created network assembly, data visualization capabilities and flexibility of data import / output formats.

We used IPA to explore pathways altered in parthenogenetic swine conceptus tissues, similar to the approach taken by Jincho *et al.* (2008). An example of an interactome depicting

genes affected in the parthenote samples compared to normal conceptus samples is shown in Fig. 6. Pathway analysis indicated that cell cycle regulation, growth and proliferation, and cellular assembly pathways were among the most common and most affected pathways in each of the tissues profiled. Our functional analysis of parthenogenetic swine conceptus tissues is in agreement with the parent-conflict hypothesis, as one might expect growth pathways to be asymmetrically affected with a reduction in biogenesis, growth and proliferation pathways.

Finally, biological text mining has also become more attractive due to its ease of use and availability. For the exploration of alternative transcript isoforms, AceView (Thierry-Mieg & Thierry-Mieg 2006) is particularly handy. Recently, the community authorship or wiki concept inspired WikiGenes (Hoffmann 2008) (http://www.wikigenes.org/), WikiPathways (http://www.wikipathways.org) and regulatory networks based on biomedical discipline (i.e. pathways defining stem cell pluripotency using the PluriNET network (Muller *et al.* 2008 ; http://www.openstemcellwiki.org/). The main advantage of wiki concept is dynamic, collaborative forum for scientists to engage in sharing data and publishing ideas.

microRNA (miRNA) and target mRNA

MicroRNAs are known to critically regulate many developmental processes by translational inhibition or destabilizing target mRNAs and are often evolutionarily conserved (Grun *et al.* 2005 , Chen & Rajewsky 2006 **)**. Comprehensive arrays are available containing a large number of known mouse and human miRNAs. In species such as swine, exploration of miRNAs is difficult due to the absence of full sequence information that would permit identification of conserved miRNAs and thus the use of these cross-species platforms, although recent reports suggest that the degree of microRNA conservation is such that other species platforms can be used to globally examine swine miRNAs (Huang *et al.* 2008). An alternate approach is to utilize microarray data as a way to predict which microRNAs are affected. This approach is facilitated by the existence of novel bioinformatic tools such as gene set enrichment analysis (GSEA).

Gene set enrichment analysis is a robust method which utilizes gene sets (Molecular Signature Database, MSigDB; http://www.broad.mit.edu/gsea/msigdb) to analyze microarray data. The GSEA-P software distinguishes whether genes in known biochemical pathways or coexpression patterns tend to be at the top or bottom of the ranked genome-wide expression dataset or randomly distributed. An enrichment score is provided, which is a value of statistical significance after correction for multiple testing. A full description of the method is available in (Subramanian *et al.* 2005).

Gene set enrichment analysis of the top hundred differentially expressed genes between biparental and parthenote placentas were used as inputs to determine whether there were common microRNAs that are dysregulated. This GSEA approach was able to identify five microRNAs predicted to be differentially expressed in the parthenote samples. Two of these microRNAs have been implicated as ligands for angiotensin receptor II type 1 (AGTR1), a gene responsible for angiogenesis (Sasaki *et al.* 2002), vasoconstriction, and increased pregnancy complication by preeclampsia (Wallukat *et al.* 1999). Gross morphological examination of swine parthenote placentas showed reduced number of blood vessels, and this observation is also supported by differential expression of AGTR1 in placental tissues ($p < 0.0009$) from our microarray datasets. At the time of writing, a single report of miRNAs surveyed in swine fetal tissues observed on day 33 and day 65 of gestation has been reported (Huang *et al.* 2008). While at this point we have not confirmed the differential expression of these miRNAs in our samples by Q-PCR, we have previously used this method to identify and confirm miRNAs affected in human intrauterine growth restriction (manuscript in preparation).

Conclusion

Swine are an attractive model to study fetal growth because their placental morphology is relatively simple—(diffuse, epitheliochorial, non-invasive)---and may provide clues to physiological defects of epigenetic gene dysregulation. The swine parthenote model is already yielding important insights into fetal growth retardation. Our collective analyses of these datasets will contribute a greater understanding of the role of epigenetic mechanisms critical to swine placental function and will hopefully aid our understanding of the formative interactions among fetus, placenta and mother, which are depicted and summarized in Fig. 7.

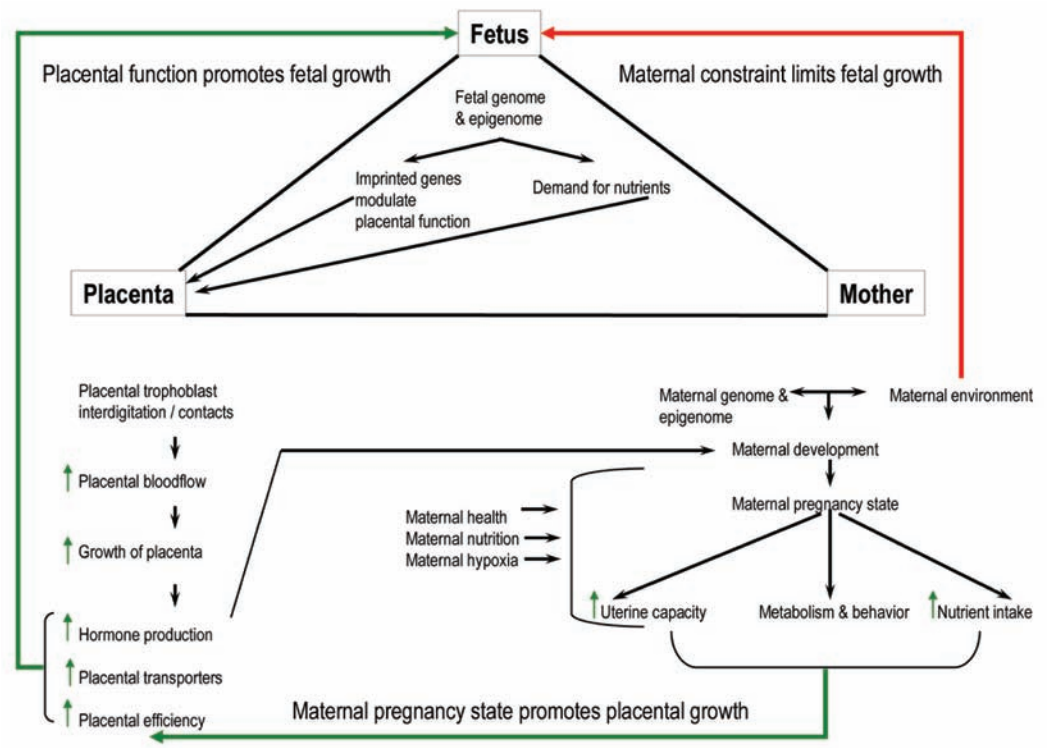


Fig. 7 Signaling between Fetus, Placenta and Mother. The diagramm highlights interactions between fetus, placenta and mother in swine pregnancy and was modified from its original version as described in Murphy et al 2006 (Murphy, Smith et al. 2006). The placenta is the nexus between fetus and mother and its function in nutrient exchange is critical for fetal growth and pregnancy outcome as outlined by the various physiological crosstalk. For example, epithelial folds of chorionic trophoblasts create interdigitation and increase placental surface area, which ultimately promotes fetal blood flow, placental and fetal growth, and enhances transport of nutrients across the non-invasive swine placenta. Imprinted genes affect mammalian pregnancy outcome and functional studies by gene-targeting have described intrauterine growth restriction (IUGR) as one disease state by their perturbation. Knockout (KO) studies in mice have shown that the paternally expressed imprinted gene family, such as IGF2 and PEG10, results in placental hypotrophy, while KO conceptus of imprinted maternally expressed growth suppressor PHLDA2 results in placentomegaly. Maternal and fetal genotypes also affect conceptus size and placental efficiency, respectively (Biensen, Wilson et al. 1999). Growth retardation is not limited to placental insufficiency, as severe maternal caloric restriction results in preterm loss and postnatal runting (Martin-Gronert and Ozanne 2007; Vuguin 2007).

Modern genomic approaches can greatly facilitate the study of physiological phenomena by providing a broad overview of the system, followed by the ability to focus on those pathways/systems that vary. Thus, while genomic analyses are not hypothesis driven, they greatly facilitate the development of hypotheses that have the most likelihood of yielding important biological information. We view genomic approaches as an initial unbiased screening step that can be followed up with more targeted functional experiments. They are not, by themselves typically conclusive, but are extremely useful for hypothesis generation. By comparison, many times candidate gene approaches suffer from too narrow a view of the biological system being studied, and fail to uncover novel interactions and pathways.

Acknowledgments

Portions of the work presented here were supported by a National Research Initiative Grant (2005-35604-15343) from the USDA Cooperative State Research, Education, and Extension Service to JP and BF, by grants HL 51587 and HD 048510 from the National Institutes of Health to JP, by a National Science Foundation IGERT fellowship to ST, a North Carolina State College of Veterinary Medicine doctoral fellowship to SB, and by a NIEHS training grant to NH. This work was performed as part of an initiative from the Center for Comparative Medicine and Translational Research (CCMTR) at the North Carolina State University College of Veterinary Medicine.

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Development of the pig placenta*

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Placental insufficiency results in fetal loss, low birth weight, stillbirth, preweaning mortality and poor growth. Placental development begins at conceptus elongation, which is a primary factor controlling the size of the placenta. After elongation, the allantois develops outward from the embryo to establish the allantochorion, which defines the size of the functional placenta. During implantation, chorionic trophoblasts adhere to endometrial epithelial cells. Placental structures known as areolae develop at the openings of the endometrial glands and take up endometrial gland secreted products (histotrophe). Between day 30 and 35 of gestation, the adhered trophoblast-endometrial epithelial bilayer undergoes microscopic folding. Fetal and maternal capillaries develop adjacent to the bilayer and blood flows are arranged in a cross-countercurrent manner. Except for nutrients secreted by the glands, nutrient exchange takes place between these capillaries within these folds. By day 85, the folds deepen and become more complex, increasing surface area. The epithelial bilayer thins and capillaries indent the plane of each layer (but do not penetrate), reducing distance between capillaries. The folded bilayer is surrounded by endometrial stroma on the maternal side and placental stroma on the fetal side. The fetal-placental stroma is partially composed of glycosaminoglycans, the most abundant being hyaluronan and heparan sulfate. Changes in both hyaluronoglucosaminidase and heparanase during placental development suggest that these enzymes play a role in placental development. In addition to structural modifications, various nutrient specific transport mechanisms exist. These mechanisms are likely to be as important to transport of specific nutrients as placental size or structure.

Introduction

The number of piglets weaned per sow on a yearly basis is a useful measure of sow productivity in breeding herds. The number of fully formed piglets and the stillbirth and preweaning mortality rates influence the number of piglets weaned. Placental function influences each of these components. At its worst, placental insufficiency results in fetal loss during pregnancy. However, even when placental insufficiency is not severe enough to cause death of the fetus, it still reduces piglet birth weights, which is strongly associated with both stillbirth

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*Mention of trade names or commercial products in this paper is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

rate (Tuchscherer *et al*. 2000) and preweaning mortality (Lay *et al*. 2002). In addition, most preweaning mortality occurs within 2 days of birth (England 1974), supporting the concept that poor fetal development is the root cause of most preweaning mortality. If piglets survive, their growth rates are permanently impaired (Tilley *et al*. 2007).

The weights of the fetus and the fetal components of the placenta (allanto- and amniochorion, hereby called "fetal-placenta") can vary widely even within the same litter, and are correlated when fetal-placental weight decreases below a threshold weight (Vallet 2000). This clearly indicates that fetal-placental weight is a primary factor contributing to the generation of runt piglets. The residual variance of fetal weights after fitting the effect of fetal-placental weight partially represents the effect of other characteristics of the placenta that determine its function (i.e., surface area, structure, vascularity). Because of the poor performance of low birth weight piglets, the goal of the swine producer is for every sow to give birth to a large litter of large, uniformly developed piglets. Because the weight of the piglet is correlated with the weight and function of the fetal-placenta, meeting this goal requires that we understand how the fetal placenta develops in terms of both its final weight and its functionality.

Fetal-placental weight and fetal weight

The relationship between fetal-placental weight and fetal weight is curvilinear (Vallet 2000), indicating that beyond a certain size fetal placenta, the fetal-placental weight ceases to influence fetal weight. Thus, fetal-placental weight is relevant primarily to underweight fetuses. Within the fetal-placental weight range that affects fetal weight, differences in fetal-placental weight do not result in fully proportional changes in fetal weight. This can be demonstrated using the relative growth rate equation of Huxley (1932) to examine relationships between fetal-placental and fetal weights. Using the data reported in Freking *et al*. (2007), the slopes of the relationships between log fetal-placental weights and log fetal weights were 0.20 on day 25, 0.26 on day 45, 0.44 on day 65, 0.57 on day 85, and 0.69 on day 105. A slope of 1 indicates fully proportional growth relationships. Because these slopes are uniformly less than 1, they indicate that compensatory mechanisms exist during gestation to minimize decreases in fetal weight when fetal-placental weight is decreased (i.e., a fetal sparing effect). They also indicate that these mechanisms become progressively less effective with advancing gestation. These compensatory mechanisms need not be confined to the fetal placenta, mechanisms within the uterus or the fetus could also compensate for reduced fetal-placental weight.

Relationship between conceptus elongation and fetal-placental size

It is well established that swine conceptuses undergo elongation at about the time of maternal recognition of pregnancy (Perry & Rowlands 1962, Geisert *et al*. 1982), reaching lengths ~1 m, but occupying ~30 cm of uterine space due to extensive folding of the endometrial lining. Elongation of the conceptus likely is a primary determinant of the amount of uterine space and maternal resources available to each developing conceptus. It also appears that most pig blastocysts cease elongation when they come into proximity with another conceptus, because individual conceptuses typically do not overlap with each other within the uterus until later in gestation (Perry & Rowlands, 1962, Ashworth *et al*. 1998), although exceptions to this have been noted (Crombie 1972, Bjerre *et al*. 2008) and this mechanism can clearly be exceeded. The initiation of elongation of blastocysts within a litter is relatively synchronous. However, a small subset of blastocysts often do not elongate with the majority of littermates. Experiments in which conceptuses were recovered just after initiation of elongation report obtaining elongated

blastocysts along with a minority population of blastocysts that remain spherical (Geisert *et al*. 1982, Pope *et al*. 1988). The mechanism that initiates elongation is not known. It has been suggested that the diversity in initiation of elongation may be explained by differences in the timing of ovulation of individual ova (Pope *et al*. 1988). However, later work using ultrasound measurement of ovulation suggests that the difference in time of ovulation from the first to last ovum does not explain the differences in elongation initiation (Soede *et al*.1992). On the other hand, it is not clear whether ova that are late to ovulate are as developmentally competent as those that ovulate earlier. Thus, although the timing of ovulation may not explain the diversity in conceptus elongation, differences in developmental competence between earlier and later ovulating ova may play a role in conceptus diversity during elongation (Xie *et al*. 1990).

Once initiated, elongation of the pig blastocyst occurs very rapidly, and the rate has been estimated to be ~45 mm/h (Geisert *et al*. 1982). Because this estimate was made by comparing the elongation of different blastocysts at two different times, this rate is approximate. It seems likely that elongation rate could vary between individual blastocysts, and that this variation contributes to variation in elongation success. However, methods are lacking to assess individual conceptus variation in elongation rate because once removed from the uterus for observation, the process of elongation is interrupted.

Another aspect of elongation that affects conceptus length is its spontaneous termination. The effect of this is clearly evident in Meishan pigs. Meishan conceptuses are smaller at all stages of development and terminate elongation at smaller placentas, resulting in smaller placenta (Ford 1997). This potentially limits individual conceptus interaction within the uterus, improving the outcome of elongation for that portion of the litter experiencing delays. Curiously, this would result in better uniformity of conceptus size after elongation has occurred, which has been reported in Meishans (Bazer *et al*. 1988). The reduction in conceptus size in Meishans is associated with reductions in uterine protein secretion during early pregnancy (Vallet *et al*. 1998a). Interestingly, treatment of Meishan pigs during early pregnancy with estrogen, which stimulates uterine protein secretion, increased placental size (Wilson & Ford 1999), but estrogen had no effect on placental size in European breed pigs (Vallet & Christenson 2004). This is also consistent with a lack of effect of treatment of gilts with estrogen on day 9 and 10 of gestation on elongation (Blair *et al*. 1991), although this treatment does result in complete embryo loss within a few days after elongation, possibly due to changes in the luminal endometrial epithelial cell glycocalyx.

Thus, the size of the uterus, the number of conceptuses, the relative timing of initiation of elongation, the relative speed of elongation, and the length of termination of elongation all interact to determine the ultimate sizes of each resulting placenta in the litter. Ideally, uniform initiation and speed of elongation would result in the uniform division of the available maternal resources among the members of any size litter. However, in the extreme, this could lead to uniform failure of all conceptuses in the litter if the division of the uterine space was such that no conceptus received an adequate amount. The diversity that occurs in elongation may provide a mechanism whereby some successful pregnancies occur even in this case.

Fetal-placental growth after elongation

A logical question to ask is whether the eventual size of the fetal placenta is fixed at elongation. The fetal placenta continues to grow in weight until day 60 to 70 of gestation, after which its growth rate decreases (Freking *et al*. 2007). Vonnahme *et al*. (2000) reported that crushing every other fetus on day 40 of gestation increased fetal-placental weights of the remaining conceptuses on day 112 of gestation, but did not affect fetal weights. In our own recent experiment (Vallet

et al. 2009), fetuses at the ovarian or cervical ends of the uterine horns were crushed on day 35 of pregnancy and adjacent fetal placenta and fetuses were compared to nonadjacent fetal placenta and fetuses on day 105. Results indicated that both the fetal placenta and fetuses were heavier adjacent to a crushed fetus, but the increase was only about 10%. In addition, fetal placentas were asymmetrical, the side of the fetal placenta adjacent to the crushed fetus was longer from umbilical junction to tip compared to the nonadjacent side. These results clearly demonstrate that (1) most of the eventual weight of the fetal-placenta is established before day 35 of pregnancy, and (2) there is a limited ability of conceptuses to benefit from the demise of an adjacent conceptus if it occurs between day 35 to 40 of gestation.

Fetal-placental structure related to function

There is clearly more to placental development than changes in size during gestation. The conceptus begins to attach to the endometrium beginning around day 13 (Keys *et al*. 1986, Stroband & Van der Lende 1990) with formal implantation occurring around day 18 (Bielanska-Osuchowska 1979). The first placental structure that develops is the yolk sac placenta, which then regresses. This is followed by the development of the allantois, which migrates into the elongated chorion. That portion of the chorion where the allantois does not reach becomes the "necrotic tip". The portion of the chorion that fuses with the allantois becomes vascularized and is the functional fetal placenta (Friess *et al*. 1980). Very little is known about factors controlling the migration of the allantois outward into the chorion, including why it stops. The chorioallantois then fills with allantoic fluid, which presses the chorioallantois against the walls of the uterus. This most likely aids in the process of implantation and also provides a mechanical stimulus for growth (Vogel & Sheetz 2006) of both the uterus and the fetal placenta until the fluid peaks around day 30 of gestation, after which the volume of allantoic fluid recedes to day 45 (Knight *et al*. 1977). Fluid volumes again increase to peak at day 60 and recede again until the volume is relatively minimal by the end of gestation. At the mouths of the uterine glands, the placental areolae develop beginning about day 15 of gestation (Bielanska-Osuchowska 1979, Leiser & Dantzer 1994), and are specialized regions of the trophoblast epithelium that take up secreted products from the uterine glands. Elsewhere on the surface of the chorioallantois, the attached trophoblast-endometrial epithelial bilayer develops microscopic folds beginning ~day 35 of gestation (MacDonald 1976, Dantzer 1984). Using microcorrosion casts, Leiser & Dantzer (1988) reported that the maternal and fetal capillaries are arranged in net-like structures on the maternal and fetal sides, respectively, of the folded bilayer and that they are arranged in a cross-countercurrent fashion. Maternal blood enters the folded structure near the top of the folds and leaves from the bottom. Fetal blood enters the folded structure near the bottom and leaves from the top. Thus, maternal-fetal exchange takes place within the folded bilayer, because this is the area of closest contact between the maternal and fetal capillaries.

Maternal-fetal exchange given this arrangement is maximized by increased folded bilayer surface area, increased capillary density on either side of the folded epithelial bilayer, and reduction in the distance between the capillaries. During later gestation, secondary folds in the epithelial bilayer develop, increasing the maternal-fetal interaction surface area (Friess *et al*. 1980). In addition, a recent study in our laboratory (Vallet & Freking 2007) indicated that the depths of the folds increased during later gestation, accounting for much of the increase in surface area, and is greater in the fetal placentas of small fetuses, suggesting a possible compensatory mechanism for small placental size/lack of uterine space.

In regards to capillary density, microcasting of the fetal capillaries indicates that their density increases with advancing gestation (see plates 4 and 5 of MacDonald 1976). A visual appraisal of

these images suggests that little room for improvement in capillary density during late gestation may be possible. Nevertheless, other reports indicate that placental vascularity is greater in Meishan fetal placentas compared to Yorkshire placentas (Wilson *et al*. 1998) although it is not clear from this report whether the vascularity measured was only those capillaries adjacent to the folded bilayer.

Regarding the distance between capillaries, it has been reported that the capillaries adjacent to the folded bilayer become indented within the planes of both the maternal and trophoblast epithelial layer during late gestation, although both epithelial layers remain intact even during late gestation (Friess *et al*. 1980). This report indicated that the intercapillary distance is as little as 2 μm. Thus, it appears that all of the functionally relevant physical characteristics of the pig fetal placenta improve with advancing gestation.

At the same time that the folded bilayer widens, the thickness of the fetal-placental stroma outside the folds decreases (Vallet & Freking 2007). In the fetal placenta of the smallest fetuses within a litter, the average thickness of the fetal-placental stroma outside the folds is reduced at all stages of gestation compared to larger littermates and, in some cases, there is no remaining fetal-placental stroma outside the folded bilayer. We have suggested that the fetal-placental stroma external to the folded bilayer represents room for further deepening of the placental folds to further increase bilayer surface area. A consequence of this hypothesis is that in fetal placenta of small fetuses where no additional fetal-placental stromal area exists external to the folds, no further widening of the folds may be possible. This provides a possible explanation for intrauterine crowding induced fetal losses that occur during late gestation (Freking *et al*. 2007).

These ideas suggest that fold development may be a primary contributor to placental efficiency, making an understanding of fold development key to improvements in pig placental function. Interestingly, the gross morphology of the trophoblast epithelial cells within the folded bilayer of the placenta is not uniform. In this review, all references to direction and position of cell types will be after orienting sections with the fetal placenta toward the top of the image, and the maternal placenta (i.e., endometrium) toward the bottom. In this orientation, trophoblast cells at the top of the folds of the bilayer are tall columnar. Along the sides and at the bottom of the folds, trophoblast epithelial cells are cuboidal (Friess *et al*. 1980). This difference in morphology suggests differences in function, but these differences have not been explored. Friess *et al*. (1980) suggested that the tall columnar cells at the tops of the folds are involved in macromolecular transport due to their secretory appearance. Electron microscope studies by Dantzer *et al*. (1981) support this possibility, but there is no other evidence to support this suggestion. Our investigation of expression of membrane-bound folate binding protein (Kim & Vallet 2007) suggests relatively uniform expression of this gene in both cell types. A detailed exploration of gene expression and protein production by these cell types is needed to resolve differences in function between these cells. However, their position at the tops of the folds, nearest to the thinning fetal-placental stroma, suggests that these cells may play a central role in fold development. Also interesting is the proximity of these cells to incoming maternal blood given the structure of the vasculature reported by Leiser & Dantzer (1988). Do the columnar cells at the tops of the folds develop in response to the nutrient rich environment provided by preexisting or developing maternal blood vessels, or do the columnar cells induce the underlying maternal vasculature? This is a key question, because it will determine whether strategies should focus on placental vascular development or generation of tall columnar trophoblast epithelial cells.

We hypothesized that generation of the folded structure at a minimum requires modification of the fetal-placental stroma. How this is accomplished depends on its composition. Stromal extracellular matrix is composed of various proteins and glycosaminoglycans (GAG), and a previous study of fetal-placental GAG content indicated that the most abundant GAG were

hyaluronan and heparan sulfate (Steele & Froseth 1980). We confirmed and extended these observations by measuring hyaluronan in fetal-placental tissues from day 25 to 105 of gestation and localizing hyaluronan in fetal-placental tissues. Hyaluronan content of fetal-placental tissue is low on day 25 of gestation, increases dramatically by day 45, and remains high to day 105. Hyaluronan content increases further from day 85 to 105 of gestation in placenta of small fetuses. Localization indicated that hyaluronan was a component of the entire fetal-placental stromal tissue (Vallet & Freking 2006).

Given that hyaluronan is a component of the fetal-placental stroma, we hypothesized a role for hyaluronoglucosaminidase (HYAL) in the changes in the fetal-placental stroma that would be necessary for bilayer folding (Vallet *et al*. 2008). Specific primers were developed to amplify several known HYAL (Csoka *et al*. 2001) and we were successful in cloning two cDNA corresponding to pig HYAL 1 and one cDNA corresponding to HYAL 2. One of the HYAL 1 cDNAs and the HYAL 2 cDNA obtained were similar to previously described forms of HYAL 1 (Frost *et al*. 1997) and HYAL 2 (Lepperdinger *et al*. 1998). The other HYAL 1 cDNA appeared to be a novel truncated form. The truncated region is reported to contain an EGF-like domain (Chao *et al*. 2007). The consequences of this truncation in terms of function of the resulting proteins require further investigation.

Expression of each form of HYAL mRNA was examined using RT-PCR (Vallet *et al*. 2008). The full length form of HYAL 1 was similar across gestation. The truncated form of HYAL 1 increased with advancing gestation. The HYAL 2 mRNA decreased with advancing gestation. For all forms examined, expression was greater in the fetal-placental tissue of small fetuses compared to larger fetuses. We also used hyaluronan zymography (Miura *et al*. 1995) to measure hyaluronoglucosaminidase activity in placental homogenates. Two molecular weight forms were observed, one similar to those previously reported and a second, more abundant, lower molecular weight form. Both molecular weight forms of hyaluronoglucuronidase activity increased with advancing gestation and were greater in the fetal-placental tissue of small fetuses. These results are consistent with a role for hyaluronan metabolism by Hyal 1 and 2 in the development of the pig fetal placenta during gestation.

Heparan sulfate was another abundant GAG present in fetal-placental tissue (Steele & Froseth 1980). In humans, only a single functional heparanase gene exists, located on human chromosome 4 (McKenzie 2007). Human chromosome 4 is homologous to swine chromosome 8 (Rohrer 1999) in a region where a QTL for litter size is located (King *et al*. 2003). Heparan sulfates are components of proteins that make up epithelial cell basement membranes (Lopes *et al*. 2006), and we hypothesized that modifications to the structure of the epithelial bilayer would require heparanase activity because the basement membranes would be altered as part of this process (Miles *et al*. 2008). Amplification of fetal-placental total RNA using primers specific for heparanase resulted in three cDNAs related to heparanase. One of these forms corresponded to the previously described active form of heparanase (Toyoshima & Nakajima 1999), the other two coded for truncated forms. Both truncated forms are likely to be enzymatically inactive, and their role in the fetal placenta will require further experimentation. Analysis of fetal-placental total mRNA for the putative active form of heparanase indicated that expression was relatively high on day 25, was less on days 45 and 65, and increased again on days 85 through 105 of gestation (Miles *et al*. 2008). In situ hybridization localized heparanase mRNA to the cuboidal trophoblast epithelium lining the sides and bottom of the placental folds (JR Miles, unpublished observations). A closer examination of the folds indicates that the interiors of the folds are also undergoing morphological changes with the area of fetal-placental stroma within the folds becoming more extensive. Thus, it seems likely that heparanase plays a role in these changes, as well as possibly being involved in the indentation of capillaries into the plane of the epithelial bilayer that results in reductions in the maternal-fetal intercapillary distance.

Placental angiogenesis

It is obvious that extensive angiogenesis must take place on the fetal and maternal sides of the pig placenta. One of the reasons we chose to study placental hyaluronan and heparan sulfate metabolism is that fragments of both are known to be angiogenic (West *et al*. 1985, Jakobsson *et al*. 2006). Thus, expression of these genes could couple changes in fold morphology with capillary development surrounding the bilayer. There is a constellation of other genes reported to participate in one or more aspects of angiogenesis in a variety of tissues (Liekens *et al*. 2001, Bouis *et al*. 2006, Lamalice *et al*. 2007, Otrock *et al*. 2007, Ribatti *et al*. 2007). These include fibroblast growth factors (FGF; Presta *et al*. 2005), vascular endothelial growth factors (VEGF; Dai & Rabie 2007), transforming growth factors (TGF; Lebrin *et al*. 2005), and the angiopoietins (Fiedler & Augustin 2006). In addition, numerous other genes and metabolites interact with these known angiogenic factors and modulate their activity. Focusing on hyaluronan and hyaluronoglucuronidases, several proteins are known to bind hyaluronan including CD44 (Knudson *et al*. 2002), receptor for hyaluronan mediated motility (RHAMM; Savani *et al*. 2001), and other hyaladherens (Turley *et al*. 2002). Numerous proteins inhibit hyaluronoglucuronidase activity (Mio & Stern 2002). Turning to heparanase, activity requires proteolytic cleavage (Shafat *et al*. 2006). Heparan sulfate is involved in receptor binding for both basic FGF (Schlessinger *et al*. 2000) and VEGF (Jakobsson *et al*. 2006), partially explaining its role in angiogenesis. The presence of VEGF (Winther *et al*. 1999) and TGFβ (Gupta *et al*. 1998) in the pig placenta have been reported. Placental VEGF expression is correlated with vascularity of the placenta during gestation (Vonnahme & Ford 2004). Given the complexity of these interacting systems, we have just scratched the surface of regulation of placental angiogenesis in the pig.

Nutrient specific transport mechanisms

Because the pig placenta is epitheliochorial, nutrients must pass through either two sets of epithelial cell tight junctions or four sets of cell membranes and two epithelial cell cytoplasms to get from the maternal blood supply to the fetal blood supply. The cytoplasmic distance is greatly reduced during late gestation (Friess *et al*. 1980). Tight junctions are selective barriers to the passage of solutes, thus passage depends on their permeability for different solutes (Schneeberger & Lynch 2004). The phospholipid bilayer composition of cell membranes means that each membrane is a hydrophobic barrier, which prevents passage of polar molecules like sugar. One way this is dealt with is by incorporating transport proteins in the membrane to allow passage of molecules that are incompatible with the lipid environment of the plasma membranes. Generally, membrane passage can take place with (active transport) or without (facilitated diffusion) expending cellular energy. Another way to accomplish passage is through incorporation of the solute into endometrial epithelial protein, which is then secreted. This requires uptake by the fetal-placental epithelium and transport across the cell, to be released to the fetal blood stream. The following describes some of the known mechanisms facilitating transport of oxygen, glucose, amino acids, lipids and vitamins and minerals.

Oxygen

Molecular oxygen is small and nonpolar and readily diffuses. Nevertheless, the low oxygen affinity of maternal hemoglobin and the higher oxygen affinity of fetal hemoglobin facilitates the transfer of oxygen from mother to fetus (Comline & Silver 1974). In the pig fetus, embryonic hemoglobin production gives way to adult hemoglobin around day 30 of gestation, the pig does

not produce a fetal hemoglobin similar to that of humans (Weber *et al.* 1987). Nevertheless, the hemoglobin present in fetal red blood cells has a higher affinity for oxygen compared to maternal hemoglobin (Zhang *et al.* 2006). The difference in oxygen affinity is the result of differences in 2,3-diphosphoglycerate concentrations in maternal and fetal red blood cells (Sasaki & Chiba 1983). Increased concentration of 2,3-diphosphoglycerate reduces hemoglobin oxygen affinity (Kim & Duhm 1974). Concentrations of 2,3-diphosphoglycerate remain low throughout fetal life in the pig and rapidly increase after birth (Gluszak 1979). A single enzyme, 2,3 bisphosphoglycerate synthase/phosphatase, catalyzes the synthesis and degradation of 2,3-diphosphoglycerate in red blood cells (Mulquiney *et al.* 1999). The factors controlling the activity of this enzyme during fetal life and the mechanism of the dramatic increase in 2,3-diphosphoglycerate levels after birth are not known.

Glucose

Glucose and other monosaccharides are polar compounds that do not diffuse readily through lipid bilayer membranes. Two groups of glucose transporter genes exist to overcome this problem, sodium-dependent glucose transporters (gene SLC5A, protein SGLT) and facilitative glucose transporters (gene SLC2A, protein GLUT; Zhao & Keating 2007). The SGLT proteins transport glucose from low to high concentrations through the coupling of glucose with sodium transport from high to low sodium concentrations. The sodium is subsequently pumped from the cell by Na/K/ATPase, thus glucose transport by this mechanism expends cell energy. The GLUT proteins are bidirectional transport proteins that transfer solute from high to low concentration and do not expend cell energy. There appears to be no report of the investigation of either of these gene classes in the swine placenta. Nevertheless, fetal blood glucose concentrations are considerably lower than maternal blood glucose concentrations (Pere 1995, Fowden *et al.* 1997), indicating that glucose transport occurs down a concentration gradient and pointing to GLUT protein mediation of glucose transfer. Examination of the swine gene index (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=pig) indicates that expressed sequence tags (EST) corresponding to sodium-glucose cotransporter 10 (SGLT5) and facilitative glucose transporters 1 (GLUT1), 2 (GLUT2), and 3 (GLUT3) have been isolated from placenta, and EST corresponding to sodium-glucose cotransporter 3 (SGLT3) and facilitative glucose transporters 1 (GLUT1), 3 (GLUT3) and 5 (GLUT5) have been isolated from uterus. The GLUT1 and 3 proteins transport glucose (Olson & Pessin 1996); GLUT2 transports glucose and fructose (Colville *et al.* 1993) but has the highest affinity for glucosamine (Uldry *et al.* 2002). The GLUT5 transporter is specific for fructose (Zhao & Keating 2007). Together, glucose transporters present in endometrial and trophoblast epithelial cells allow glucose to cross all four cell membrane barriers.

The pig placenta is fructogenic (Rama *et al.* 1973), converting a portion of the incoming glucose into fructose, and fetal plasma levels of fructose are actually higher than glucose (4.5 versus 2 mM; Pere 1995). It has been suggested that this placental ability sequesters glucose in the fetus because fructose does not escape back into the maternal circulation (Pere 1995). Conversion of glucose to fructose would also serve to maintain lower fetal glucose concentrations, widening the maternal to fetal glucose concentration gradient, and improving glucose transport to the fetus. However, the fetus does not appear to use fructose because umbilical artery and vein fructose concentrations do not differ appreciably (Pere 1995). This calls into question the utility of sequestration of glucose in the form of fructose. However, it appears that the fetal liver is also fructogenic (White *et al.* 1979). Fetal circulation in the pig is such that all the blood returning from the placenta passes through a structure similar to a ductus venosus, and has relatively intimate contact with fetal liver tissue (Dickson 1956). In addition

blood returning to the placenta is actually a mixture of blood processed by the fetus and blood returning from the placenta (Kiserud 2005). Thus fructose concentrations in the umbilical artery may not fully reflect fructose metabolism by the fetus. The fructogenic activity of the liver may mask any substantial use of fructose by the fetus because fructose may be replaced as it is metabolized. On the other hand, ^{14}C-fructose tracer studies in sheep conceptuses, which are also fructogenic, indicate that the rate of usage of fructose by the sheep conceptus is one-fifth that of glucose (Meznarich *et al.* 1987).

Two alternative pathways exist to generate fructose from glucose (Rama *et al.* 1973). The first is the Embden-Meyerhoff-Cori pathway in which glucose is first phosphorylated to glucose-6-phosphate by hexokinase, which is then converted to fructose-6-phosphate by isomerase. Alternatively, glucose can be converted to fructose through the sorbitol pathway, involving aldose reductase and ketose (sorbitol) reductase. The latter pathway has the advantage that ATP is not consumed. It has been reported that the pig placenta lacks ketose reductase (Rama *et al.* 1973), but ESTs for both aldose reductase and sorbitol reductase have been obtained from pig placenta. Thus, it is likely that both pathways exist in the placenta so the primary enzyme pathway controlling conversion of glucose to fructose requires investigation.

Curiously, fructose-6-phosphate participates in a pathway that generates the N-acetylglucosamine needed for hyaluronan and other GAG synthesis (Holden *et al.* 2003, Moussian 2008). Fructose-6-phosphate is converted to glucosamine-6-phosphate in a reaction catalyzed by glutamine fructose-6-phosphate amidotransferase (GFAT). This reaction consumes glutamine, which has the highest concentration of any amino acid in fetal plasma (Wu *et al.* 1995). Wen *et al.* (2005) indicated that the GFAT enzyme, through generation of glucosamine from fructose-6-phosphate, activates mammalian target of rapamycin (mTOR), which increases the proliferation of a human trophoblast cell line. In addition, the affinity of GLUT-2 for glucosamine is an order of magnitude higher than for glucose and fructose (Uldry *et al.* 2002), suggesting that glucosamine concentrations may regulate glucose and fructose transport by this transporter through competition for binding. It seems possible that the pathways that generate glucosamine from fructose, and fructose from glucose, could link GAG synthesis, trophoblast proliferation and glucose transport, and may play a role in changes in placental structure in response to reduced placental size. Clearly, these pathways and their role in placental development require further study.

Amino acids

Wu *et al.* (1995) reported the concentrations of amino acids in fetal and maternal circulations. Only the concentrations of cysteine, glycine, histidine, leucine, phenylalanine, and tryptophan were lower in fetal umbilical venous plasma compared to maternal plasma, and total α-amino acid concentration was greater in fetal plasma compared to maternal plasma. This suggests that amino acid transport must involve one or more active transport mechanisms. Because of interconversions among amino acids and the fact that some amino acids can be synthesized from other metabolic substrates, concentrations of individual amino acids do not allow firm conclusions on transport mechanisms.

Of the amino acids, glutamine concentrations display the greatest fetal maternal difference, and the placenta is capable of synthesizing glutamine from other amino acids (Self *et al.* 2004). Arginine concentrations are also unusually high in fetal fluids (Wu *et al.* 1996). Interestingly, arginine supplementation has been reported to improve litter size (Mateo *et al.* 2006, Hazeleger *et al.* 2007) and has been proposed to play a role in numerous functions during pregnancy (Wu *et al.* 2004). These include nitrous oxide (NO) generation, polyamine synthesis and creatine generation (Morris 2007, Brosnan & Brosnan 2007). While, NO (Wu *et al.* 1998) and polyamine

synthesis (Wu *et al.* 2005) have received some attention, creatine is thought to be responsible for shuttling ATP from the mitochondria to the cytoplasm (Paddon-Jones *et al.* 2004) and is thus essential for energy utilization in the fetus. Because low birthweight piglets have decreased survival within the first two days of life, the ability to utilize energy efficiently at birth could have a profound effect on piglet survival.

Finch *et al.* (2004) have investigated leucine uptake by placental trophoblast vesicles. They report that both sodium-dependent and independent mechanisms are present in the pig placenta, and that the sodium-dependent transport capacity of placentas of small fetuses was less than that of larger littermates. Because sodium-dependent transport requires energy to maintain the sodium gradient, this could be an adaptation to decreased availability of energy in small fetuses. Clearly, the mechanisms responsible for amino acid transport and the generation, maintenance, and reasons for the high concentrations of certain amino acids in fetal plasma warrant further investigation.

Lipid transport

Lipids are higher energy substrates than sugars, thus transport of lipids would be advantageous to the developing pig fetus. Unfortunately, when lipid transport has been measured, it has been found to be low or nonexistent (Thulin *et al.* 1989, Ramsay *et al.* 1991). However, Thulin *et al.* (1989) infused radiolabelled free fatty acids to determine transport by the placenta, which is unlikely to present lipids to the placenta in the same form that lipids typically circulate in the blood. Lipids are typically bound to lipoproteins and are delivered to tissues through receptors for the lipoprotein-lipid complexes (Herrera 2002). Thus, it may be that the capacity of the pig placenta to deliver lipids to the fetus may be underappreciated. In further support of this, certain essential fatty acids do not appear to be synthesized by animal tissues, so some mechanism for delivery of these essential fatty acids must exist (Youdim *et al.* 2000). Although piglet fat reserves are typically low at birth, one aspect of development that requires lipid is brain myelination (Youdim *et al.* 2000). Myelin membranes are specialized plasma membrane structures of nerve peripheral cells composed primarily of phospholipid (Deber & Reynolds 1991). Brain myelination begins before birth in the piglet and likely affects piglet survival after birth (Dickerson *et al.* 1971). Thus, while the currently available results suggest that lipid transport by the placenta is poor, the opportunity represented by improvements in brain function and piglet survival would seem to be good justification for further exploration of lipid transport by the pig placenta.

Vitamins and minerals

It is likely that the transport of various vitamins and minerals by the pig placenta differs with each individual nutrient. However, some general concepts can be inferred from the vitamins and minerals for which the transport mechanisms have been explored. Iron is transported to the pig fetus as a component of uteroferrin (Roberts & Bazer 1988). Pig endometrial glands secrete copious quantities (2 g per day) of uteroferrin (Buhi *et al.* 1982), which is picked up by fluid phase pinocytosis by the areolae (Raub *et al.* 1985). Substantial amounts of the iron in uteroferrin are metabolized by the placenta because circulating concentrations of acid phosphatase activity are quite low in fetal plasma (Vallet *et al.* 1996). However, the fetal liver is also capable of binding uteroferrin by mannose-6-phosphate receptors (Saunders *et al.* 1985). Finally, circulating plasma uteroferrin can also be cleared by the fetal kidney into the allantois where substantial amounts of uteroferrin are present (Baumbach *et al.* 1986). A mechanism

exists in the allantoic fluid to transfer iron from uteroferrin to transferrin, which can then return to the fetus (Buhi *et al.* 1982). The handling of uteroferrin by the pig conceptus in this way may prevent the oxidizing activity of reduced uteroferrin (Vallet 1995). Thus, iron transport via uteroferrin as a model suggests that the endometrial glands and areolae participate in the delivery of nutrients to the developing pig conceptus, and that the allantoic sac may serve as a storage and protective chamber for metabolites.

Retinol is delivered to the developing pig conceptus bound to retinol binding protein (Clawitter *et al.* 1990). Retinol plays a role in cell signaling (Duester 2008) and may also participate in providing antioxidant activity for the developing conceptus (Vallet 1995). It is likely that retinol is delivered to the fetus via the areolae in a manner similar to that of uteroferrin (Dantzer & Winther 2001).

In contrast to uteroferrin and retinol, folate transport to the developing pig conceptus likely takes place through the concerted activities of secreted and placental membrane-bound forms of folate binding protein (Kim & Vallet 2004). Uterine flushings on days 13 and 15 of the cycle or pregnancy contain large amounts of secreted folate binding protein (Vallet *et al.* 1998b). Immunohistochemical localization of the secreted form of folate binding protein indicated that it was present in endometrial glands beginning about day 13 of pregnancy until day 20 and disappears by day 35 of pregnancy (Kim & Vallet 2004). Endometrial mRNA levels for this protein do not follow this pattern (Vallet *et al.* 1999), and it has not been possible to demonstrate synthesis of secreted folate binding protein by endometrial tissue (J.L. Vallet & J.G. Kim, unpublished observations). Thus, it seems highly likely that the secreted folate binding protein found in the endometrial glands and in uterine flushings during early pregnancy may originate from serum. The placenta expresses an mRNA for a membrane-bound form of folate binding protein (Vallet *et al.* 1999), and in situ hybridization indicates that this mRNA is present in trophoblast epithelium of the placental folds (e.g., present in both tall columnar cells and in cuboidal cells) throughout gestation (Kim & Vallet 2007). Kim & Vallet (2004) have hypothesized that folate transport is thus accomplished by the sequential effects of secreted folate binding protein during early pregnancy, followed by placental membrane-bound folate binding protein during later gestation. Conceptually, transport with these characteristics is likely to be sensitive during the switchover period (i.e., during early placental development). This period of pregnancy is unusually sensitive to intrauterine crowding (Vonnahme *et al.* 2002, Freking *et al.* 2007), and it seems possible that nutrients like folate that transition from histotrophic to placental transport could contribute to the sensitivity of this period.

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Growth, body state and breeding performance in gilts and primiparous sows

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Optimizing gilt management is a critical point to improve breeding herd efficiency. This review describes the effects of growth rate (GR) and body state at onset of puberty stimulation or at first mating on gilt puberty attainment, productivity and sow longevity. Traditional management practices should be re-evaluated with attention to different modern genotypes. It is difficult to discern the real effects of age, weight, backfat depth and estrus number at first insemination on longevity and reproductive performance, because these variables affect one another. GR interacts with age at boar exposure to influence age at puberty. Higher lifetime GR gilts (>700 g/d) attain puberty earlier and have a lower anoestrus rate. If gilts attain a target weight (135-150 kg), are adapted to herd health status and have at least one previously recorded estrus, they can be inseminated. Overweight at first breeding and throughout gestation should be avoided. There is no advantage in breeding gilts heavier than 150 kg; at first farrowing the target weight is 180-185 kg. Piglet production at first parity may be increased in gilts with a high GR but the number of stillborn piglets can also be increased. The culling rate over 3 parities for locomotion problems, which is one of the major risk factors for reduced herd retention rate, can be increased in overweight gilts at first breeding (>150-170 kg).

Introduction

Gilts and primiparous sows are the largest farrowing group in pig farms. Implementing an effective gilt pool management strategy is a fundamental tool to achieve targets for body condition and physiological maturity. The efficiency with which gilts are introduced to the breeding herd has a major impact on breeding herd efficiency. A constant flow of high quality and well managed gilts into the breeding herd will increase sow longevity, stabilize the parity structure of the breeding herd and allow the farm to achieve weekly breeding targets.

Modern genotypes are more sensitive to nutritional mismanagement than their predecessors, as they have less appetite and lower energy reserves at the beginning of their productive lives (Whittemore 1996). To achieve breeding targets and to make gilts as highly productive as possible, it is important to recognize the key physiological characteristics of contemporary dam-line females, and particularly their exceptional lean growth potential. Unfortunately, the

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major changes in lean growth potential and associated changes in overall tissue metabolism of contemporary dam-line sows are still not adequately recognized (Foxcroft *et al.* 2005).

Traditional management practices that were established even 20 years ago need to be re-evaluated if we are to capture the full economic potential of the modern breeding sow and her offspring, in terms of greatly improved nutrient utilization (Foxcroft *et al.* 2005). Even after re-evaluating these practices for modern genotypes, some peculiarities among the wide variety of animals available from the various breeding companies are expected.

Potential efficiency of the breeding herd increases as the number of non-productive days associated with gilt development decreases. To choose the right moment to introduce and breed gilts, the cost of production, potential first and second parity total born, farrowing rate, retention rate, sow longevity and lifetime productivity must be taken into account.

Although replacement gilts can enter a herd at various ages and with different management practices, they need to be managed correctly from birth through their first lactation. The successful development of replacement gilts requires the integration of health, nutrition, genetics, housing and management practices. Thus, an excellent gilt development program will reduce replacement costs of multiparous sows. The objective of this paper is to review the effects of growth rate (GR) and body state on puberty onset, productivity and sow longevity.

Growth rate and age at puberty

The occurrence of puberty can be stimulated at younger ages by several environmental and management factors, but associations between puberty and a critical weight (Prunier *et al.* 1987, Newton & Mahan 1992), GR (King 1989, Beltranena *et al.* 1991, Beltranena *et al.* 1993), lean tissue (Patterson *et al.* 2002) and backfat depth or body fat percentage (Newton & Mahan 1992, Rozeboom *et al.* 1995, Gaughan *et al.* 1997) are still controversial. The interaction among corporal characteristics and other factors such as breed, age, boar effect, season and environment, which also affect puberty attainment (Evans & O'Doherty 2001), is not clearly understood. An increased potential for lean tissue deposition and an associated lack of fatness in the modern genotypes could change the minimum level of leanness or fat, or ratio of fat to lean to attain puberty. Such a selection trend indicates that traditional management practices established with old genotypes should be re-evaluated.

Earlier data (Beltranena *et al.* 1991) suggested that only when GR was below 0.55 kg/day from birth to boar stimulation onset at 160 days of age was there a delay on pubertal estrus. Recent data support that with unrestricted feeding during the prepubertal period (growth/finish phase) and recommended space allocations during development, it is unlikely that GR in modern genotypes will limit age at onset of first estrus (Foxcroft *et al.* 2005, Amaral Filha *et al.* 2009). It should be emphasized that boar contact at an appropriate age will exert a great influence and play a critical role on puberty attainment. The onset of boar contact at a younger age decreases age at puberty but requires a longer period of stimulation. On the other hand, older gilts at boar exposure onset are typically older at puberty, require fewer days of stimulation and are more synchronized at first heat (Van Wettere *et al.* 2006, Amaral Filha *et al.* 2009).

In a well managed farm, approximately 85% of gilts should attain puberty within 3 or 4 weeks after boar exposure (Foxcroft *et al.* 2005). Kummer *et al.* (2009) observed that higher GR gilts (723 g/day) stimulated at approximately 144 days of age showed puberty nine days earlier (155 vs 164 days of age) than lower GR gilts (577 g/day) with more of them attaining puberty by 190 days of age (95% vs 76%). The fact that 95% of the heavier gilts showed estrus by 190 days of age indicates the importance of pre-pubertal GR as a factor influencing puberty mainly when gilts are exposed to boars at an early age. Amaral Filha *et al.* (2009) described the effect

of the interaction between age at boar exposure (142 vs 157 days of age on average) and GR on puberty attainment. It was shown that puberty onset of high GR females (> 725 g/d) when gilts were stimulated at 130-149 days of age was earlier than low GR females (550-649 g/d). In contrast, if gilts were submitted to boar exposure at an older age (150-170 days), no influence of GR on puberty age was observed (Table 1). High GR gilts are likely to be physiologically more mature at younger ages in terms of possible minimums in threshold weight, lean:fat ratio, fatness or rate of body reserve accumulation (Hughes 1982, Kirkwood & Aherne 1985), which are necessary for responsiveness to boar contact.

Table 1. Cumulative percentage of gilts showing the pubertal estrus and age at puberty according to growth rate and age at the onset of boar exposure.

	Age at boar exposure					
	130–149 days			150–170 days		
Growth rate, g/d	550-649	650-725	726-830	550-649	650-725	726-830
Number of gilts	170	400	181	201	349	185
Estrus by 10 d, %	27.6a	29.0a	38.1b	44.3b	45.3b	43.2b
Estrus by 20 d, %	48.2a	48.7a	59.7b	63.7b	67.3b	63.8b
Estrus by 30 d, %	70.6ab	67.5a	76.2bc	83.6c	81.7c	82.7c
Estrus by 40 d, %	82.3ac	79.2a	85.6ab	88.6bc	89.7b	89.2bc
Pubertal females, %	90.0a	89.2a	91.7a	93.0a	93.1a	96.2a
Puberty age, d (± SE)	164.8a (1.11)	162.2ab (0.76)	159.6b (1.03)	172.1c (1.07)	171.5c (0.81)	174.0c (1.17)

Different letters, in the row, indicate significant differences ($P < 0.05$).

(Amaral Filha *et al*. 2009).

It seems possible that feed restriction may influence the relationship between GR and age at puberty (Beltranena *et al*. 1991; Rozeboom *et al*. 1995). Beltranena *et al*. (1991) reported a quadratic relationship between lifetime GR and age at puberty, suggesting that gilts that gained more than 600g/d may have late puberty. However, age at puberty was not quadratically or linearly related to GR from birth to puberty in another study (Rozeboom *et al*. 1995). Discrepancy between studies may be related to the fact that the curve of Beltranena *et al*. (1991) was derived using both restricted and full-fed gilts, whereas all gilts were provided *ad libitum* access to feed in the study of Rozeboom *et al*. (1995). Indeed, when considered separately, average daily gain of full-fed gilts was not related to age at puberty in the study of Beltranena *et al*. (1991). Although low, Kummer *et al*. (2009) found a negative correlation between age at puberty and GR from birth to 165 days ($r = -0.36$, $P = 0.0002$). There was also a low association in the work of Amaral Filha *et al*. (2009), being significant only for gilts stimulated prior to 150 days of age. GR from birth to 134 kg of body weight (BW), in Landrace x Yorkshire gilts, was also negatively correlated ($r = -0.40$, $P < 0.001$) with age at first observed estrus (Tummaruk *et al*. 2009). However, puberty was attained at about 200 days of age and average GR was 583 ± 56 g/d, which is in agreement with previous findings in the same breed of gilts (Tummaruk *et al*. 2007), but with a larger variation on puberty age and GR compared with other studies (Amaral Filha *et al*. 2009, Kummer *et al*. 2009). These variations could be explained by differences in the genetics between experiments and also by many management factors, specially the boar stimulation protocol.

Taken together, these results show that GR close to 800 g/d has no detrimental effect on puberty onset. Other authors also suggest that when GR is higher than 620 g/d (Kirkwood & Thacker 1992) or at commercially acceptable GR (550 to 800 g/d) from birth to 100 d of age, puberty seems not to be limited by GR (Foxcroft *et al*. 2005).

Effect of growth rate on conception rate and litter size

Increasing GR by 100g/d, in the first 100 days of life, has resulted in an increase of 0.3 piglets born, an earlier return to estrus after weaning and a greater farrowing rate (Tummaruk *et al.* 2001). Comparing two groups of gilts according to their GR from birth to 144 days of age (average GR of 577 and 724 g/d), no differences were observed in the pregnancy rate, ovulation rate, total and viable embryos, and embryo survival rate at 32 days of gestation (Kummer *et al.* 2009). However, it should be pointed out that 19% of gilts with lower GR were culled for anoestrus compared with 3.4% of high GR gilts ($P < 0.01$). In commercial farms, these anoestrus gilts would probably have been reallocated or hormonally treated to stimulate their first estrus, resulting in a possible drop in reproductive performance and increase of non productive days.

Using the minimum GR (726g/d) and weight (95 kg) observed in high GR gilts (Amaral Filha *et al.* 2009) combined with boar stimulation at an early age (130-149 days), the expectation is that even the lightest gilt will still reach a weight around 139 kg by its third estrus, and remain within the range of 135-150 kg considered as ideal for the first mating (Williams *et al.* 2005). Conversely, the weight of high GR gilts receiving boar stimulation later (150-170 days) ranged from 110 to 134 kg at the start of boar contact, implying that most of these gilts could become overweight by the time of breeding at their second or third estrus. It has been suggested that high GR may result in negative effects on the physical fitness of replacement gilts, and on welfare and culling rates of higher parity sows (Foxcroft & Aherne 2001). To overcome the problem of excessive weight, gilts with a high GR could receive boar stimulation earlier. Since culling rate and reproductive performance over three parities are not compromised in gilts with a GR ≥700g/d when mated 25 days younger (Kummer *et al.* 2006), the advancement of first mating in high GR gilts can be suggested. Therefore, healthy and adapted gilts which have a BW of 130 to 150 kg and two estrous cycles can be mated regardless of age or backfat thickness (BT). It has been reported that there is no advantage in breeding gilts at greater than 150 kg body weight. From a practical standpoint, high GR gilts should be bred earlier (Kummer *et al.* 2006) or nutritional strategies could be used during the growing phase to reduce their GR. In situations where gilts are bred at greater than 150 kg, strategies can be used during gestation to manipulate the GR of the sows up to first parity.

Recently, Amaral Filha (2009) categorized gilts that were inseminated at an average of 3.5 ± 0.6 estrous cycles and about 220 days of age into three groups according to the GR from birth until first mating (Table 2). Farrowing rate and return to estrus were not affected by GR groups. GRII and GRIII females had greater litter size compared to GRI gilts, but there was no difference among GR groups in the number of piglets born alive. Nevertheless, GRIII females had a higher percentage of stillborns than GRI and GRII females (Table 2). Young *et al.* (2008) also verified that gilts with highest GR (>860 g/d) had more total piglets born but more stillborns than gilts with GR <680, 680–770 and 770–860 g/d (0.96, 0.64, 0.64, and 0.74 stillborns, respectively). A higher number of intrapartum stillborns in high GR gilts could be related to their excessive weight at farrowing being associated with prolonged farrowing (Muirhead & Alexander 1997). Another possible reason for increasing intrapartum stillborns could be variation in birth weight. The higher coefficient of variation (CV) for birth weight, the higher percentage of litters with CV of birth weight >20% and the greater number of piglets with weight <1200g observed in highest GR gilts probably contributed to their greater stillbirth rate (Table 2). Because birth weight is an important indicator of pre-weaning piglet survival and birth weight variation has been associated with changes in weaning weight (Milligan *et al.* 2002, Quiniou *et al.* 2002, Gondret *et al.* 2005, Wolf *et al.* 2008), it seems reasonable to avoid an excessive weight at mating to minimize birth weight variation.

Table 2. Reproductive and production traits for gilts grouped according to growth rate (GR) from birth until first mating (± SE)

	Groups of growth rate, g/d		
	GRI (600-700 g/d)	GRII (701-770 g/d)	GRIII (771-870 g/d)
Return to estrus rate, % (n/n)	6.4a (22/345)	6.2a (44/710)	6.0a (22/366)
Adjusted farrowing rate*, % (n/n)	92.6a (315/340)	92.7a(651/702)	93.6a(339/362)
Total born, n	12.0 ± 0.16a	12.5 ± 0.11b	12.9 ± 0.15b
Born alive, n	10.9 ± 0.16a	11.3 ± 0.12a	11.3 ± 0.17a
Total stillborn, %	5.5 ± 0.61a	6.1 ± 0.44a	8.7 ± 0.83b
Prepartum Stillborn, %	0.8 ± 0.15a	1.0 ± 0.12a	1.3 ± 0.21a
Intrapartum Stillborn, %	4.7 ± 0.59a	5.1 ± 0.41a	7.2 ± 0.74b
Mummified, %	2.8 ± 0.28a	3.2 ± 0.25a	3.7 ± 0.38a
Females weighed at farrowing	282	551	290
Weight at farrowing, kg	196 ± 0.71a	206 ± 0.56b	217 ± 0.75c
Net weight gain during gestation, kg	49.2 ± 0.68a	46.4 ± 0.51b	44.8 ± 0.75b
Backfat thickness at farrowing, mm	16.6 ± 0.16a	17.0 ± 0.12ab	17.3 ± 0.16b
Litters weighed, n	280	553	286
CV** for birth weight (BW), %	15.9 ± 0.38a	17.1± 0.28b	18.1 ± 0.37b
Litters with CV for BW >20, % (n)	25.0 (70)a	29.3 (162)a	36.4 (104)b
Piglets with weight <1200g	2.5 ± 0.17a	2.8 ± 0.12ab	3.1 ± 0.17b

a, b, c different letters indicate difference in the same row ($P<0.05$).
* Calculated by excluding dead females and females removed for non reproductive reasons.
** Coefficient of Variation
(Adapted from Amaral Filha 2009).

Effect of weight at first mating or at farrowing on performance over multiple parities and lactational catabolism

Because gilts bred with less than 135 kg have less total piglets born over 3 parities than gilts weighing over 135 kg, this weight seems to be the minimum necessary at first service to allow gilts have a proper body mass at first farrowing, assuming a weight gain of 35 to 40 kg during first gestation (Williams *et al.* 2005). Kummer *et al.* (2006) observed that high GR gilts (≥ 700g/day) could be inseminated at an early age (185 to 210 days) without impairing their productive performance over three parities. After grouping gilts according to their weight at first service (130-150 kg, 151-170 and 170-200 kg), no differences were observed in total born and born alive over 3 parities (Amaral Filha *et al.* 2008). However, the heavier gilts at first service had decreased farrowing rate in parity 2 (Table 3). On the other hand, Tummaruk *et al.* (2007) observed that age, BW and BT at first estrus influenced subsequent reproductive performance over the first three parities. The mean litter size was greater in gilts that had a first observed estrus between 181 and 200 days of age with 110-120 kg BW and 13-15 mm BT. It must be pointed out that these authors worked with crossbred Landrace x Yorkshire gilts that expressed their first standing estrus on average at 195 days of age, later than the usual age at puberty observed in modern commercial genotypes (Amaral Filha *et al.* 2009, Kummer *et al.* 2009).

Based on experimental data with modern genotypes and cost-benefit analysis, gilts should be inseminated at a target weight of 135-150 kg and at least at the second estrus (Williams *et al.* 2005, Kummer *et al.* 2006). In this way, primiparous sows should reach approximately 180 kg of BW at farrowing, this target minimizing the effects of the loss of protein mass that is still seen in many genotypes during the first lactation (Williams *et al.* 2005).

Table 3. Performance over three parities for sows grouped according to growth rate or weight at first artificial insemination (AI).

	Kummer *et al.* (2006)			Amaral Filha *et al.* (2008)		
	Growth rate and age at first mating			Weight at first mating, kg		
	≥700 g/d <210 d	≥700 g/d ≥210 d	<700 g/d ≥210 d	130–150	151–170	171–200
Number of females	164	165	239	298	1007	421
Weight at AI, kg	149±9a	164±8b	147±8c	143±5a	160±5b	177±6c
Age at AI, d	198±6a	223±8b	223±8b	211±9a	219±9b	225±8c
Parity 1						
Farrowing rate, %	88.4a	87.9a	88.7a	89.9a	90.7a	92.9a
Total born, n	11.7±3.0a	12.8±3.0b	11.8±3.4a	12.1±2.8a	12.4±2.9a	12.8±3.1b
Born alive, n	10.5±3.1a	11.3±2.9a	10.5±3.3a	11.1±2.8a	11.1±3.0a	11.3±3.2a
Parity 2						
Farrowing rate, %	84.6a	86.2a	89.4a	88.2a	79.3b	72.5c
Total born, n	11.0±3.6a	11.5±3.6a	10.6±3.2a	9.6±3.5a	9.8±3.3a	9.8±3.7a
Born alive, n	10.6±3.5a	10.7±3.2a	10.1±2.9a	9.1±3.5a	9.3±3.2a	9.2±3.5a
Parity 3						
Farrowing rate, %	88.2a	91.1a	90.2a	88.1a	91.5a	88.9a
Total born, n	12.0±3.7a	12.3±3.6a	12.4±3.4a	11.7±2.9a	11.7±3.2a	12.0±3.3a
Born alive, n	11.2±3.6a	11.4±3.3a	11.5±3.1a	11.0±2.9a	10.8±3.2a	11.0±3.2a

Values presented as means ± SD
a, b, c in the row indicate differences among groups within each reference ($P<0.05$).

Modern genotypes have less voluntary feed intake (Whittemore 1996), which compromises the demands for maintenance and higher milk production, resulting in body reserve mobilization. First parity sows are the most susceptible to these losses. Heavy gilts at first service tend to be heavy at farrowing with more demands for maintenance during lactation. Highest body reserve mobilization occurs in females that are heavier (Quesnel *et al.* 2005a) or fatter (Young *et al.* 2004) at farrowing. This is explained by reduced feed intake in fat sows (Revell *et al.* 1998, Young *et al.* 2004, Quesnel *et al.* 2005b), mainly during the first two weeks of lactation, which can be related to the circulating concentration or oxidation of nonesterified fatty acids and glycerol (Revell *et al.* 1998) or to an insulin resistance state in heavy sows (Quesnel *et al.* 2005b). Greater weights at first parity are associated with greater losses in BW and protein during lactation, however, percentages of fat loss were similar regardless of the weight at farrowing (Table 4). It has been proposed that changes in amino acid profile according to the degree of muscle protein mobilization can affect oocyte development and maturation (Clowes *et al.* 2003, Quesnel *et al.* 2005a). Moreover, responses such as decreased RNA-to-DNA ratio, increased protease gene expression and reduction of essential amino acid levels in *triceps brachii* muscle were magnified in primiparous sows fed to have a higher degree of protein mobilization during lactation (Clowes *et al.* 2005). These findings help to explain the effect of lactational catabolism on the reduction of embryo survival without affecting ovulation rate or weaning-to-estrus interval (Vinsky *et al.* 2006). To understand the reduction in the subsequent farrowing rate but not in the number of total born piglets that is observed in heavy females (Table 4), it would be necessary to suppose that their degree of protein mobilization had such a great impact on oocyte quality that fertilization or early embryo survival was impaired resulting in higher return to estrus rate. However, to cause return to estrus, the reduced quality of a sufficient fraction of oocytes would be required so that the number of viable embryos in the uterus would be insufficient for an appropriate signal for pregnancy recognition. Alternatively,

breeding outcome may be influenced by an asynchrony between uterine milieu and embryonic development, which could result from changes in the embryonic or uterine proteome under the influence of protein catabolism.

Table 4. Body characteristics of primiparous sows at farrowing, body reserve mobilization during lactation and reproductive performance after weaning for gilts grouped according to weight at first farrowing (± SE).

	Groups of weight at first farrowing		
	Light	Intermediate	Heavy
Number of primiparous sows	376	805	379
Characteristics at farrowing			
Body weight, kg	189 ± 0.33a	207 ± 0.19b	225 ± 0.33c
(Range)	(167.5-197.0)	(197.1-217.0)	(217.1-245.0)
Body fat, kg	43.1 ± 0.22a	48.7 ± 0.14b	53.8 ± 0.23c
Body protein, kg	30.1 ± 0.06a	33.3 ± 0.04b	36.6 ± 0.07c
Backfat depth, mm	15.9 ± 0.13a	17.1 ± 0.09b	17.9 ± 0.14c
Lactation length, d (range 15-26 d)	19.5 ± 0.11a	19.7 ± 0.07a	19.6 ± 0.11a
Total born, n	12.8 ± 0.14a	12.5 ± 0.10a	11.9 ± 0.17b
Born alive, n	11.7 ± 0.13a	11.4 ± 0.10a	10.8 ± 0.16b
Body reserves mobilization			
Body weight loss, %	7.9 ± 0.22a	9.3 ± 0.15b	10.1 ± 0.21c
Body fat loss, %	17.2 ± 0.44a	17.9 ±0.30a	17.6 ± 0.40a
Body protein loss, %	7.3 ± 0.26a	8.8 ± 0.17b	9.8 ± 0.25c
Backfat depth loss, mm	2.9 ± 0.11a	3.2 ± 0.08a	3.2 ± 0.13a
Weaned piglets, n	10.5 ± 0.05a	10.5 ± 0.04a	10.6 ± 0.05a
Reproductive performance after weaning			
Weaning to estrus interval, d	6.1 ± 0.18a	6.2 ± 0.14a	5.9 ± 0.19a
Return to estrus rate, %	11.4a	16.5b	17.7b
Adjusted farrowing rate, %	84.9a	80.0b	76.4b
Total born, n	9.3 ± 0.19a	9.8 ± 0.14ab	10.3 ± 0.20b
Born alive, n	8.9 ± 0.18a	9.3 ± 0.13ab	9.6 ± 0.19b

a, b, c ($P < 0.05$). (Schenkel *et al.*, unpublished observations).

Effect of weight at insemination on skeletal soundness

Gilt body condition at first mating has a significant effect on lifetime productivity. Productive performance and herd longevity can be compromised if gilts have an insufficient or excessive weight at first mating. Useful production targets might be that 85% and 75% of selected gilts should reach the first and second farrowing, respectively.

With leaner modern genotypes, pubertal BW is increased and physiological maturity is reduced. Fast growing gilts becoming overweight by the first service may be one of the major risk factors for poor retention in the herd (Williams *et al.* 2005). Excessive weight gain during the rearing phase may result in increased culling rate, mainly due to osteochondrosis, reducing sow longevity (Jongbloed *et al.* 1984, Sorenson *et al.* 1993). Nevertheless, this issue is still controversial, because it has been confirmed that fast growth in rearing does not impair skeletal integrity compared with slower growing pigs (Crenshaw 2003, Ytrehus *et al.* 2004). Most studies on osteochondrosis in pigs are performed as evaluation of lesions in slaughter animals and in some of them daily GR is not similar to that observed in the modern dam-line genotypes. Ytrehus *et al.* (2004) evaluated animals in different age groups and the oldest one had a gain of 600 g/d from birth to 15 weeks of age. They found no correlation between

osteochondrosis and weight or daily gain. However, these authors (Ytrehus *et al*. 2004) report that associations between osteochondrosis and rapid growth may be related with inherited traits in some populations.

Even for females comprising the breeding pool, the association between GR and removal due to lameness is still controversial. Overall retention rate over three parities was similar (75.2 vs. 72.4) in females with high and low GR ($\geq$700 g/d vs. $<$700g/d) until first mating (Kummer *et al*. 2006) and removal for lameness at the end of three parities was not affected by GR in rearing (Young *et al*. 2008). However, culling rate for locomotion problems over three parities increased (Table 5) as the weight at the first mating increased (Amaral Filha *et al*. 2008).

Table 5. Culling reasons over three parities for sows grouped according to body weight at first mating.

Culling reason, n (%)	Groups of weight at first mating, kg		
	130–150 (n = 298)	151–170 (n = 1007)	170–200 (n = 421)
Locomotion	18 (6.0)a	104 (10.3)b	64 (15.2)c
Reproductive	37 (12.4)a	104 (10.3)a	52 (12.4)a
Others	41 (13.8)a	109 (10.8)a	48 (11.4)a

a, b, c in the same row indicate significant differences ($P < 0.05$).
(Amaral Filha *et al*. 2008).

Fatness and breeding performance

The effect of fatness on puberty attainment and productive performance is still a controversial issue. Fatness seems to be less critical than a targeted weight for puberty attainment (Foxcroft *et al*. 2005). Taking into account that increases in fatness are transient and any residual effects disappear by weaning of the first litter (Gill 2006), fatness at first mating seems less important than a target weight for lifetime productivity (Foxcroft *et al*. 2005).

While the results of some studies (Newton & Mahan 1992, Rozeboom *et al*. 1995) show that the onset of puberty seems not to be controlled by a specific amount of body fat, another study (Gaughan *et al*. 1997) indicated that more gilts reached puberty in the group with higher BT define at selection (16-18 mm) compared to the group with a low BT (10-12 mm). Recently, Tummaruk *et al*. (2009) found no correlation between BT at 90 or 134 kg of BW with age at first estrus. In terms of productivity, whereas a clear impact of BT, measured at 100 kg body weight (Tummaruk *et al*. 2001) or at first mating (Rozeboom *et al*. 1996; Williams *et al*. 2005), on the number of piglets born in the first litter or over three parities has not been observed, gilts with a BT between 13 and 15 mm at the first estrus had more piglets than gilts with a BT between 11 and 13 mm (Tummaruk *et al*. 2007). More piglets were born in gilts with 16-17 mm of BT (12.7 ± 0.11) than in BT 10-15 mm gilts (12.2 ± 0.16) but no increase in total piglets born was observed in high (18-23 mm) BT gilts (12.4 ± 0.14; Amaral Filha 2009).

Altogether, these observations show that BW rather than BT should be used as criterion to breed swine females. Furthermore, BW cannot be predicted by measuring BT because the pattern of increase in lean body mass differs from that of changes in BT (Williams *et al*. 2005, Gill 2006), which is usually weakly correlated with BW (Williams *et al*. 2005). Indeed, the correlation between BT and BW at mating is not significant in low GR gilts and it is lower than 0.5 in high GR gilts (Table 6). Tummaruk *et al*. (2009) also reported a low significant correlation between BT and BW ($r = 0.27$, $P < 0.001$) when gilts were approximately 230 d of age.

Table 6. Relationship between body weight and backfat thickness at mating in gilts with different growth rates.

	Kummer *et al*. (a)		Amaral Filha *et al*. (b)		
	492-656 g/d n = 43	658-800g/d n = 55	600-700g/d n = 345	701-770g/d n = 710	771-870g/d n = 366
Correlation coefficient	0.27	0.45	-0.016	0.090	0.11
P value	0.079	0.0005	0.771	0.017	0.038

(a) Growth rate from birth until approximately 144 d of age.
(b) Growth rate from birth until first mating.
(Amaral Filha *et al.* and Kummer *et al.*, unpublished observations)

Conclusions

It is important to recognize that physiological characteristics of contemporary females are different from old genotypes thereby traditional management practices that were established in the past should be re-evaluated. The comprehension of the interaction among body characteristics and puberty attainment, productivity performance and longevity is still limited. It seems that these interactions change with time and it is also expected that some peculiarities exist among the wide variety of genotypes. It is difficult to discern the real effects of age, weight, backfat and estrus number at first insemination on reproductive performance and longevity, because these variables are associated among themselves. Age at boar exposure interacts with GR to influence age at puberty. Higher lifetime GR gilts (>700 g/d) attain puberty earlier and have a lower anoestrus rate after boar exposure for puberty stimulation. Gilts that attain the breeding target weight (135-150 kg) with at least one previously recorded estrus and that are adapted to herd health status can be inseminated irrespective of age and backfat level. Piglet production at first parity may be increased in gilts with a high GR but the number of stillborns can also increase. Excessive weight at first breeding or first farrowing should be avoided. The culling rate for locomotion problems over 3 parities can be increased in these gilts.

Acknowledgements

The authors acknowledge the support of Brazilian National Council of Technological and Scientific Development (Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq) for their financial support.

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Genetic selection for lifetime reproductive performance

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Genetic improvement of sow lifetime reproductive performance has value from both the economic perspectives of pork producers and the pork industry, but also from the perspective of ethical and animal welfare concerns by the general public. Genetic potential for piglets produced from individual litters is a primary determinant of lifetime prolificacy, but females must be able to sustain productivity without injury or death beyond the achievement of positive net present value. Evidence exists for between- and within-line genetic variation in sow lifetime performance, suggesting that improvements may be made by both line choices and genetic selection within lines. However, some of the same barriers to accurate within-line selection that apply to individual litter traits also present challenges to genetic selection for sow lifetime prolificacy: generally low heritabilites, sex-limited expression, expression after the age that animals are typically selected, and unfavorable genetic correlations with other traits in the profit function. In addition, there is an inherent conflict within the genetic nucleus herds where selections take place between the goal of shortened generation interval to accelerate genetic progress and the expression of sow lifetime traits. A proliferation in the industry of commercial multipliers with direct genetic ties and routine record flows to genetic nucleus herds provides a framework for accurate estimates of relevant genetic variances and covariances, and estimation of breeding values for sow lifetime traits that can be used in genetic selection.

Introduction

Female prolificacy is a primary determinant of commercial success in the pork industry and, accordingly, selection for improved prolificacy has long been recognized as a high priority in pig breeding strategies (Tess *et al.* 1983). Due to the overhead costs of female development to an active stage of reproduction, the effects of parity on number of piglets produced, and animal welfare concerns associated with a high turnover rate for females in the breeding herd, there is significant value in improvement of sow lifetime reproductive performance in the industry, and great interest in the impact that genetic selection may have on this goal.

The amount of genetic variation in litter size traits has been well-documented and successfully exploited in experimental and commercial selection to increase the number piglets produced per litter (Rothschild & Bidanel 1998). More recently, there have been reports of significant genetic variation in sow longevity and lifetime output (Serenius & Stalder 2006; Arango *et al.* 2005) that suggest that effective selection for sow lifetime reproductive performance may be

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possible. But questions remain regarding the complete genetic covariance architecture among all economically important traits and how to optimize selection for both lifetime prolificacy and efficient market hog performance (Young *et al*. 1977; Rutledge 1980; Foxcroft *et al*. 2006). Moreover, at a practical level there is the inherent conflict between intentional replacement of breeding females in genetic nucleus herds to minimize generation interval and accelerate genetic progress, and the need to allow expression of lifetime performance of each sow if these traits are to be measured. This conflict may affect parameter estimates from commercial pig populations, and represents a significant challenge to implementation of effective commercial breeding strategies for lifetime traits.

The objectives of the present paper are to provide a brief review of the literature on sow lifetime reproduction as related to genetic selection, and to suggest a strategy for both estimation of genetic parameters and selection for sow lifetime performance in a typical commercial pig population.

The economics and dynamics of sow lifetime performance

Although the number of piglets with the potential for full market value that are produced by a sow in her lifetime is clearly an important factor in the determination of profitability, the economics of sow lifetime performance is a complex function of investment and opportunity costs, changes in biological output over time, the value of the output and the salvage value of the sow. A definition of optimum sow lifetime performance and associated optimum parity and replacement rate for commercial herds have been the focus of several studies of which a few are highlighted herein.

Stalder *et al*. (2003) used a deterministic model of a breed-to-wean system to identify the average parity at which a sow achieved a positive net present value (NPV). PigCHAMP records from 1996 to 2000 were used as a basis for assumptions of input costs, performance levels and output value, and sensitivity to changes in replacement gilt cost, number of live piglets born per litter, and weaned-pig price were modeled. Using the average base assumptions, a sow reached a positive NPV at parity three. Since the average parity in all reporting herds over that same time period was 3.36, the authors concluded that the average sow in an average breed-to-wean system left the herd just after reaching profitability and that significant profit potential was not realized. The average parity at which positive NPV was achieved was more sensitive to a decrease in number born alive per litter and changes in weaned-pig price than changes in replacement gilt costs.

Commercial data from 32 farms in central Illinois, collected from 1995 to 2001 and including representation of eight primary genetic lines, were used to model the sensitivity of NPV of enterprise income to parity at culling, litter income per parity, and discount rates which reflect a combination of inflation, interest rates and risk (Rodriguez-Zas *et al*. 2003). Average parity at culling ranged from 2.95 to 4.00 across the eight genetic lines. When income per litter was assumed for all lines at either $50 or $10, it was impossible for a positive NPV to be achieved in any of the lines at discount rates greater than 4% and income per litter of $10, emphasizing the need to maximize net income per litter to realize the economic benefits of longevity. When genetic lines were compared at these fixed incomes per litter, the economic advantage of lines with greater longevity was reduced as discount rates increased, but the results in general also demonstrated that herds with a greater average longevity were subject to less economic risk than those with lesser average longevity.

The commercial data reported by Rodriguez-Zas *et al*. (2003) were also the basis for a subsequent application of a model for optimum replacement of assets in an infinite planning

horizon (Burt 1965) to sow replacement and salvage value scenarios in breed-to-wean systems (Rodriguez-Zas *et al*. 2006). The economic inputs were discount rate, building and equipment costs per sow, sow replacement costs, revenue per piglet weaned and sow salvage value. By optimization of returns in a continuous system per fixed unit of time, this approach accounted for the opportunity cost of sow space. The optimum parity at replacement using base biological and economic inputs was 5 parities when voluntary replacement rates were low and there was no sow salvage value, and 4 parities when voluntary replacement rates were high and a percentage of sows had salvage value, the latter considered most similar to typical breed-to-wean production. In sensitivity analyses of economic factors, sow replacement costs and salvage value had the greatest effect on optimum parity number at replacement; revenue per piglet weaned had a significant but lesser effect. Huirne *et al*. (1991) reported similar results from earlier application of dynamic modeling to optimization of sow replacement, both in terms of the impact of sow costs and salvage value, and the optimum parity at replacement (5.5).

Although these economic models of sow lifetime performance provide a basis for evaluation of commercial systems and guidance for the optimum emphasis to put on longevity and biological output in assessment of individual animals in the herd, solutions are clearly complex and system specific, and there remains opportunity for improvement in resolution of the tools. Considering average parity at replacement and replacement rates observed in commercial production (Stalder *et al*. 2004), it is clear from reported economic analyses that there is currently greater risk of lost profit due to premature removal from the herd than due to late removal from the herd (Rodriguez-Zas *et al*. 2006). Even though profitability in some analyses was less sensitive to number of piglets weaned per litter than to cost of gilt development, price per weaned pig and sow salvage value (e.g., Rodriguez-Zas *et al*. 2006), practical models will be more useful when extended to account for line differences and genetic trend in biological output. As work continues to provide effective models for system and genetic line comparisons, interactions between lines in average number of piglets per litter across parities (Moeller *et al*. 2004) may also be considered.

Animal welfare has also been mentioned as a justification for improving sow longevity (e.g., Stalder *et al*. 2004). The economic modeling work reviewed in this section focused primarily on the optimum use of voluntary culling of sows in commercial herds, in other words optimum culling of sows from the herd because of factors such as a small litter weaned or cumulative number weaned, inadequate estimated breeding value, poor rebreeding performance or simply because of age. While minimization of involuntary culling creates the greatest opportunity for optimization as defined in these economic models (i.e., the greatest opportunity for voluntary culling), it is also the minimization of involuntary culling and optimization of voluntary culling in tandem that should result in improved sow longevity from an animal welfare perspective. A short life in the herd that concludes with voluntary culling because of a small litter produced contributes to a need for more animals to produce the same amount of output and for that reason is undesirable from both an economic and animal welfare perspective. A scenario in which a female leads a long reproductive life, but leaves the herd because of structural injury or death may fit optimization in a purely economic model but is undesirable from an animal welfare perspective. Thus, understanding and managing factors that contribute to involuntary culling is also important to both economic and animal well-being goals.

Stalder *et al*. (2004) summarized a large body of literature published over the time period from 1960 to 2000 on factors that resulted in removal of sows from the breeding herd in commercial production systems. Reproductive failure was consistently the predominant category (ranging from 8.8 to 39.2% removed), but significant percentages of females were also removed because of feet, leg and locomotion problems (ranging from 6.1 to 15%) and

death (ranging from 3.0 to 12.3%). The tendency in these same reports was for reproductive failure and feet and leg soundness to be a likely cause of removals at early parities, and for poor performance (presumably piglet output) or death to be a frequent cause for removal at later parities. Among the mortalities, arthritis, mastitis, cystitis, gastritis, heart failure and gastric torsion each accounted for significant proportions in specific populations. The same general predominance of reproductive failure, and tendency for changes in cause for removal as parity increases, has also been reported in more recent studies (Arango *et al*. 2005; Mote *et al*. 2009). In addition, recent summaries of commercial performance in United States systems include similar rates of sow mortality (PigCHAMP, 2007; overall average = 8.74%, bottom 10% of herds average = 12.5%).

Genetic variation and selection for lifetime performance

Genetic improvement of sow prolificacy has traditionally been focused on the component traits with known economic value that are most readily and routinely measured, primarily number of piglets produced in a litter. Significant differences between breeds and lines for litter traits can be the basis for relatively rapid changes in prolificacy and have been reported extensively dating back to the breed evaluation and crossbreeding studies coordinated in the US through regional research projects (Craft 1953, Johnson 1981). Even greater between-line variation in domestic swine was observed in breed evaluations after the importation of Chinese breeds into the US (Young 1995, Rothschild & Bidanel 1998). More recently, evaluation of commercial crosses for reproductive output through four parities has been reported (Moeller *et al*. 2004); the range of averages for number born alive across the six commercial crosses tested was 1.45 piglets per litter.

Within-line genetic variance in litter size traits has also been suggested for many years as a means for genetic improvement of sow prolificacy (e.g., Dickerson & Hazel 1944). Although the heritabilities for total number born and number born alive tend to be low (average 0.11 and 0.09, respectively; Rothschild & Bidanel 1998), large phenotypic coefficients of variation provide the potential for genetic progress. Early attempts at controlled, direct selection for litter size in pigs were relatively unsuccessful (Rutledge 1981, Bolet *et al*. 1989), but selection on estimated breeding values for litter size traits in both males and females (Avalos & Smith 1987) has become standard practice in industry nucleus herds and has resulted in significant increases in piglets born per litter.

Selection for biological components of litter size has also been pursued as a way for efficient genetic improvement of litter output (Johnson *et al*. 1984, Christenson *et al*. 1987). Direct selection for litter size was successful in a line of pigs previously selected for greater ovulation rate (Lamberson *et al*. 1991), suggesting that the previous selection had created a line in which ovulation rate was no longer a limiting factor on average and that subsequent selection for number born was effective by putting upward pressure on uterine capacity. Selection on an index of ovulation rate and embryo survival to day 50 of gestation, index selection followed by direct selection for litter size, and two-stage selection for ovulation rate followed by number born, have each been demonstrated to effectively improve number of live piglets born per litter in a long-term selection experiment at the University of Nebraska (Johnson *et al*. 1999, Ruiz-Flores & Johnson 2001, Petry & Johnson 2004).

Only recently have similar estimates of between- and within-line genetic variance in sow lifetime prolificacy traits been reported, and reports of correlated responses in lifetime reproductive performance to selection for first parity litter traits have been limited. There have been no reports in the scientific literature of response to selection for sow lifetime prolificacy.

The evaluation of six commercial maternal lines for reproductive performance through parity four (Moeller *et al*. 2004) was extended to six parities in an effort to estimate between-line differences in sow longevity (Serenius *et al*. 2006). Removal from the project through parity four only occurred if a female failed to conceive within 50 days after weaning, was culled by a veterinarian because of health, or died. Of 3,251 total gilts studied, 17% had right-censored data because they lived longer than six parities. Results from a survival analysis revealed that sows from one of the six maternal products lines had a significantly less risk of being removed from the herd than sows from the other five product lines, which were similar in survival distributions. On average, the five lines similar in longevity had 1.43 times greater risk of being culled than the line with lowest risk. The product line with lowest risk for culling was also the line with greatest number born alive per litter in the first four parities (Moeller *et al*. 2004). The previously described work on development of economic models for sow longevity by Rodriguez-Zas *et al*. (2003) also revealed significant differences between commercial maternal lines in average parity at culling (range of between-line averages = 1.05 parity). These reports suggest that relatively rapid changes in sow longevity and lifetime prolificacy can be made by exploiting between-line variation.

Methods applied to analyses of within-line genetic variance have included use of linear models to length of productive life or binary (0/1) records of survival to a given point in time ("stayability" to a given parity). Alternatively, to account for the inherent censored nature of lifetime data, survival analyses have been applied based on proportional hazard models. While survival analysis methods are more attractive from a theoretical perspective, there have not been practical methods to extend beyond univariate analysis of right-censored survival traits. More recently, methods have been reported for multivariate analyses of Gaussian (normally-distributed), right-censored Gaussian, ordered categorical and binary traits (Korsgaard *et al*. 2003; Damgaard & Korsgaard 2006), thereby allowing for modeling of genetic and environmental correlations and hence the potential for more accurate estimation of parameters, breeding values and predicted responses to selection for lifetime traits.

A review by Serenius & Stalder (2006) included a summary of estimates of within-line genetic variance for traits of sow longevity and productive life. Average heritability for length of productive life was 0.13 and 0.17, respectively, when linear and survival models were applied, and average heritability for stayability was 0.08. Serenius *et al*. (2008) used a multivariate Bayesian approach based on Korsgaard *et al*. (2003) in a subsequent study of Finnish Landrace national field records and reported a heritability of 0.22 for length of productive life. The categories for sow removal reported by Arango *et al*. (2005) were the basis for estimation of genetic variances using a linear censored model. Estimated heritabilities for the categories of removal due to reproductive, non-reproductive, and other reasons were 0.18, 0.13, and 0.15, respectively. In total, these reports of within-line genetic variation for sow longevity and lifetime performance in current industry populations indicate that there exists the potential for response to effective genetic selection.

Some of the challenges that exist in genetic improvement of output per litter may also apply to selection for lifetime prolificacy. In addition to the generally low heritability for litter size and number born alive, selection accuracy is reduced because the traits are expressed only in females and at an age that is typically after selection decisions are made. Hence, selection is based largely on pedigree information. These barriers to accurate selection also certainly apply to sow lifetime reproductive performance.

Unfavorable correlations between direct genetic effects for traits of prolificacy and market pig performance also present a challenge to breeders to optimize selection emphasis on these trait areas and maximize genetic trend in profitability. Although much of the early literature

reported non-significant genetic correlations between litter traits in early parities and growth performance, estimates of correlations and correlated responses to selection in more modern populations indicate unfavorable associations (Clutter & Brascamp 1998, Holm *et al*. 2004). The review by Serenius & Stalder (2006) of parameter estimates associated with sow lifetime performance also suggested unfavorable relationships with market pig traits. Average genetic correlations of stayability or length of productive life with leg conformation, litter size, average daily gain and backfat thickness were 0.13, 0.26, -0.15 and 0.14, respectively. They suggested based on this summary that leg conformation and litter size could be used as early indicators of longer productive life, but questioned if the relationship between litter size and longevity was an autocorrelation due to culling for small litter size.

A negative daughter-dam regression has been widely reported in swine, in which females from large litters tend to produce litters of below average size (Revelle & Robison 1973, Rutledge 1980), leading to recommendations for standardization of litter size in selection herds (e.g., Van Der Steen 1985) and estimation of both maternal genetic and additive genetic effects (Roehe & Kennedy 1993) to achieve greatest response to selection. Negative correlations between maternal and direct effects on growth and efficiency have also been reported in swine (Robison 1972) and maternal effects on growth in swine and litter-bearing laboratory animals have been reported to extend to ages far beyond weaning (Clutter *et al*. 1996, Johnson *et al*. 2002). Foxcroft *et al*. (2006) describe a process through which intrauterine competition in highly prolific females results in a reduced number of muscle fibers by day 90 of gestation that may cause a permanent retardation of growth in surviving offspring. Population studies designed to quantify associations of maternal environment with subsequent growth, development and lifetime reproduction of offspring are needed to more fully and clearly understand these phenomena in the context of lifetime performance, and to better optimize lifetime reproduction and efficient lean meat production in commercial breeding strategies. The extent of linkage disequilibrium in the pig along with availability of densely-spaced, genome-wide markers (Du *et al*. 2007, Rothschild 2009) now also provides the opportunity to map the genetic architecture underlying these genetic variances and covariances by genotyping pedigreed populations that have had relevant phenotypes recorded.

An additional factor that must be considered when collecting new, or using historical, lifetime reproduction data from commercial pig populations is the amount of intentional emphasis put on sow replacement as a means of shortening generation interval and thereby accelerating genetic progress, versus culling of females from the breeding herd based purely on commercial profit drivers. Serenius *et al*. (2008) discussed the large amount of variation due to farm-year effects in the Finnish field dataset that they analyzed and suggested the usefulness of estimates of those effects over time as a management tool, but it is not clear in general how well the current statistical tools being applied to sow lifetime traits account for variation in censoring due to different culling policies among farms. These intentions may be more readily discernable in systems that have well-defined genetic nucleus, multiplier and commercial segments, than in systems in which the functions are less distinct, but in either case this factor must be accounted for adequately if useful estimation of genetic parameters is to be achieved. Moreover, this inherent conflict in a genetic nucleus herd between generation interval and expression of lifetime reproductive performance adds another level of challenge to genetic selection for a sex-limited trait that takes significant time to express, by requiring that the trait also be measured outside the genetic nucleus in animals more genetically distant to selection candidates.

An alternative for estimation of genetic merit in commercial breeding systems

The desire to minimize the risk of new pathogens in commercial pork production systems by limiting the introduction of live animals, and minimize the genetic lag between genetic nucleus and commercial herds, has resulted in the widespread implementation of maternal multiplier systems in which, after the system is initially populated with females, only semen is introduced.

This structure may create a pedigreed population in which one segment is part of a genetic nucleus herd subject to intentionally high female replacement rates as a means of rapid generation turnover, and another segment has the goal of multiplication and female replacement is typical of commercial production. In this latter segment, sow lifetime performance can be expressed and recorded.

In Fig. 1 is a scheme for one such system that exists at Newsham Choice Genetics (NCG) and some of its customers. At the top of the pyramid at NCG are two distinct, closed Landrace nucleus populations (Internal Genetic Nucleuses GN-LR1 and GN-LR2). For any of these customers, an initial population of females from GN-LR1 is used to create an external nucleus (EGN-LR1), after which that multiplier is closed to new animal introductions. A proportion of the EGN-LR1 females each week are bred with semen from GN-LR1, and the remaining matings of EGN-LR1 females that week is with semen from GN-LR2. Thus, enough LR1 x LR1 matings are made to produce candidate females for replacement to maintain the EGN-LR1, and the remainder of the matings are made to produce LR2 x LR1 litters. Selected females from the LR2 x LR1 litters ("LR3") are bred with semen from a Large White line to produce parent-stock gilts for commercial production. A key point is that all EGN-LR1 females are fully pedigreed and genetically tied to GN-LR1.

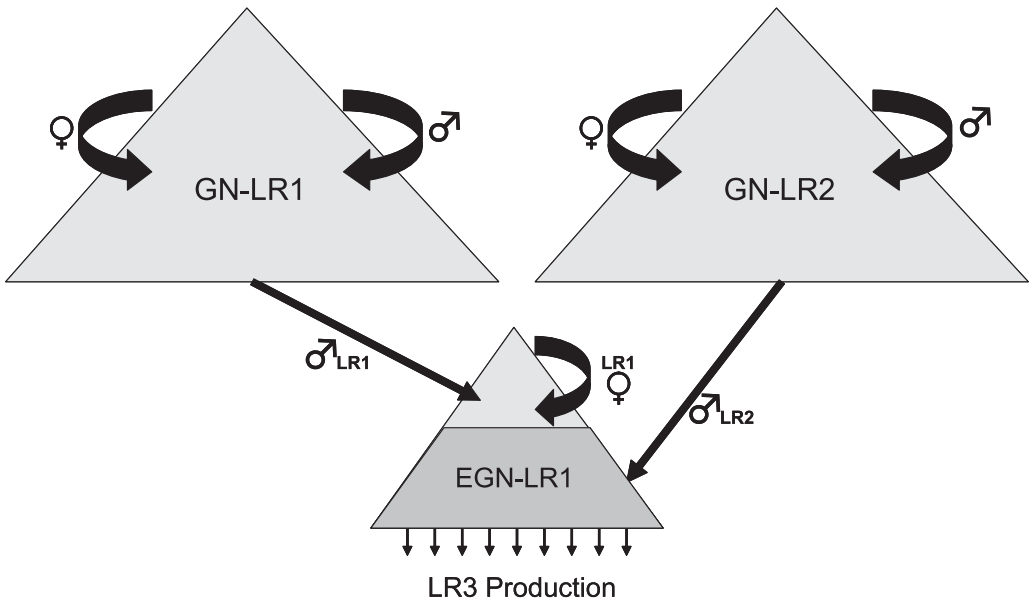


Fig. 1 Schema for external multiplication of hybrid Landrace females (LR3) to be used in production of parent-stock commercial gilts. GN-LR1 is the closed genetic nucleus for Landrace population 1. GN-LR2 is the closed genetic nucleus for Landrace Population 2. EGN-LR1 depicts one of the external customer herds of Landrace 1 females that produces its own replacement females and LR3 females.

The maximum parity achieved for 59,268 females across all EGN-LR1 herds is summarized in Fig. 2a. These numbers include right-censored data (females still in the herd) and data from herds that have not fully matured (i.e., relatively new herds). In Fig. 2b are the proportions of females in each of the categories for maximum parity achieved, both for GN-LR1 and EGN-LR1herds. These data provide a basis for estimation of genetic variance in sow lifetime traits in GN-LR1, and of genetic covariances between growth, body composition, and early parity litter traits measure in GN-LR1 and sow lifetime traits measured in EGN-LR1. In addition, estimated breeding values for sow lifetime prolificacy can be generated for GN-LR1 animals through multi-trait genetic evaluation and used in selection for sow lifetime reproductive performance.

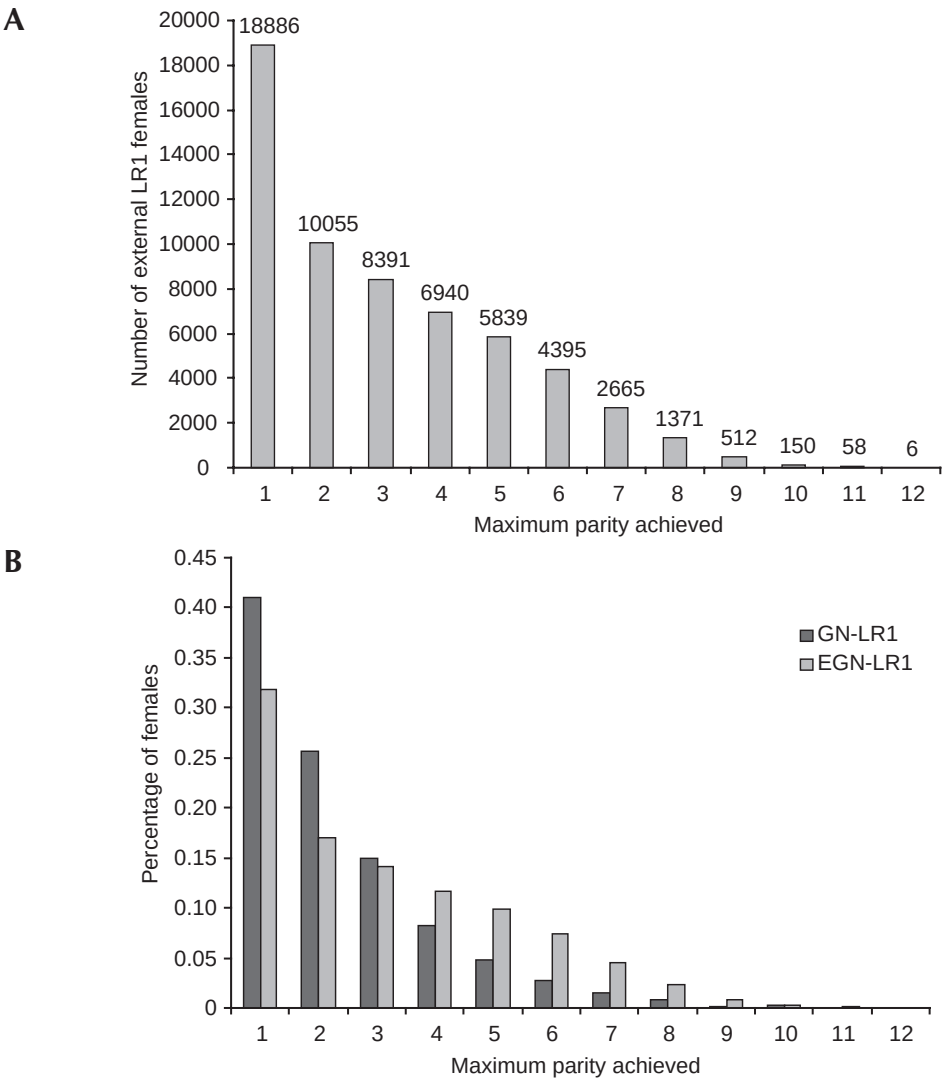


Fig. 2a-b The distribution of maximum number of parities achieved among sows from external herds of Landrace 1 in the multiplication system depicted in Fig. 1 (i.e., EGN-LR1 herds): a) actual numbers of external herd females in each category; b) percentage of females in each category in external herds relative to the internal genetic nucleus for Landrace 1 (GN-LR1).

Conclusions

Studies of the bioeconomics of sow lifetime performance reveal that selection goals for breeding programs in this area must emphasize not only longevity but consistent and prolonged reproductive output. Estimates of heritabilities for sow lifetime prolificacy indicate that genetic selection may be successful, but work remains to effectively overcome unfavorable genetic correlations that likely exist among traits in the overall profit function, as well as the challenge of how best to measure a commercial lifetime trait for use in selection within a genetic nucleus population. Commercial multiplier systems with direct genetic and record-keeping ties to genetic nucleus herds may provide a framework for more accurate parameter estimation and practical selection tools.

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Responses to exogenous gonadotrophin treatment in contemporary weaned sows

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The principal goal of commercial breeding herds is to consistently meet weekly breeding targets. Weaned sows failing to return to estrus within 7 d after weaning contribute to missed breeding targets and increased non-productive sow days. Treatment with low doses of exogenous gonadotrophins (GT) has traditionally been used to advance and synchronize estrus in weaned primiparous sows. However, in well managed contemporary commercial sow farms, more than 90% of sows may return to estrus within 7 d after weaning, posing questions about the likely efficacy of exogenous GT treatment. Therefore, the primary objective of the present study was to determine the response to GT treatment at weaning in contemporary parity 1 commercial sows with lactation lengths typical of the North American swine industry.

Primiparous crossbred sows (PIC C22, n = 275; and PIC C29, n = 131) from a 5,000 sow commercial farrow-to-wean facility (Wildcat, Carthage Veterinary Service, Carthage, IL) were blocked by estimated farrowing weight and genetic line and then randomly allocated to either receive a combination dose of 400 IU eCG and 200 IU hCG (PG600, Intervet, USA, De Soto, KS) I.M. in the neck on the morning of weaning (PG group; n = 189), or to be untreated controls (CON group; n = 218). From the day after weaning, all sows were provided twice daily fence-line contact with mature boars for stimulation and detection of estrus. Sows were bred according to established herd protocols with doses of 3.0×10^9 sperm cells/insemination. Reproductive parameters analyzed were estrus synchronization rate (ESR), determined as the number of sows with observed standing heat within 7 d after weaning, weaning-to-estrus interval (WEI), proportion of sows bred over a 3-d period (BRD3), proportion of sows bred that farrowed (FR), total litter size (TB), and total live born piglets (BA) at farrowing. Based on WEI, sows were retrospectively grouped into 4 categories (WCAT); 1) Target Breed Week: sows bred within 7 d post-weaning, 2) "Tail-enders" sows with an extended 8-19 d WEI , 3) "Missed heats" sows detected in heat $\geq$ 20 d post weaning, or 4) No detected heat by 30 d post weaning.

Estimated farrowing (193.6 $\pm$ 1.5 vs 192.2 $\pm$ 1.6 kg) and weaning (189.4 $\pm$ 1.3 vs 188.0 $\pm$ 1.4 kg) weights were similar in CON and PG sows, respectively, and marginal weight loss in lactation was recorded across all sows (4.2 kg). Considering data from all sows assigned to treatment (Table 1), treatment did not affect the proportion of sows bred within 7 d after weaning (ESR), or within a 3-d breeding window (BRD3). However, the timing of this 3-d breeding window (d 4, 5 and 6 for CON vs d 3, 4 and 5 for PG sows) reflected a shorter ($P < 0.001$) WEI in PG compared to CON sows. PG treatment also eliminated the incidence of sows with an extended WEI (classified as "tail enders"), and reduced variance ($P < 0.05$) in WEI in PG (0.07) compared to CON (0.14) sows when considered over the 19-d period after weaning. For those sows bred within 7 d after weaning, farrowing rate (82.8 vs 86.4%), total born (12.2 $\pm$ .3 vs 12.9 $\pm$ .3) and born alive (11.6 $\pm$ .3 vs 12.1 $\pm$ .3) were

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not different (P >0.05) between PG and CON sows, respectively, and a combined Fertility Index (as defined in Table 1) indicated no gain in productivity in response to GT treatment.

Consistent with earlier reports (Kirkwood *et al*. 1999; Knox *et al*. 2001; Vargas *et al*. 2006), exogenous GT treatment in the present study shortened the WEI, but as also reported by Bates *et al*. (2000) had no effect on estrous synchronization rate, farrowing rate or subsequent litter size born. These results contrast earlier reports of a negative relationship between a shorter WEI and subsequent litter size born (Kirkwood *et al*.. 1999; Knox *et al*. 2001; Vargas *et al*. 2006). These differences in the response to GT treatment may reflect the very synchronous return to estrus seen in the Control sows and good lactational management as reflected in the marginal overall loss in estimated sow body weight at weaning.

Table 1. Effects of treatment (T; exogenous GT at weaning (PG) or untreated controls (CON)), genotype (G), and their interaction on the distribution of observed estrus in all sows on experiment, and productivity of those sows bred within 7 days after weaning.

Treatment	CON	PG	P-Value of effect		
			T	G	T x G
Sows assigned (n)	218	189			
ESR (%)	88.1	92.1	0.22	0.07	0.38
WEI (d)	4.5 ± .07	4.0 ± .07	0.001	0.42	0.88
BRD3 (%)	79.2	85.2	0.27	0.34	0.13
Sows farrowed (n)	166	144			
FR (%)	86.5	82.8	0.71	0.28	0.32
TB	12.9 ± .3	12.2 ± .3	0.11	0.45	0.24
BA	12.1 ± .3	11.6 ± .3	0.20	0.38	0.22
Fertility index[1]	983	930			
	CON	PG	T	WCAT	T x WCAT
Sows per WEI category (1/2/3/4)%	88.1/2.8/2.3/6.9	92.1/0.5/3.1/4.2	0.34	0.001	0.14

[1] Fertility index = sows bred in < 7 days after weaning and farrowed per 100 sows weaned x TB

The absence of sows classified as "tail enders" in the PG group may be of practical significance. Assuming that the incidence of "tail enders" will increase at more adverse times of year due to seasonal effects on lactation feed intake and overall reproductive performance, the apparent ability of GT treatment to induce an early ovulation in these sows is of interest. Those GT treated sows showing a silent first heat and then detected in estrus at 19 or more days after weaning will have active corpora lutea at days 17-19 after weaning. These sows should therefore respond to luteolytic prostaglandin treatment and could then either be bred if they show a clear standing estrus or be reliably culled as either showing persistent silent heats or as being totally anovulatory. The return on investment of being able to impose a standardized protocol for reaching a culling decision based on reliable indicators of reproductive performance after weaning, and thus reduce unwarranted non-productive sow days seen in many herds, may be substantial.

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Benefits of synchronizing ovulation with porcine luteinizing hormone (pLH) in a fixed time insemination protocol in weaned multiparous sows

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Insemination protocols are usually based on the onset of estrus, with a view to inseminating within a 24 hour window before or up to 16 hours after ovulation (Kemp & Soede 1996). However, use of transrectal ultrasonography has shown that the time of ovulation relative to onset of estrus is highly variable, and dependent on weaning-to-estrus interval (WEI), duration of estrus and frequency of estrus detection (Weitze *et al*. 1994). Variation in the timing of the pre-ovulatory LH surge relative to onset of estrus is assumed to cause these differences in the time of ovulation during estrus (Tilton *et al*. 1982) and injection of porcine luteinizing hormone (pLH) induces ovulation approximately 38 h after administration (Cassar *et al*. 2005). Using pLH at the onset of estrus, timed insemination 24 or more hours after pLH then ensures optimal fertility outcomes. The efficacy of combining pLH administration at first detection of standing heat in weaned multiparous sows in a commercial operation, followed with fixed-time double insemination, was initially compared to results from sows conventionally managed and inseminated multiple times until no longer in estrus.

Sows (parity 2-9) were assigned to treatment at weaning on d 21 of lactation. From the day of weaning, twice-daily boar exposure at 8am and 2pm facilitated estrus detection. Onset of behavioural estrus was defined as the time that sows first showed a standing response to back pressure in the presence of a boar. In accordance with normal farm practice, untreated control sows (CON; n = 156) were inseminated at 6-, 18- and 24-h intervals based on their WEI and the time (am or pm) they were first recorded in estrus (See Table 1). After the second insemination, additional inseminations were employed at 24-h intervals until sows were no longer in standing heat. Sows assigned to LUT (n = 163) were administered 5mg pLH (Lutropin-V, Bioniche Animal Health, Belleville, ON; I.M. in 4 ml of vehicle) concomitant with the first detection of standing heat. If sows were first detected in estrus in the morning (8am) they were inseminated at 8am and 2pm the next day (24 and 30 h after pLH injection). If estrus was first detected in the afternoon (2pm), sows were inseminated at 2pm the next day and 8am the following day (24 and 42 h after injection; see Table 2). Extended semen was < 3 days old and contained a minimum of $3x10^9$ live spermatozoa per dose. Data from sows that had a weaning to last insemination interval (WLII) < 2 or > 10 d were removed from the analysis. Parities 7, 8 and 9 were analyzed as one group (Parity 7+). The effect of treatment, parity, and their interaction on number of inseminations, age of semen, WLII, litter size and weight born, and individual piglet weight were analyzed using a linear mixed effect model, with lactation length as a covariate. Proportions of sows bred that were pregnant and farrowed were analyzed using a chi-squared test.

There were significant effects of treatment and parity but no treatment by parity interaction for any variable measured. For the purposes of this presentation only the main effects of treatment

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are reported. The last insemination occurred sooner after weaning (5.3 ± 0.07 vs. 5.8 ± 0.08d; $P < 0.001$), the number of inseminations was fewer (2.0 ± 0.02 vs.2.2 ± 0.02; $P < 0.001$) and farrowing rate tended to be higher (87.4 vs. 82.3%; $P = 0.1$) in LUT compared to CON sows, respectively. Total pigs born and born alive was also greater ($P < 0.01$) in LUT (12.9 ±0.3 and 11.7 ± 0.3, respectively) compared to CON (11.8 ± 0.3 and 10.6 ± 0.3, respectively) sows. Treatment had no affect on average piglet weight or on piglet removal rate due to low viability before cross fostering at d 3 after farrowing.

Table 1. Untreated control sow insemination schedule

WEI (d)	Estrus onset	1st insemination time / Estrus to AI interval (h)	2nd insemination* / Estrus to AI interval (h)
≤ 4 days	am	Same day pm (6 h)	Next day am (24 h)
	pm	Next day am (18 h)	Following day am (42 h)
> 4 days	am	Same day am (0 h)	Next day am (24 h)
	pm	Same day pm (0 h)	Next day am (18 h)

* then inseminated at 24-h intervals until no longer in standing estrus

Table 2. Lutropin treated sow insemination schedule

Estrus onset / pLH administration	1st insemination time / pLH to AI interval (h)	2nd insemination time / pLH to AI interval (h)
am	Next day am (24 h)	Next day pm (30 h)
pm	Next day pm (24 h)	Following day am (42 h)

These results indicate that administration of pLH at onset of behavioural estrus to control time of ovulation enables a double fixed-time insemination protocol to be employed, which resulted in reduced semen usage and labour devoted to estrus detection, and improved sow productivity. These data also provide preliminary information suggesting that administration of pLH concomitant with onset of estrus could be used to facilitate a fixed-time insemination protocol in which a single insemination is performed 24 or 30 hours after pLH administration.

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Antiluteolytic mechanisms and the establishment of pregnancy in the pig

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Extended exposure of progesterone and conceptus estrogen influences the vascular compartment of the uterus and expression of many factors, such as prostaglandins (PGs), growth factors, extracellular matrix and adhesion molecules, cytokines and transcription factors. One of the supportive mechanisms by which the conceptus inhibits luteolysis is by changing PG synthesis in favor of luteoprotective PGE2. Alteration in PG synthesis may result from increased PGE synthase (mPGES-1) expression in the trophoblast and endometrium on days 10-13 of pregnancy with simultaneous down-regulation of PGF synthase (PGFS) and prostaglandin 9-ketoreductase (CBR1). Conceptus and endometrial, rather than luteal, synthesis of PGE2, is involved in the process of maternal recognition of pregnancy. However, complex (direct and indirect) actions of estrogen on the CL, including decreased luteal VEGF soluble receptor on day 12 of pregnancy, are important for luteal maintenance. Moreover, conceptus signals affect another lipid signaling component - lysophosphatidic acid receptor (LPA3), as well as HoxA10 and Wnt in the endometrium, to create the appropriate uterine environment for establishment of pregnancy and implantation.

Antiluteolytic mechanisms

In pigs, corpora lutea (CL) regression on days 15-16 of the estrous cycle results from an increase in pulsatile endometrial secretion of prostaglandin F2α (PGF2α) (for review, see Bazer *et al.* 1982). Basal PGF2α release by endometrium increases 3-fold between day 5 and 14 of the estrous cycle (Stepien *et al.* 1999). According to Krzymowski & Stefanczyk-Krzymowska (2008), the PGF2α pulsatile elevation in the blood outflowing from the uterus during luteolysis and shortly afterwards, results from decreased blood flow in the endometrium and excretion of PGF2α and its metabolites to lymph, blood and tissue fluids which accumulate PGs. However, oxytocin, luteinizing hormone (LH) and tumor necrosis factor α should be considered as potential modulators of endometrial PGF2α production during luteolysis (Uzumcu *et al.* 1998, Blitek & Ziecik 2006, Blitek *et al.* 2007).

The porcine CL is unusual among domestic animals because it does not display a luteolytic response to exogenous PGF2α until days 12-13 of the estrous cycle. The insensitivity of early CL to exogenous PGF2α is explained partially by low number of luteal PGF2α receptors (FPr) (Boonyaprakob *et al.* 2003) and a deficiency in post-FPr signaling (Zorrilla *et al.* 2009). Lower FPr concentration on day 14 of pregnancy/pseudopregnancy in comparison to the estrous cycle suggests that conceptus signals may inhibit FPr expression in the CL (Gadsby *et al.* 1993). The

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role of oxytocin in controlling PGF2α secretion is not as well defined as in ruminants (for review see, Ziecik *et al*. 2006). Systemic infusions of oxytocin-antagonist between days 12 and 20 of the estrous cycle reduce amplitude of PGFM (PGF2α metabolite) pulses, but does not prevent luteolysis (Kotwica *et al*. 1999). Surprisingly, oxytocin concentration in the uterine lumen significantly increases on days 12-14 of pregnancy when compared to corresponding days of the estrous cycle (Vallet *et al*. 1998). Moreover, oxytocin is not luteolytic when administered locally to the uterine lumen as it is when administered systemically (Sample *et al*. 2000) and intra-uterine infusion of oxytocin decreases plasma concentrations of PGFM on day 16 after estrus (Sample *et al*. 2004).

Maternal recognition of pregnancy (days 11-13) and implantation (days 14-19) are critical for CL maintenance and pregnancy establishment. Maternal recognition of pregnancy occurs simultaneously with rapid transformation of trophoblast from spherical to tubular then filamentous forms between days 10-12 just prior to implantation. During this period, conceptuses secrete elevated levels of estrogens, mainly estradiol-17β. A second, more sustained increase of estrogen secretion is observed between days 15 and 25-30 of pregnancy (Geisert *et al*. 1990). Inhibition of luteolysis and establishment of pregnancy in pigs require this biphasic pattern of estrogen secretion that results in prolonged luteal life span and progesterone secretion (for review, see Geisert *et al*. 1990). Systemic estrogen administration on days 11-15 of the estrous cycle prevents CL regression and extends CL life span (Frank *et al*. 1977). The luteoprotective action of estrogen is complex (Fig. 1). Estrogen stimulates luteal progesterone secretion directly (Conley & Ford 1989) as well as by increasing luteal LH receptor (LHR) concentration (Garverick *et al*. 1982) and by decreasing PGF2α release from uterus into peripherial circulation (Moeljono *et al*. 1977, Bazer & Thatcher 1977).

Estrogen receptor (ESR) expression in luminal (LE) and glandular epithelium (GE) of the endometrium (Geisert *et al*. 1993) and in the conceptus (Kowalski *et al*. 2002) coincides with estrogen secretion from the conceptus, which suggests both autocrine and paracrine responses. Both sufficient conceptus estrogen secretion (less than 2 conceptuses in each horn results in pregnancy failure) and timing of endometrial exposure to estrogen is critical for establishment of pregnancy. Early administration of estrogen on days 9-10 of pregnancy results in embryonic loss and altered endometrial expression of many genes, during later pregnancy, probably causing desynchronization of the uterine environment and conceptus implantation (Geisert *et al*. 2006). Included among the altered genes is an enzyme involved in PG synthesis (prostaglandin-endoperoxide synthase 2; PTGS2).

Endometrial function and conceptus development during the peri-implantation period of pregnancy are uniquely regulated through interacting effects of progesterone from the CL and estrogen from the conceptus. Estrogen is suggested to be involved in changes of uterine secretory activity (Geisert *et al*. 1982), increased blood flow (for review see, Bazer *et al*. 1982), endometrial edema (Laforest & King 1992), and regulation of endometrial expression of many factors (Fig. 1). However, other reports indicate that concentrations of many proteins within the uterine lumen during early pregnancy are independent of the presence of the conceptus (Vallet *et al*. 1998, Kayser *et al*. 2006).

Factors involved in PG synthesis and signaling during inhibition of luteolysis and pregnancy establishment

Concentrations and peak amplitude of PGF2α in utero-ovarian vein plasma are higher in cyclic than in pregnant gilts on days 12-17 (Bazer & Thatcher 1977, Moeljono *et al*. 1977). Moreover, uterine flushings of pregnant pigs contain higher amounts of PGF2α than those from

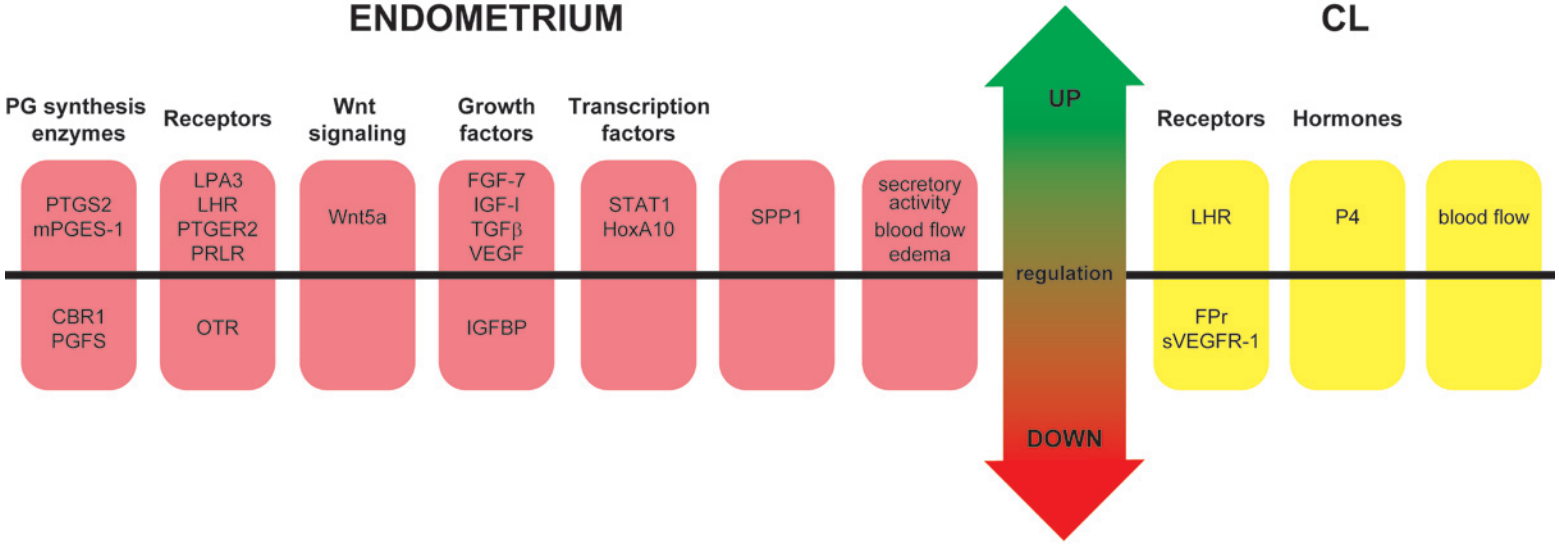


Fig. 1 Changes induced in the endometrium and the CL by conceptus estrogen and/or occurring simultaneously with increased conceptus estrogen secretion. PTGS2 - prostaglandin-endoperoxide synthase 2, mPGES-1 – PGE synthase, CBR1 - prostaglandin 9-ketoreductase/carbonyl reductase, PGFS - PGF synthase, LPA3 - lysophosphatidic acid receptor, LHR - luteinizing hormone receptor, PTGER2 – PGE2 receptor, PRLR – prolactin receptor (Young *et al*. 1990), OTR - oxytocin receptor, Wnt5a - wingless-type MMTV integration site family member, FGF-7 - fibroblast growth factor-7, IGF-I - insulin-like growth factor-I, TGFβ - transforming growth factor β, VEGF - vascular endothelial growth factor, IGFBP – IGF binding protein, STAT1 - signal transducer and activator of transcription 1 (Joyce *et al*. 2007), HoxA10 – homeobox A10, SPP1 – secreted phosphoprotein 1, FPr – PGF2α receptor, sVEGFR-1 – VEGF soluble receptor, P4 -progesterone.

cyclic animals (Zavy *et al*. 1980). This may be explained by the proposed theory that during maternal recognition of pregnancy, conceptus estrogen redirects PGF2α secretion from the uterine venous drainage (endocrine) to the uterine lumen (exocrine) (Bazer & Thatcher 1977). A part of the putative antiluteolytic mechanism could also be the retrograde transfer of PGF2α from venous blood and uterine lymph into the uterine lumen and ability of uterine veins and arterial walls to accumulate PGF2α (Krzymowski & Stefanczyk-Krzymowska 2004). However, Hunter & Poyser (1982) suggested that exocrine redirection of uterine PGF2α secretion may not provide a full explanation for maintenance of the CL in pregnancy in pigs, nor may this route of sequestering the luteolytic hormone always be effective.

Conceptus and/or endometrium PGE2 was suggested to have a role in CL protection against luteolytic PGF2α. In contrast to estrogen, intraluteal administration of PGE2 protects individual CL against luteolytic effect of simultaneously administered PGF2α (Ford & Christenson 1991). PGE2 is capable of extending CL life span (Akinlosotu *et al*. 1986). Interestingly, progesterone content is elevated in the blood plasma or luteal tissue after uterine infusions of PGE2 (Akinlosotu *et al*. 1986) or when increased secretion of PGE2 was observed in the gravid uterus (Christenson *et al*. 1994). The infusion of PGE2 into the ovarian artery increases the concentration of progesterone in ovarian venous blood on day 13 and 14 of pregnancy (Stefanczyk-Krzymowska *et al*. 2006). Moreover, higher PGE2/PGF2α ratio protects against the luteolytic effect of PGF2α on luteal cells collected on days 10-12 of the estrous cycle and stimulates progesterone and estradiol-17β secretion by these luteal cells (Gregoraszczuk & Michas 1999).

On days 11-13 of pregnancy, at the time of maternal recognition of pregnancy, the PGE2/PGF2α ratio in the uterus and uterine vein increases, suggesting that PGE2 helps to overcome the luteolytic effect of PGF2α, thus preventing CL regression (Davis & Blair 1993, Christenson *et al*. 1994). Therefore, another potential mechanism by which the conceptus helps to prevent luteolysis is by changing PG synthesis in favor of the luteoprotective PGE2 (Fig. 2), since the endometrium and trophoblast synthesize elevated amounts of PGE2 before implantation (Waclawik *et al*. 2006, Waclawik & Ziecik 2007).

PGs are synthesized by PTGS and specific terminal prostaglandin synthases: PGE synthase (mPGES-1) and PGF synthase (PGFS). Moreover, PGE2 can be converted into PGF2α by prostaglandin 9-ketoreductase/carbonyl reductase (CBR1). Our recent results *in vitro* indicate that estrogen stimulates PGE2 synthesis through increase of PTGS2 and mPGES-1 gene expression and decreases the content of enzymes involved in PGF2α production (PGFS and CBR1) in the endometrium on days 11-12 after estrus (Waclawik *et al*. 2009). It is consistent with findings that mPGES-1 expression is relatively high whereas PGFS and CBR1 expression is low in the endometrium between days 10-13 of pregnancy compared to the implantation period (Waclawik *et al*. 2006, Waclawik & Ziecik 2007). Similar, but more pronounced alterations of PG enzyme expression were found in conceptuses (Fig. 3, Waclawik & Ziecik 2007). Selective changes in PG synthesis enzymes during early pregnancy correspond with estrogen secretion by the conceptus. Endometrial mPGES-1 expression exhibits a biphasic profile, similar to conceptus estrogen. By contrast, rapid increase of PGFS and CBR1 after initiation of implantation corresponds with the decline in conceptus estrogen synthesis. Accordingly, estrogen elevates PGE2 content in the uterus (Geisert *et al*. 1982) and therefore may be an important factor regulating the PGE2/PGF2α ratio during early pregnancy.

Our recent studies indicate the existence of a putative PGE2 autoamplification loop in the endometrium that can additionally contribute to the increase of PGE2/PGF2α ratio during the peri-implantation window. PGE2 acting through endometrial PGE2 receptor (PTGER2) elevates secretion of this prostanoid and expression of the enzymes involved in PGE2 synthesis in the endometrium. Interestingly, endometrial PTGER2 is higher during implantation and can be regulated by estrogen (Waclawik *et al*. 2009).

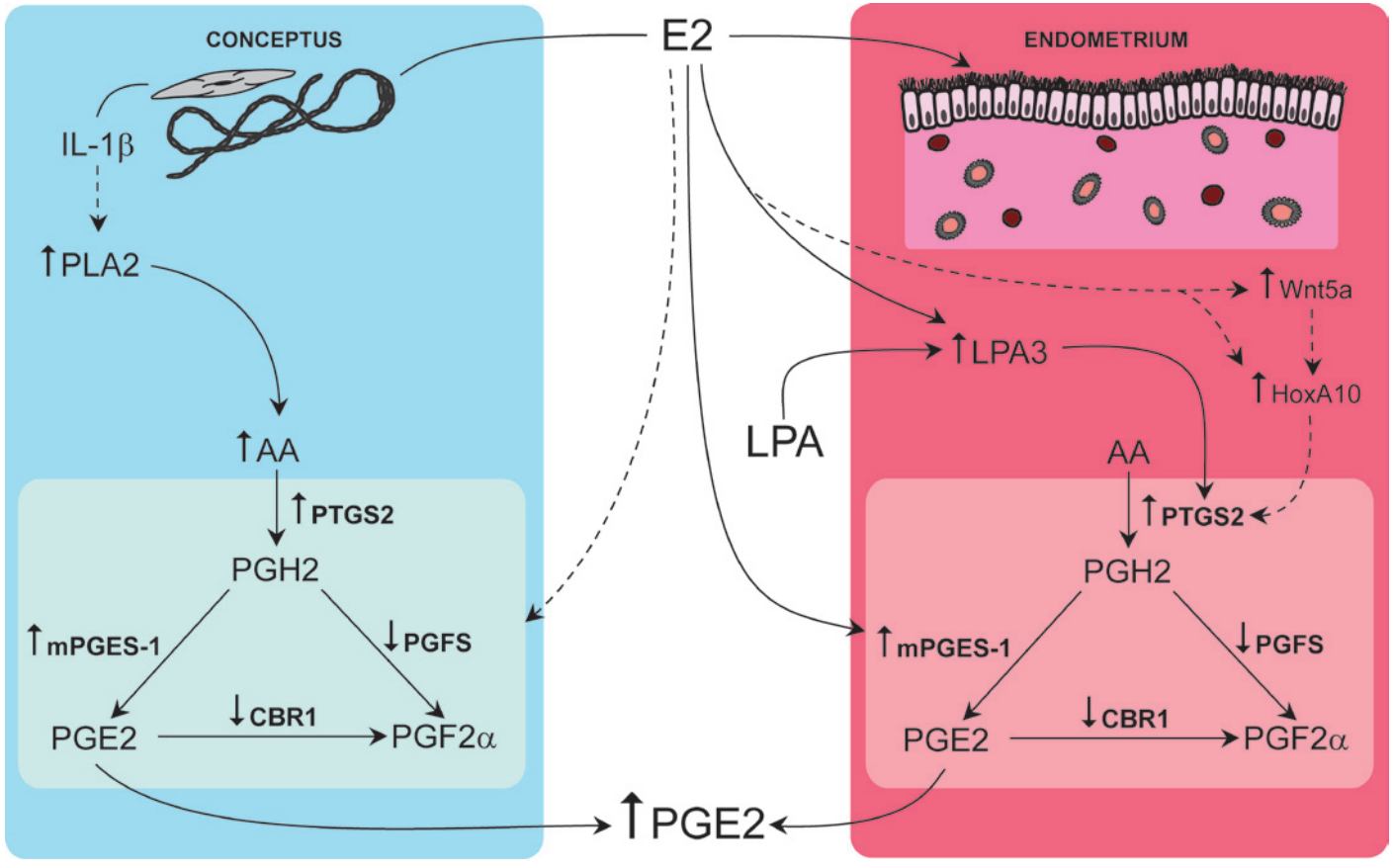


Fig. 2 Proposed concept of the role of the conceptus in the increase of PGE2 level and the PGE2/PGF2α ratio during the maternal recognition of pregnancy in the pig. Antagonistic changes in the expression of PGE synthase (mPGES-1) and the enzymes involved in PGF2α synthesis - PGFS and CBR1 in the endometrium or/and conceptus on days 10-13 of pregnancy may be a result of conceptus estrogen. LPA present in the uterus, activates PTGS2 expression via LPA3 receptors which are up-regulated by estrogen during maternal recognition of pregnancy. Abundant levels of IL-1β secreted by the conceptus induce phopholipase A2 (PLA2) and increase arachidonic acid (AA) release from cell membrane. Additionally, up-regulation of Wnt5a and HoxA10 in pregnancy may contribute to increase of PTGS2. PGH2 – prostaglandin endoperoxide H2.

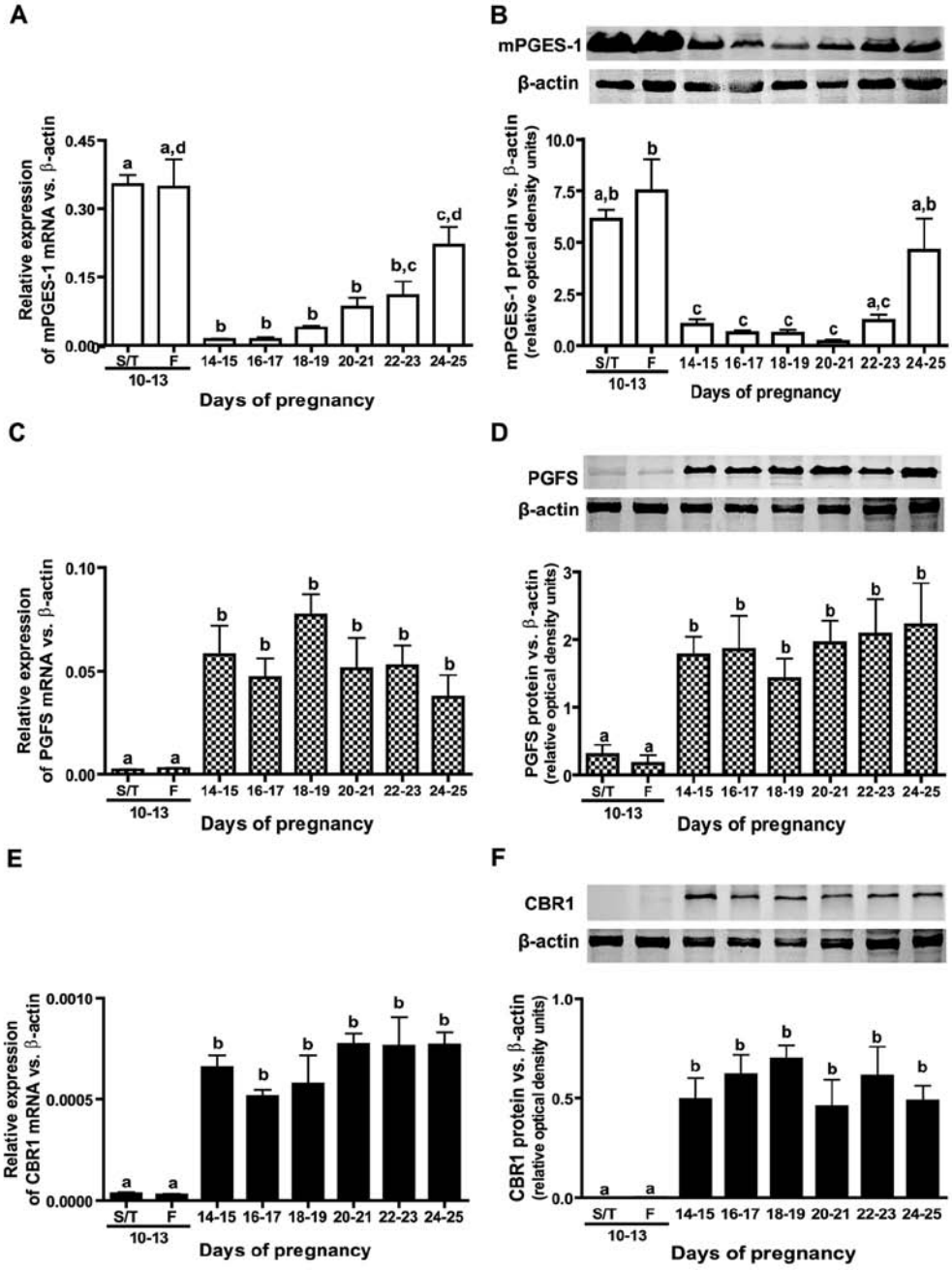


Fig. 3 Expression of PGE synthase (**A, B**), PGF synthase (**C, D**) and prostaglandin 9-ketoreductase/carbonyl reductase (**E, F**) in the porcine conceptus/trophoblast during early pregnancy (Waclawik & Ziecik 2007).

Although actions of luteal PGE2 in autoregulation of CL function cannot be excluded, synthesis of PGE2 in the conceptus and the endometrium likely contributes to the process of maternal recognition of pregnancy (Waclawik *et al.* 2008, Wasielak *et al.* 2008, Ziecik *et al.* 2008).

Lysophosphatidic acid (LPA)

LPA influences PG synthesis by increasing endometrial PTGS2 expression (Seo *et al.* 2008). Content and type of this phospholipid-derived mediator in uterine lumen differs between day 12 of the estrous cycle and pregnancy. LPA acts through specific G-protein coupled receptors LPA1-LPA4 and LPA3 is the dominant receptor in the porcine endometrium. Ye *et al.* (2005) indicated a critical role of LPA3 mediated signaling in mouse conceptus implantation. LPA3 is localized to LE and GE and its expression is elevated by estrogen in the pig (Seo *et al.* 2008). Endometrial LPA3 expression is increased during early pregnancy with the highest levels on days 11-12 and its mRNA content is higher in endometrium from the gravid compared to the non-gravid uterine horn (Kaminska *et al.* 2008).

HoxA10

Hox (for human HOX) genes are vertebrate homologs of *D. melanogaster* homeotic genes, that determine the identity of specific body segments. Recently, HoxA10 gene has attracted more attention, because it plays a role in uterine development. In mice, targeted disruption of expression of this transcription factor results in implantation failure (Benson *et al.* 1996). Moreover, this gene was implicated in the control of endometrial PTGS2 expression and PG synthesis in mice (Paria *et al.* 2002). In human, ovarian steroid-regulated HOXA10 expression in endometrium and peak expression of HOXA10 during the window of implantation suggests an important role for this gene in controlling uterine receptivity (Daftary & Taylor 2006). Our latest studies showed up-regulation of endometrial HoxA10 during implantation in the pig (Kaczmarek *et al.* 2009a) and the stimulatory effect of estradiol-17β on its expression in endometrium (A Blitek, unpublished).

Wnt

HoxA10 gene expression may be regulated by Wnt (Bartol *et al.* 2006). Wnt may mediate trophoblast-epithelial interactions critical for uterine receptivity to implantation (Hayashi *et al.* 2007). Our recent research indicates that Wnt4 mRNA expression does not change significantly in the endometrium during the estrous cycle and early gestation whereas level of Wnt5a is higher during early pregnancy (Kiewisz *et al.* 2009). Moreover, expression of Wnt4 is down-regulated and Wnt5a and E-cadherin up-regulated in the CL during early pregnancy (J Kiewisz, unpublished).

Luteinizing hormone receptors

LH is able to increase PGE2 secretion and PTGS2 expression in the endometrium (Blitek *et al.* 2007). Additionally, estrogen administration between days 11-15 after estrus results in an increase of LH-induced PGE2 release from endometrium *in vitro* (G Bodek, A Ziecik, unpublished). Thus, it is possible that conceptus estrogen maintains endometrial LHR expression ensuring a higher output of PGE2. However, only simultaneous action of estradiol-17β and

progesterone results in increased PGE2 secretion in response to LH in stromal cells (Blitek *et al*. 2007). Therefore, the endometrial expression of LHR during early pregnancy may also be involved in the luteoprotective action of LH.

Uterine receptivity and the establishment of pregnancy

Pigs have a true epitheliochorial placenta in which LE remains intact throughout pregnancy. Hormonally regulated signals from the ovary induce essential changes manifested by proliferation, differentiation, and spatiotemporal expression of specific biological molecules, leading to a temporary state of uterine receptivity for implantation. This state may be further enhanced by additional factors secreted by conceptus. However, some changes are independent of conceptus presence and controlled mainly by progesterone (for review, see Bowen & Burghardt 2000). Sustained stimulation of the endometrium by progesterone over 7-8 days causes a loss of progesterone receptors (PR) from LE and GE by day 10 of the estrous cycle and pregnancy, which is highly associated with the development of endometrial receptivity for conceptus implantation (for review, see Geisert *et al*. 2006). Since PR are maintained in stroma and myometrium, the effects of ovarian progesterone on expression of many factors in LE may be mediated indirectly by either progesterone-induced progestamedins produced by PR-positive stromal cells or by induction of molecules in LE that causes loss of PR to regulate expression of endometrial genes (Geisert *et al*. 2006, Ka *et al*. 2007).

Progesterone induces down-regulation of PR from LE and GE resulting in the decrease in mucin-1 from the apical surface of LE and exposure of integrins for trophoblast attachment (Bowen *et al*. 1996). Expression of α4, α5, β1 integrin subunits increases on days 11–15 in both cyclic and pregnant gilts and is regulated by progesterone alone or together with estrogen (Bowen *et al*. 1996). Secreted phosphoprotein 1 (SPP1), induced by estrogen in LE during apposition phase of implantation, is a potent adhesion protein mediating attachment between trophoblast and LE (White *et al*. 2005).

Cytokines

Among cytokines, interleukin-1β (IL-1β) seems to play a pivotal role during maternal recognition of pregnancy, and may be involved in cell adhesion and other cytokine stimulation. Abundant levels of IL-1β secreted by the conceptus are associated with rapid trophoblast elongation (Ross *et al*. 2003). Moreover, Geisert *et al*. (2006) suggested that IL-1β, an inducer of phospholipase A2, regulates arachidonic acid release from cell membrane, thus ensuring the membrane fluidity necessary for trophoblast remodeling during conceptus elongation and contributing to PG synthesis (Fig. 2). Interferon γ (INFγ) and interferon δ (INFδ) of conceptus origin, IL-6 of conceptus and endometrial origin and leukaemia inhibitory factor (LIF) produced by endometrium (Modric *et al*. 2000, Joyce *et al*. 2007) may also serve as conceptus-maternal signaling molecules involved in the proliferation, differentiation and cell survival that is associated with conceptus growth and implantation.

Growth factors

Growth factors are also implicated in conceptus development and successful establishment of pregnancy since endometrial expression of fibroblast growth factor-7 (FGF-7), transforming growth factor β (TGFβ), vascular endothelial growth factor (VEGF) and uterine lumen content of insulin-like growth factor-I (IGF-I) are elevated on days 12-13 of pregnancy and may be

regulated by estrogen and/or progesterone (for review, see Ziecik *et al*. 2006). TGFβ stimulates expression of extracellular matrix molecules and integrins as well as proteases and protease inhibitors to facilitate conceptus-uterus connections during implantation and to limit trophoblast invasiveness (Burghardt *et al*. 2002). Additionally, IGF-I increases steroidogenesis by stimulation of P450 aromatase expression in filamentous conceptuses (Green *et al*. 1995). Transition from spherical to filamentous trophoblast is associated with the decrease of IGF binding proteins (IGFBP) in the uterine lumen that regulates IGF bioavailability (Geisert *et al*. 2006). Both IGF-I and PGE2 stimulate VEGF expression in stromal cells during maternal recognition of pregnancy, which may result in growth and remodeling of endometrial vasculature (Kaczmarek *et al*. 2008). Additionally, conceptus VEGF164 expression increases gradually with conceptus development until day 16 of pregnancy (Kaczmarek *et al*. 2009b) suggesting that VEGF164 may induce endometrial vascular permeability and edema during implantation.

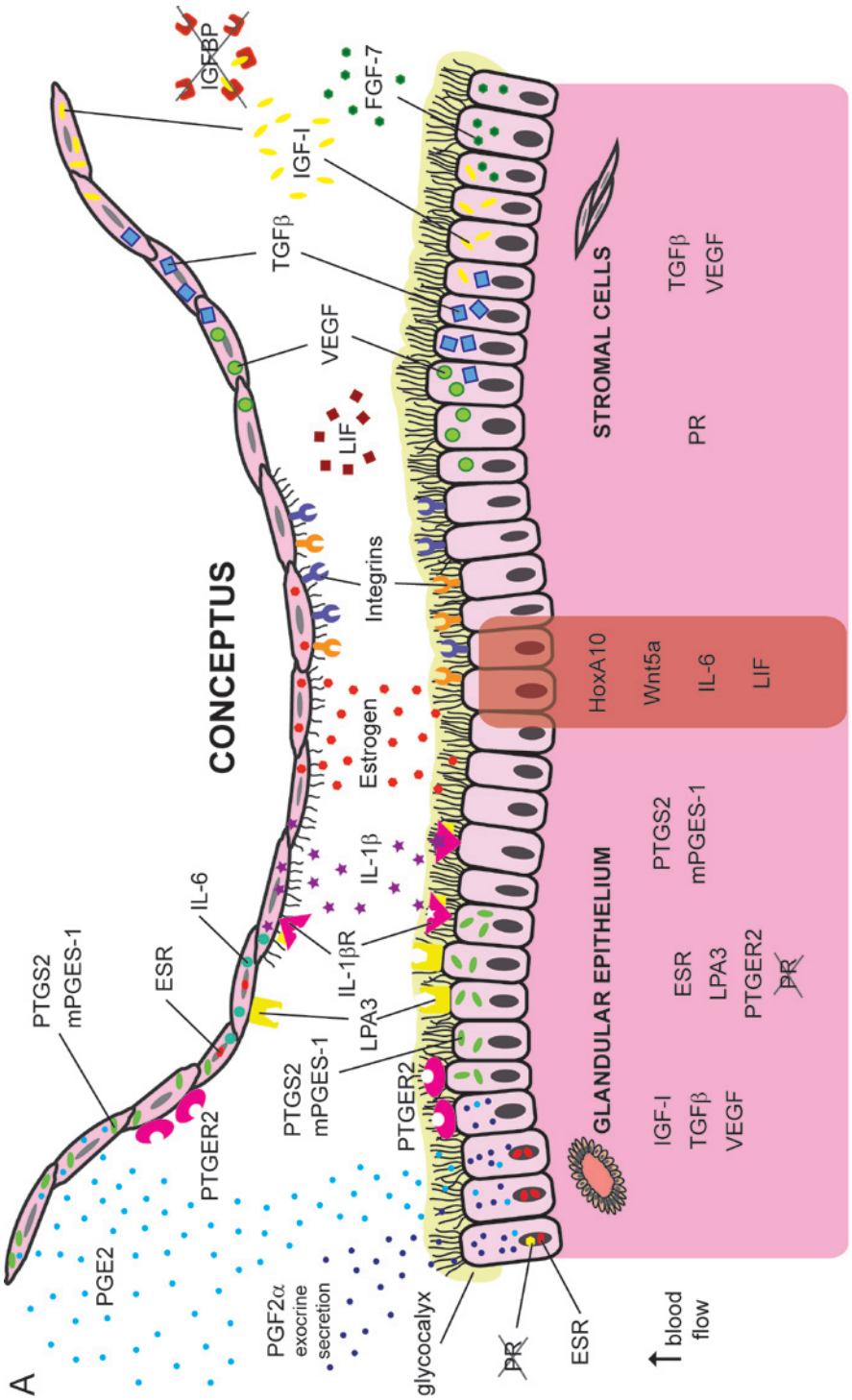
The VEGF ligand-receptor system is involved in maintenance and stabilization of the vascular bed in CL during pregnancy. PGE2-stimulated VEGF secretion by luteal cells on days 10-12 of pregnancy suggest that the luteoprotective actions of PGE2 may be partially mediated by luteal VEGF (Kowalczyk *et al*. 2008). Interestingly, VEGF soluble receptor (sVEGFR-1) mRNA levels are lower in the CL on day 12 of pregnancy compared to the estrous cycle (Kaczmarek *et al*. 2009b). Down-regulation of sVEGFR-1 (strong endogenous antagonist of VEGF) and the consequent elevation of bioavailable VEGF may sustain progesterone production by increasing luteal capillary permeability, which may aid transport of PGs from the circulation and delivery of cholesterol to the luteal cells.

Conclusions

The conceptus affects the lipid signaling system (prostaglandin and LPA) as well as HoxA10 and Wnt (factors which may be involved in PG synthesis) in the porcine endometrium in order to inhibit luteolysis and/or to create the appropriate uterine environment for conceptus development and implantation (Fig. 2). Conceptus estrogen may prevent luteolysis through modification of expression of the enzymes involved in PG synthesis, reducing release of PGF2α and favoring PGE2 release on days 10-13 of pregnancy. Down-regulation of luteal sVEGFR-1 may be supportive of CL function during maternal recognition of pregnancy. Although it is evident that estrogen and progesterone induce essential proliferative and differentiative changes in the endometrium leading to a temporary state of uterine receptivity, a number of growth factors, cytokines and extracellular matrix and adhesion molecules are required to establish the necessary dialog between the conceptus and endometrium (Fig. 4).

Acknowledgements

We thank Dr Henry N. Jabbour for reviewing the manuscript. Studies described in this manuscript are supported partially by funds from the State Committee for Scientific Research in Poland (grants NN311319135 and N31104732/2777). A. Waclawik is the recipient of Domestic Grant for Young Scientist from the Foundation for Polish Science. J. Kiewisz is funded by the President of Polish Academy of Sciences and the European Social Fund.


A
PTGS2
mPGES-1
PGE2
PTGER2
ESR
IL-6
PGF2α
exocrine
secretion
PTGS2
mPGES-1
IL-1βR
LPA3
PTGER2
IL-1β
Estrogen
Integrins
LIF
VEGF
TGFβ
IGF-I
IGFBP
FGF-7
CONCEPTUS
glycocalyx
PR
ESR
GLANDULAR EPITHELIUM
IGF-I
TGFβ
VEGF
PTGS2
mPGES-1
ESR
LPA3
PTGER2
PR
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IL-6
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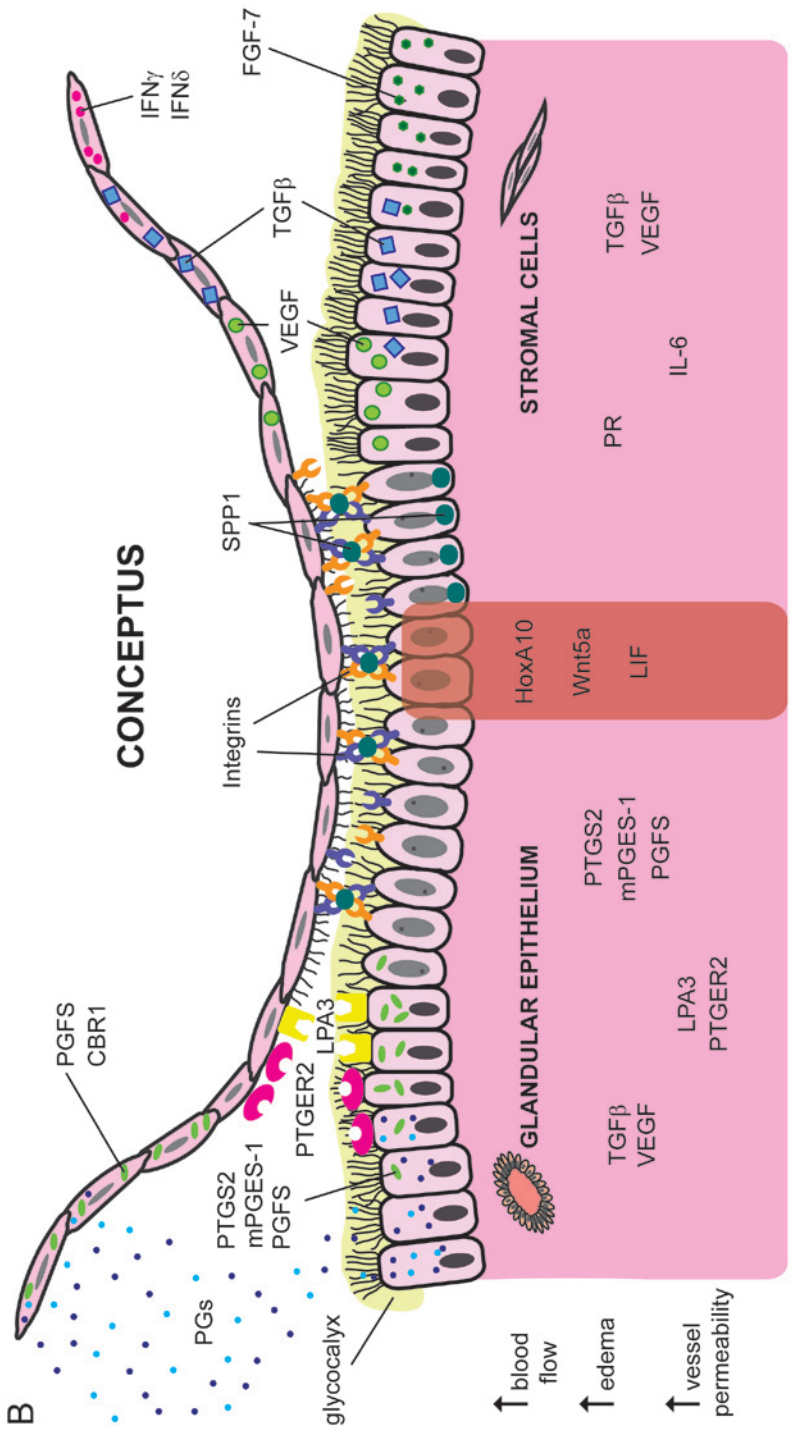


Fig. 4 Maternal recognition of pregnancy on days 11-12 (**A**) and the initial attachment of conceptus to the endometrium on days 14-16 (**B**) is dependent on various biological molecules produced by the endometrium and conceptus. Factors for which localization of expression in specific endometrial cells remains undetermined, are indicated in a red box.

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Conceptus-uterus interactions in pigs: endometrial gene expression in response to estrogens and interferons from conceptuses

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This review highlights information on conceptus-uterus interactions in the pig with respect to uterine gene expression in response to estrogens and interferons (IFNs) secreted from elongating conceptuses. Pig conceptuses release estrogens for pregnancy recognition, but also secrete IFNs that do not appear to be antiluteolytic. Estrogens and IFNs induce expression of largely non-overlapping sets of genes, and evidence suggests that pig conceptuses orchestrate essential events of early pregnancy including pregnancy recognition signaling, implantation and secretion of histotroph by precisely controlling temporal and spatial (cell-specific) changes in uterine gene expression through initial secretion of estrogens, followed by cytokines including IFNG and IFND. By Day 12 of pregnancy, estrogens increase the expression of multiple genes in the uterine luminal epithelium including SPP1, STC1, IRF2 and STAT1 that likely have roles for implantation. By Day 15 of pregnancy, IFNs upregulate a large array of IFN responsive genes in the underlying stroma and glandular epithelium including ISG15, IRF1, STAT1, SLAs and B2M that likely have roles in uterine remodeling to support placentation.

Introduction

This review assembles information on the regulation of endometrial gene expression by conceptus estrogens (Geisert *et al*. 1982a), and interferons (IFNs) delta (IFND) and gamma (IFNG) (La Bonnardière *et al*. 1991, Lefèvre *et al*. 1998) during the peri-implantation period of pregnancy. The estrogens and IFNs regulate cell-type specific expression of endometrial genes responsible for the complex interplay between endometrium and conceptus required for pregnancy recognition signaling and implantation.

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Pig conceptus estrogens and interferons

Early pregnancy in pigs is complex and is influenced by the overlapping events of conceptus elongation (Fig. 1A), endometrial remodeling for implantation (Fig. 1B) and pregnancy recognition signaling. In pigs, oxytocin released in a pulsatile manner by the uterus binds oxytocin receptors to stimulate pulsatile release of prostaglandin $F_{2\alpha}$ (PGF) from endometrial luminal epithelium (LE) (Mirando *et al*. 1995). PGF is the uterine luteolysin in pigs as corpus luteum (CL) regression correlates with pulsatile release of PGF into the uterine venous drainage beginning on Day 15 or 16 of the estrous cycle and hysterectomy extends CL lifespan to about 120 days (Mirando *et al*. 1995). However, roles of PGs in the pig uterus remain to be clarified. Inhibitors of PG synthesis fail to protect the CL from luteolysis (Kraeling *et al*. 1985), amounts of PGF and PGE2 in the uterine lumen are greater in pregnant than cyclic pigs (Bazer and Thatcher 1977), uterine PGF is processed into an inactive metabolite through a utero-ovarian countercurrent vascular pathway within the broad ligament (Krzymowski *et al*. 1990), and PGE2 synthase:PGF synthase ratios are higher in CL from pregnant than cyclic pigs, but not between CL ipsilateral or contralateral to the pregnant uterine horn. Therefore, it has been suggested that compounds from the conceptus are transported within the mesometrium to the ovaries to enhance CL maintenance (Wasielak *et al*. 2008).

Pregnancy recognition is the result of conceptus secretion of estrogens on Days 11 and 12 of pregnancy to redirect PGF secretion from the uterine vasculature to the uterine lumen (Fig. 2A & 2B). The theory of estrogen-induced maternal recognition of pregnancy in pigs is based on the following evidence: (i) the uterine endometrium secretes luteolytic PGF; (ii) pig conceptuses secrete estrogens which are antiluteolytic; (iii) PGF is secreted toward the uterine vasculature (endocrine) in cyclic gilts to induce luteolysis; and (iv) secretion of PGF in pregnant gilts is into the uterine lumen (exocrine) where it is sequestered from the corpora lutea and/or metabolized to prevent luteolysis (Bazer and Thatcher 1977). In addition to pregnancy recognition, conceptus estrogens modulate uterine gene expression thought to be required for implantation (Geisert *et al*. 1982b). The importance of estrogen to implantation of pig conceptuses is underscored by the fact that premature exposure of the pregnant uterus to estrogen on Days 9 and 10 results in degeneration of all pig conceptuses by Day 15 (Ross *et al*. 2007). It should be noted that PGE2, as well as lysophosphatic acid (LPA) have proposed roles in pregnancy signaling. Expression of PGE2 synthase by trophoblast and endometrium decreases production of PGF in favor of PGE2 to support CL maintenance (Ziecik *et al*. 2008). In addition LPA: (i) increases in uterine luminal fluids of pigs; (ii) its receptor, EDG7, is expressed by pig conceptuses; and (iii) its expression is increased by estrogen in endometrial epithelia during early pregnancy (So *et al*, 2008). Indeed, LPA3 is critical for embryo migration and spacing in mice (Ye *et al*. 2005), events that are critical to implantation and placentation in pigs.

Pig trophectoderm is unique in secreting both Type I and Type II IFNs during the peri-implantation period (Figs. 2A & 2B). Cultured conceptuses from Day 11 of pregnancy secrete proteins that cross react with antiserum against INF alpha (IFNA) (Cross & Roberts 1989), but peak antiviral activity is not measured in uterine flushings or conceptus culture media until Day 14 of pregnancy (Mirando *et al*. 1990). The major IFN species, constituting 75% of antiviral activity in pig conceptus secretory proteins (CSP), is type II IFNG. The other (25%) is the novel type I IFND (La Bonnardière *et al*. 1991, Lefèvre *et al*. 1998). Abundant IFNG mRNA is detectable in porcine trophectoderm between Days 13 and 20 of pregnancy, whereas IFND mRNA is detectable in Day 14 conceptuses only by RT-PCR analysis (Joyce *et al*. 2007a). On Day 15 of pregnancy, immunoreactive IFNG and IFND proteins are co-localized to peri-nuclear membranes typically occupied by endoplasmic reticulum and Golgi apparatus, as well as cytoplasmic vesicles within clusters of trophectoderm cells along the endometrial LE (Lefèvre

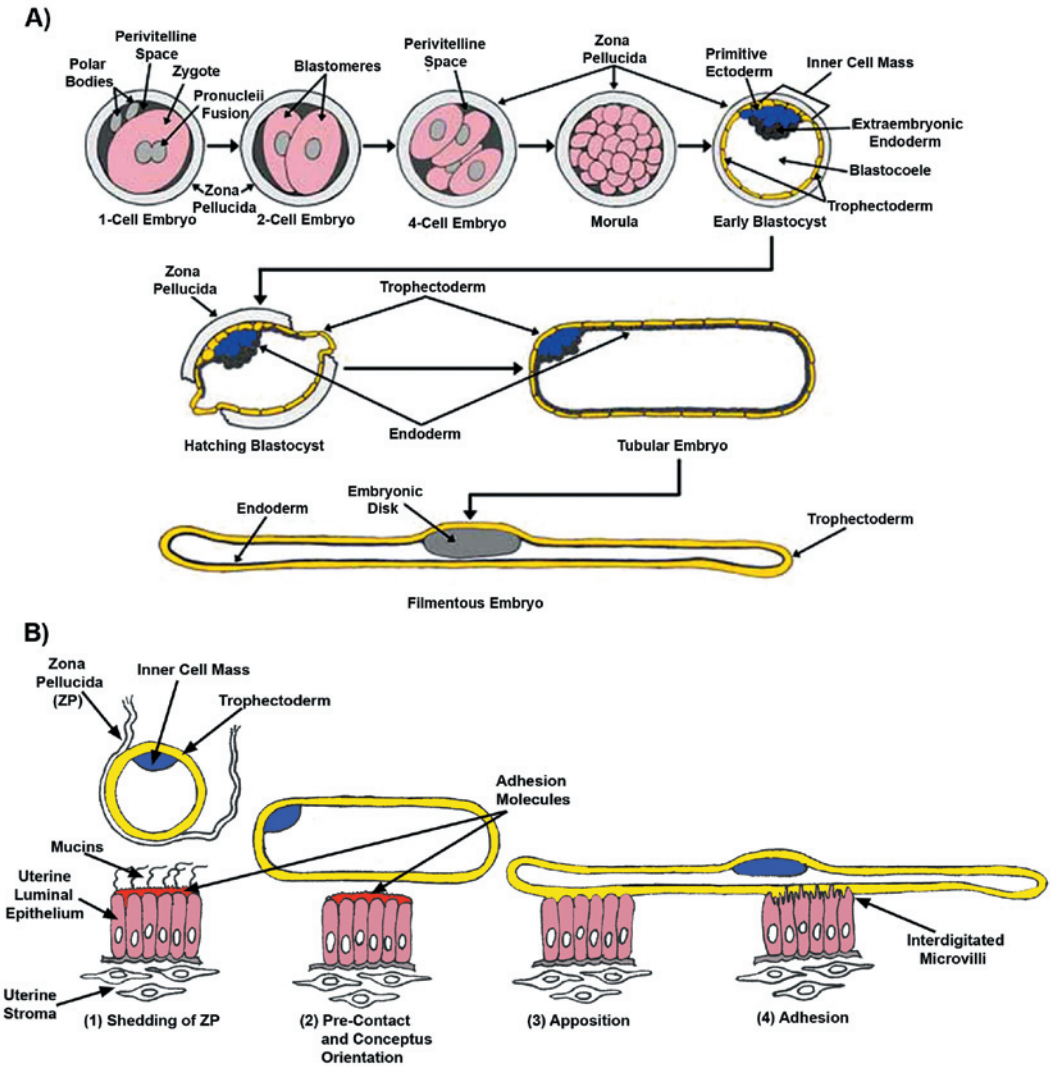


Fig. 1 Panel A. Pre-implantation development of the pig conceptus within the oviducts and uterus. The origin and organization of tissue layers within the conceptus are depicted. In pigs, the 1-cell fertilized ovum or zygote undergoes cleavage to form a 2-cell embryo by 26 h after fertilization, enters the uterus at the 4- to 8-cell stage between 48 to 56 h, differentiates into the blastocyst, "hatches" from the zona pellucida as a 0.5 to 1 mm diameter sphere, increases in size to Day 10 of pregnancy (2-6 mm), then undergoes a morphological transition to a large sphere of 10 to 15 mm diameter and then a tubular (15 mm by 50mm) and filamentous (l mm by 100-200 mm) form on Day 11. Panel B. Implantation Cascade in Pigs. Implantation in pigs extends from Days 13-25 and includes four phases that overlap and involve increasingly complex interactions between trophectoderm and uterine luminal epithelium to achieve true epitheliochorial placentation. Conceptus attachment first requires loss of anti-adhesive molecules in the glycocalyx of LE, comprised largely of mucins that sterically inhibit attachment. This results in "unmasking" of molecules, including selectins and galectins, that contribute to initial attachment of conceptus to uterine LE. These low affinity contacts are then replaced by a more stable and extensive repertoire of adhesive interactions between integrins and maternal ECM, for example secreted phophoprotein 1 (SPP1 or Osteopontin), which appear to be the dominant contributors to stable adhesion at implantation.

et al. 1998). In contrast to sheep conceptuses, in which a Type I IFN (interferon tau) is the signal for maternal recognition of pregnancy (Spencer *et al*. 2007), the IFNs produced by pig conceptuses do not appear to be antiluteolytic. Intrauterine infusion of CSP on Days 12 and 15 of the estrous cycle have no effect on interestrus interval or temporal changes in concentrations of progesterone in plasma (Harney & Bazer 1989). Although pig conceptus IFNs have yet to be shown to influence pregnancy recognition, paracrine effects for IFNs are suggested by localization of IFN receptors on endometrial epithelial cells (Lefèvre *et al*. 1998), increased secretion of prostaglandin E_2 (Harney & Bazer 1989), expression of several known IFN-responsive genes in the endometrium (Hicks *et al*. 2003, Joyce *et al* 2007a, Joyce *et al*. 2007b, Joyce *et al*. 2008), and modulation of uterine stromal and glandular epithelial (GE) gene expression by the IFNs in CSP preparations (Joyce *et al* 2007a, Joyce *et al*. 2007b, Joyce *et al*. 2008).

Estrogen- and interferon-stimulated genes in the endometrium

Estrogens and IFNs regulate endometrial genes that interact to effect communication between endometrium and conceptus during pregnancy recognition and implantation in pigs. Fig. 2 illustrates the fact that timing of estrogen secretion by the conceptus correlates with the induction of *SPP1* expression in the LE, whereas stromal induction of *STAT1* correlates with IFNG and IFND secretion by the conceptus. Indeed administration of exogenous estradiol to ovariectomized pigs induces *SPP1* mRNA in endometrial LE (White *et al*. 2005), while intrauterine infusion of CSP, which contain IFND and IFNG, into cyclic pigs treated with exogenous estrogen increases STAT1 as compared to intrauterine infusion of control proteins (Joyce *et al*. 2007a), similar to expression patterns for these genes during the peri-implantation period of pigs (Fig. 2C). Upregulation of *SPP1* within uterine LE and *STAT1* within stroma and GE in close proximity to the implanting conceptus implies paracrine regulation of genes by conceptus estrogens and IFNs. It is likely that effects of estrogen on the endometrium are restricted to regions near the conceptus due to metabolic activity of trophectoderm. During pregnancy, pig endometrium rapidly converts estradiol to estrone and then converts it to the biologically inactive estrone sulfate which is present in high concentrations within the uterine lumen of pregnant pigs (Flood 1974). The trophectoderm has sulfatase enzyme activity that restores the biological activity of estrogen, allowing for a localized effect of estrogen to up-regulate genes such as *SPP1* in LE. In contrast, it is somewhat surprising that initial increases in expression of *STAT1* in stroma are restricted to sites of contact between the conceptus and uterus, given that *IFNG* synthesis and secretion by pig conceptuses appears to be similar in magnitude to IFNT production by sheep conceptuses (Joyce *et al*. 2007a). Indeed, *STAT1* expression increases universally in the stroma and GE of pregnant sheep independently of conceptus location within the lumen, presumably due to the high levels of secretion of IFNT by conceptuses (Spencer *et al* 2007). One explanation for the spatial pattern of *STAT1* expression in the pig uterus is that IFND and IFNG act synergistically to upregulate expression of ISGs. Interactions between Type I and Type II IFNs have been demonstrated previously (Decker *et al*. 1989). It is plausible that high levels of IFNG act on uterine stroma and GE to increase intracellular stores of interferon-stimulated gene factor 3 (ISGF3) so that the much lower levels of IFND can maximally upregulate STAT1 expression in close proximity to the implanting pig conceptus. To date, only a limited number of estrogen- and IFN-stimulated genes have been localized in the pig endometrium. Table 1 summarizes gene expression in pig uteri during normal pregnancy and in response to i.m. injections of estrogen and/or intra-uterine injections of pig CSP containing IFNG and IFND (Ka *et al*. 2000, Hicks *et al*. 2003, White *et al*. 2005, Joyce *et al* 2007a, Joyce *et al*. 2007b, Ka *et al*. 2007, Ross *et al*. 2007, Joyce *et al*. 2008, So *et al*. 2008, Song *et al*. 2009). Several of these genes are discussed further in the remainder of this review.

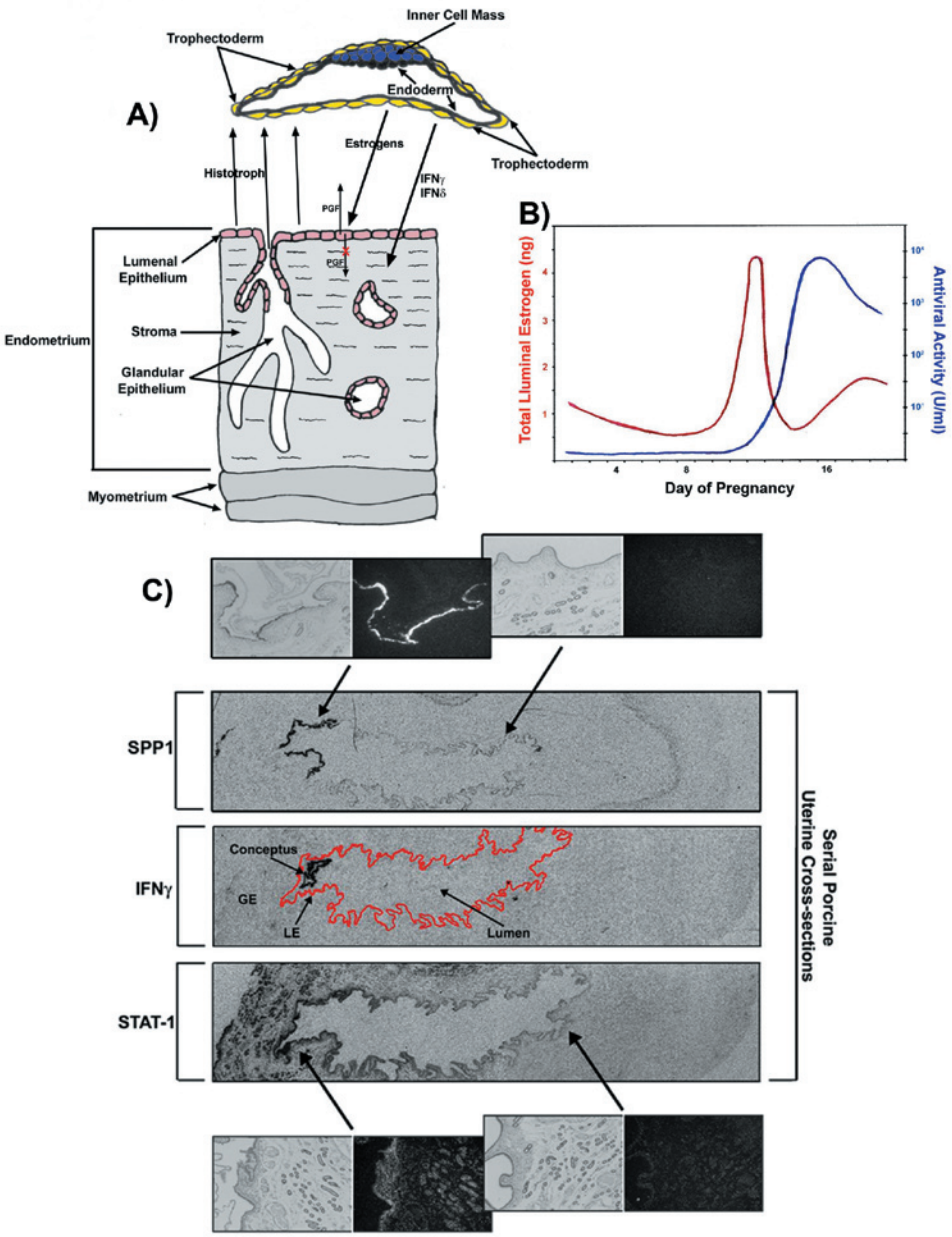


Fig. 2 Panel A. Peri-implantation signaling between the conceptus and uterus in the pig. Secretion of conceptus estrogens and IFNs elicit and support uterine responses including maintenance of the CL, production of histotroph and induction of multiple estrogen- and IFN-stimulated genes. Panel B. Graph depicting temporal changes in uterine luminal levels of conceptus estrogen and IFN antiviral activity in pigs during early pregnancy. Panel C. Conceptus estrogens (E2) induce *SPP1* in endometrial luminal epithelium (LE), and conceptus IFNs induce *STAT1* in endometrial stroma and glandular epithelium (GE) during the periimplantation period of pig pregnancy. In situ hybridization analysis of *SPP1*, *IFNG* and *STAT1* mRNAs in autoradiographic images (Biomax-MR; Kodak) showing entire serial cross-sections of the uterine wall from Day 15 of pregnancy. The luminal epithelium of tissue probed for *IFNG* mRNA has been outlined in red for histological reference. Corresponding brightfield and darkfield images from the same uterus probed with *SPP1* and *STAT1* cRNAs are also shown. Endometrial *SPP1* and *STAT1* increase in close proximity to paracrine release of E2 and IFNG from implanting pig conceptuses. Width of each field of autoradiographic images is 20 mm. Width of each field of brightfield and darkfield images is 940 μm.

Table 1. Temporal and Cell-Type Specific expression of conceptus estrogen- and interferon-regulated genes during the establishment of pregnancy in pigs. Only genes that have been spatially localized in pig endometrium and trophectoderm are listed.

Location	Regulation		Initial Day of Expression	
	By Estrogen	By IFNs	11-13	13-15
Conceptus			Estrogen	*IFNG*
			EDG7	*IFND*
Uterine lumen	FGF7		Estrogen	IFNG
	SPP1		FGF7	SPP1
	STC1		STC1	
LE	*AKR1B1*		*AKR1B1*	
	B2M		*B2M*	
	CD24		*CD24*	
	FGF7		*FGF7*	
	IRF2		*IRF2*	
	MX1		*NMB*	
	NMB		*SLA1,2,3*	
	SLA1,2,3		*SLA6,7,8*	
	SLA6,7,8		*SPP1*	
	SPP1		*STC1*	
	STC1		*EDG7*	
	EDG7			
GE		*B2M*		*B2M*
		IRF1		*IRF1*
		ISG15		*ISG15*
		SLA1,2,3		*MX1*
		SLA6,7,8		*SLA1,2,3*
		STAT1		*SLA6,7,8*
		STAT2		*STAT1*
				STAT2
ST		*B2M*		*B2M*
		IRF1		*IRF1*
		ISG15		*ISG15*
		SLA1,2,3		*MX1*
		SLA6,7,8		*SLA1,2,3*
		STAT1		*SLA6,7,8*
		STAT2		*STAT1*
				STAT2

LE, Luminal epithelium; GE, Glandular epithelium; ST, Stroma

Secreted Phosphoprotein 1 (SPP1)

SPP1, also called osteopontin (OPN), is a secreted ECM protein for which expression is upregulated during the initial stages of pregnancy in uteri of pigs (White *et al*. 2005) and other mammalian species, including humans (Johnson *et al*. 2003). SPP1 contains an Arg-Gly-Asp (RGD) sequence that mediates binding to cell surface integrin receptors, including αvβ3, α5β1, αvβ1, αvβ5 , αvβ6 and α8β1 (Johnson *et al*. 2003). Alternative binding-sequence interactions between *SPP1* and integrins such as α4β1, α9β1 and α4β7 can also occur (Johnson *et al*. 2003). As noted previously, estrogens secreted by the elongating Day 12 conceptus induce synthesis and secretion of *SPP1* specifically in endometrial LE cells in direct apposition to the

implanting conceptus (White *et al*. 2005). SPP1 protein is abundant on both the apical surface of uterine LE as well as conceptus trophectoderm (Tr) coinciding with the attachment phase of implantation in pigs (White *et al*. 2005). Recently, direct binding of αvβ6 trophectoderm and αvβ3 uterine LE integrins to *SPP1* was demonstrated. This binding stimulated trophectoderm cell adhesion and migration, but not proliferation, suggesting that *SPP1* is an excellent candidate to promote trophectoderm migration for conceptus elongation and attachment to endometrial LE for implantation in pigs (G. Johnson and D. Erikson, unpublished observations).

Stanniocalcin 1 (STC1)

STC1 is a homodimeric phosphoglycoprotein that regulates calcium and phosphate homeostasis (Wagner *et al*. 1986, Madsen *et al*. 1998). STC1 is also expressed in uteri of pregnant pigs, sheep and mice (Stasko *et al*. 2001, Song *et al*. 2006, Song *et al*. 2009). Recently, *STC1* mRNA was localized to uterine LE during the period of conceptus attachment to uterine LE for implantation in pigs i.e., Days 12-25 of pregnancy (Song *et al*. 2008). Further, *STC1* expression by uterine LE was induced by progesterone from the CL and further stimulated by estrogen from elongating pig conceptuses (Song *et al*. 2009). The presence of a 25 kDa form of STC1 in uterine luminal fluids from Days 12 through 15 suggests a role(s) in ion transport within trophectoderm and LE cells (Song *et al*. 2008). Indeed, total recoverable calcium in uterine flushings from pigs increases abruptly on Days 11 to 12, coincident with production of estrogen by elongating blastocysts (Geisert *et al*. 1982a). Therefore, estrogen may induce STC1 secretion from LE that then enhances transport of intracellular calcium to the lumen, resulting in increased levels of free calcium that mediate uterine secretion of multiple proteins that compose histotroph (Geisert *et al*. 1982b).

Interferon-Stimulated Gene 15 (ISG15)

In has been known for some time that IFNT increases expression of several IFN-stimulated genes (ISGs) in the stroma of the ruminant uterus. Over the last decade the list of ISGs known to be upregulated in endometrial stroma and GE has grown from one (*ISG15* in both cows and sheep) (Johnson *et al*.1999a, Johnson *et al*. 1999b), to over 20 proteins (see review for complete listing, Spencer *et al*. 2007). The first (Naivar *et al*. 1995) and most thoroughly studied ISG (Rempel *et al*. 2007) is *ISG15*, a functional ubiquitin homologue that has the C-terminus Leu-Arg-Gly-Gly amino acid sequence common to ubiquitin, allowing conjugation to intracellular proteins (Hass *et al*. 1987). Conjugation of proteins either targets proteins for rapid degradation in the proteasome, or stabilizes proteins for long-term modification (Wilkinson 2000). *ISG15* does indeed form stable conjugates with endometrial proteins, indicating a biologically active molecule that is responsive to conceptus IFNs and can target proteins for pregnancy-associated regulation and/or modification (Johnson *et al*. 1998, Joyce *et al*. 2005). Shown in Fig. 3 is the first evidence that *ISG15* is expressed in the stromal stratum compactum of pregnant pigs in response to conceptus IFNs. *ISG15* increases in the stroma between Days 12 and 14, then decreases gradually between Days 15 and 20 to undetectable levels by Day 35 (Data not shown).

Interferon Regulatory Factors (IRFs) 1 and 2

IRF1 is a key intermediate in the induction cascade of many classical ISGs through binding and transactivating IFN-stimulated response elements (ISREs) in their promoters (Floyd-Smith

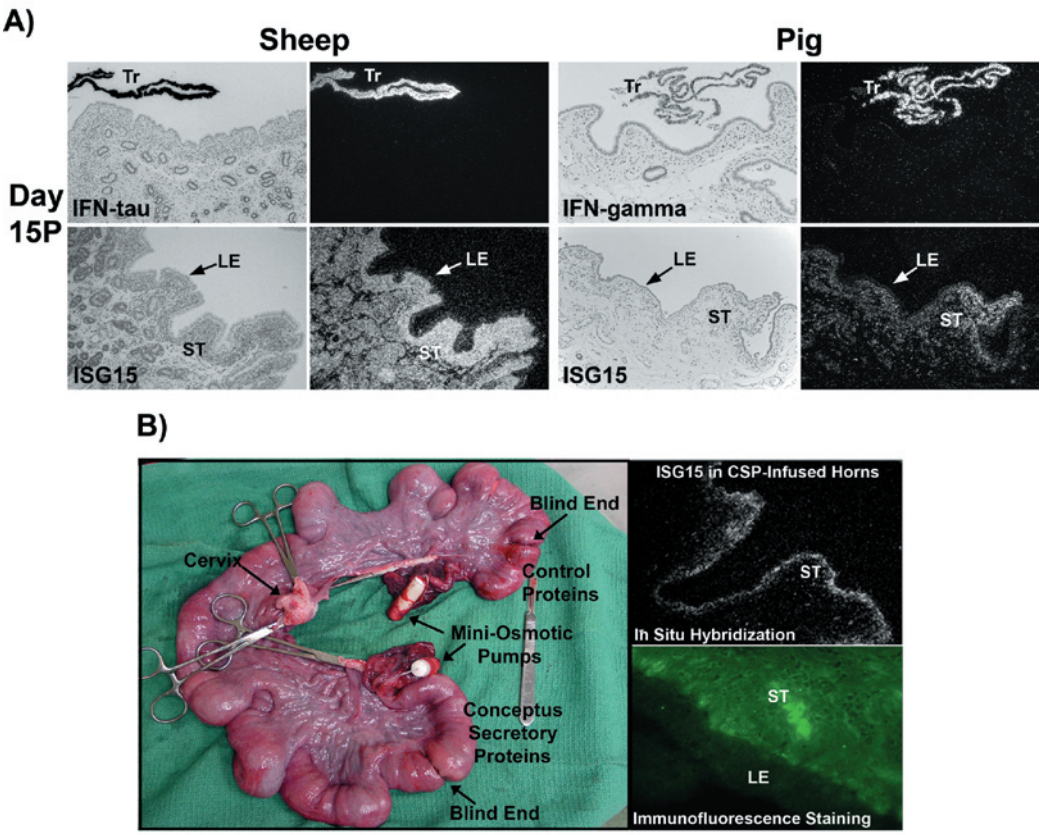


Fig. 3 Trophoblast IFNs increase expression of ISG15 within the endometrial stroma of domestic animals. A) *In situ* hybridization analyses for IFN-tau, IFN-gamma, and *ISG15* mRNA in cross-sections of sheep and pig uterui and placentae. Corresponding brightfield and darkfield images from Day 15 pregnant (P) sheep and pigs are shown. LE, luminal epithelium; ST, stroma; Tr, trophectoderm. Width of each field is 940 μm. B) Induction of *ISG15* in pig endometrium by conceptus secretory proteins containing IFNG and IFND. Left panel: On Day 12 post-estrus pigs were surgically implanted with two indwelling ALZET® osmotic pumps. For each pig, one uterine horn was infused by a pump filled with porcine serum albumin whereas the other uterine horn was infused by a pump filled with porcine conceptus secretory proteins (CSP) containing IFNs. Right panels: In situ hybridization and immunofluorescence detection of *ISG15* within the stratum compactum stroma of horns infused with CSP. Horns infused with control serum albumin did not express *ISG15* (data not shown). The rabbit polyclonal antibody against recombinant bovine ISG15 was kindly provided by Dr Thomas R. Hansen, Colorado State University.

et al. 1999, Chatterjee-Kishore *et al*. 2000, Stewart *et al*. 2002). Both Type I and Type II IFNs induce IRF1 expression (Floyd-Smith *et al*. 1999) which, in the reproductive tract of mice, has a role in placental development (Chatterjee-Kishore *et al*. 2000). In sheep, *IRF1* expression increases in stroma and GE, but not in the LE during early pregnancy. Presumably, this is due to expression of IRF2, a potent transcriptional repressor of ISGs, that is constitutively expressed in the LE and increases during early pregnancy (Stewart *et al*. 2002). Pig and sheep endometria have similar patterns of expression for *IRF1* and *IRF2*. IRF1 expression is upregulated in the stromal stratum compactum between Days 12 and 15 and remains through Day 25 of pregnancy (Joyce *et al*. 2007b). When pigs were implanted with mini-osmotic pumps that delivered conceptus secretory proteins (CSP) containing IFNs to the uterine horn (see Fig. 3)

CSP increased *IRF1* in stroma, indicating upregulation of *IRF1* by conceptus IFNs (Joyce *et al*. 2007b). In contrast, *IRF2* mRNA increased in LE after Day 12 in response to conceptus estrogen (Joyce *et al*. 2007b). The similar temporal and spatial patterns of expression for *IRF1* and *IRF2* in pigs and sheep support the idea that *IRF2* represses expression of ISGs in the LE of pigs and perhaps mammals in general.

Signal Transducer and Activator of Transcription (STAT) 1

Cell-type specific induction of *STAT1* expression in pig endometrium is differentially regulated by conceptus signals. Estrogen secretion by the conceptus on Day 12 increases *STAT1* in the LE (Joyce *et al*. 2007a). Stromal induction of *STAT1* correlates with secretion of IFND and IFNG by the conceptus, and intrauterine infusion of conceptus secretory proteins (see Fig. 3), which contain IFND and IFNG, increases *STAT1* in a manner similar to that observed on Days 15-20 of pregnancy (Joyce *et al*. 2007a). STAT1 activation generally results in transcription of genes that are antiproliferative, proapoptotic and proinflammatory that could profoundly influence endometrial remodeling for implantation and placentation (van Boxel-Dezaire *et al*. 2006). Interestingly, although Type I IFNA and Type II IFNG each induce expression of largely non-overlapping sets of genes, they can also act in concert to reinforce physiological responses (Levy *et al*. 1990). This synergy has been demonstrated for induction of STAT1. Normally relatively non-responsive to IFNG, sequential treatment of cells with IFNG followed by IFNA results in higher magnitude ISG induction (Levy *et al*. 1990). In addition, co-treatment with IFNG and IFNA increases the magnitude and extends the period of ISG expression over IFNA alone (Decker *et al*. 1989). Clearly the pig may be unique among mammalian species studied. In pigs, combined conceptus IFND and IFNG may influence uterine physiology through cooperative induction of cytokine-specific transcription factors, such as *STAT1*, that allow reinforcement of effects of distinct cell-surface ligands while maintaining the specificities of the individual inducing IFNs in their ability to induce effects such as endometrial gene expression.

Swine Leukocyte Antigens (SLA) and Beta 2 Microglobulin (B2M)

MHC class I molecules (swine leukocyte antigens (SLAs) in pigs) and beta 2 microglobulin (B2M) are membrane glycoproteins that present peptide antigens to T cell receptors, and bind to inhibitory and activating receptors on natural killer cells and other leukocytes. They are involved in the discrimination of self from non-self by the immune system, and are essential to graft rejection. Downregulation of the expression of these classical MHC class I molecules and/or expression of nonclassical monomorphic MHC class I molecules by cells of the placenta benefit pregnancy (Hunt *et al*. 1987, Huddleston & Schust 2004). Although known to be interferon response genes, MHC class I and B2M are absent from endometrial LE during the peri-implantation period (Choi *et al*. 2003, Joyce *et al*. 2008). In pigs, expression of classical *SLA1*, *SLA2* and *SLA3*, non-classical *SLA6*, *SLA7* and *SLA8*, and *B2M* increases in endometrial LE between Days 5 and 9 in response to progesterone, then decreases between Days 15 and 20 (Joyce *et al*. 2008). Downregulation of SLA class I and B2M expression in uterine LE, in coordination with a lack of expression of these genes by the placenta (Ramsoondar *et al*.1999), may be important for preventing fetal allograft rejection in species exhibiting epitheliochorial placentation. In contrast to the situation observed for LE, expression of *SLAs* and *B2M* increases in stromal cells by Day 15 of pregnancy in response to conceptus IFNs, and remains detectable through Day 40 (Joyce *et al*. 2008). Cell-type specific regulation of *SLA* and *B2M* expression by progesterone and IFNs suggests that placental secretions control expression of immune

regulatory molecules on uterine cells to provide an immunologically favorable environment for survival of the fetal-placental semi-allograft.

Conclusions and future directions

Collectively, recent evidence from our laboratory and others suggests that pig conceptuses orchestrate precise temporal and spatial (cell-specific) changes in uterine gene expression through initial secretion of estrogens, followed by cytokines including IFNG and IFND. However, only a few differentially expressed genes have been investigated. Further, the pregnancy-specific role(s) of estrogen- and IFN-stimulated genes remains largely conjectural. Researchers in pig reproduction are challenged to incorporate new technologies including discovery-based microarray analyses to determine changes in global gene expression, adenovirus, morpholino and small interference RNAs to perform gain- and loss-of-function studies of specific endometrial and trophoblast genes, and state-of-the-art cell culture and imaging techniques necessary to delineate specific mechanistic functions of conceptus and uterine proteins.

Acknowledgements

This work was supported by National Research Initiative Competitive Grants nos. 2006-35203-17199 and 2008-35203-19120 from the USDA Cooperative State Research, Education, and Extension Service.

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Effect of progesterone antagonist RU486 on uterine progesterone receptor mRNA expression, embryonic development and ovarian function during early pregnancy in pigs

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Porcine peri-implantation development and maternal recognition of pregnancy is temporally associated with down-regulation of progesterone receptor (PGR) in the endometrial epithelium on days 10 to 12 (Geisert *et al*. 2006). One theory for down-regulation of uterine epithelial PGR is progesterone stimulates epithelial PGR to induce expression of RANKL [receptor activator for nuclear factor-kappa B (NF-κB) ligand or TNFSF11]. RANKL binds to its receptor, RANK (TNFRSF11A) to activate NF-κB. NF-κB and PGR are mutually antagonistic to one another. Activation of NF-κB, therefore, may inhibit PGR expression and induce the increase in endometrial prostaglandin-endoperoxide synthase 2 (PTGS2 or COX2) expression that occurs in the endometrium of cyclic and pregnant pigs on days 10 to 12.

The PGR antagonist, RU486, could be used to determine if blocking PGR down-regulation in the uterine epithelium prevents RANKL expression and NF-κB activation. To test this hypothesis, gilts were inseminated at estrus (d 0) and assigned to one of three treatments: RU486 (400 mg/d) on d 3, 4, and 5 (T1; n = 10); RU486 on d 6 and 7 (T2; n = 9); or control (n = 9). Blood was collected daily for plasma progesterone analysis, and the uterus and ovaries were harvested after slaughter on d 8 or d 12. Endometrial total RNA was isolated and analyzed with specific primers for RANKL, PTGS2, PGR isoform B (PGR-B) or the region common to PGR isoforms A and B (PGR-AB) by real-time reverse transcriptase PCR (RTPCR). NF-κB activation was measured by immunohistochemistry and scored objectively by three independent individuals.

Gilts treated with RU486 (T1 and T2) had heavier ovaries (17.9, 19.8 and 16.1 g [SEM = 1.1]; T1, T2 and control; $P < 0.05$), greater average follicular diameters (5.6, 4.9 and 3.6 mm [SEM = 0.5]; $P < 0.01$), a tendency for a greater number of corpora lutea (16.8, 15.0 and 13.7 [SEM = 1.0]; $P < 0.07$) and greater mid-cycle plasma progesterone concentration (25.2, 28.0 and 20.6 ng/mL; $P < 0.05$; d 9 to 11). Uterine weight (g) was reduced ($P < 0.05$) for T1 (608 ± 46) compared with T2 (780 ± 49) or control (785 ± 44).

Treatment of gilts with RU486 affected early embryonic development. The proportion of gilts with normal early embryonic development was lowest for gilts in T1 (chi-square = 11.2; $P < 0.01$; Table 1). There was a treatment effect ($P < 0.01$) on log-transformed RANKL mRNA expression (fold change relative to internal assay control) because compared with the control gilts, RANKL expression was greater in T1 (d 8 and d 12) and greater in T2 on d 12. Treatment affected both endometrial PGR-B ($P < 0.001$) and PGR-AB ($P < 0.001$) mRNA abundance. The PGR-B mRNA was more abundant in T1 (9.1 ± 1.0) compared with control (3.1 ± 1.0) with mRNA expression intermediate (6.0 ± 1.0) for T2 pigs. Likewise, the PGR-AB mRNA was more abundant in T1 (10.7 ± 1.1) compared with control (3.5 ± 1.0) and the PGR-AB for T2 pigs was intermediate (7.0 ± 1.0). There was a day effect and a tendency for a treatment by day interaction ($P < 0.06$; log-transformed data) for endometrial NF-κB activation. The amount of nuclear localization for activated NF-κB increased from d 8 to d

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12 for T2 and control pigs but remained relatively unchanged in T1 pigs (d 8 to 12). The pattern of PTGS2 mRNA expression was similar to that observed for NF-κB activation (day, $P < 0.001$; treatment by day, $P < 0.01$).

Table 1. Number and percent of gilts with normal embryonic development, relative mRNA amount in endometrium and luminal epithelium NF-κB activation (0 = cytoplasmic localization to 10 = nuclear localization) for pigs (d 0 = estrus) treated with RU486 on d 3, 4, and 5 (T1), RU486 on d 6 and 7 (T2), or untreated (control) with tissue collected on d 8 or d 12.

	Day 8			Day 12		
	T1	T2	Control	T1	T2	Control
Normal development (%)	3/5 (60)	4/4 (100)	5/5 (100)	0/5 (0)	3/5 (60)	4/4 (100)
RANKL mRNA	17.8 ± 9.8	3.7 ± 10.9	1.8 ± 9.8	26.6 ± 10.9	25.2 ± 9.8	2.8 ± 10.9
PGR-B mRNA	11.0 ± 1.3	4.9 ± 1.5	1.9 ± 1.3	7.3 ± 1.5	6.9 ± 1.3	4.3 ± 1.5
PGR-AB mRNA	10.4 ± 1.3	6.1 ± 1.4	2.8 ± 1.3	10.9 ± 1.7	8.0 ± 1.4	4.2 ± 1.4
NF-κB activation	2.4 ± 0.6	2.6 ± 0.7	2.2 ± 0.6	2.5 ± 0.6	4.0 ± 0.6	4.8 ± 0.6
PTGS2 mRNA	1.0 ± 0.6	0.5 ± 0.6	0.3 ± 0.6	0.3 ± 0.6	4.4 ± 0.6	3.4 ± 0.6

RU486 treatment stimulated ovarian follicular and luteal development perhaps by removing the inhibitory effect of progesterone on the ovary or hypothalamus. Early embryonic development was compromised for gilts treated with RU486 on d 3, 4 and 5 (T1). Inhibition of progesterone action shortly after insemination, therefore, is incompatible with conceptus development and survival. There was a slight numeric reduction in the percentage of normal embryos on d 12 for gilts treated with RU486 on d 6 and 7 (T2). Blocking progesterone action with RU486 during early pregnancy increased PGR-B and PGR-AB mRNA expression on d 8 and 12. This supports the general concept that progesterone is responsible for PGR down-regulation in the uterine epithelium. Our results do not support the hypothesis that RANKL mediates NF-κB inhibition of PGR. Both RANKL and PGR expression were clearly elevated in T1 gilts on d 8; a time when activated NF-κB was low. Endometrial RANKL expression does not appear to be responsible for NF-κB activation in the uterus and it is possible that another pathway for NF-κB activation such as Toll-like receptor 4 could be involved. Activated NF-κB was only detected on d 12 in treatments that were conducive to early conceptus development (T2 and control). Activation of NF-κB, therefore, was temporally associated with PGR down-regulation and the secretion of IL-1β by the elongating porcine conceptuses on d 12 (Ross *et al*. 2003). The activation of NF-κB on d 12 coincided with greater PTGS2 expression; a response observed previously in the pig (Ashworth *et al*. 2005).

This project was supported by National Research Initiative Grant no. 2007-35203-17836 from the USDA Cooperative State Research, Education and Extension Service.

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Select nutrients and glucose transporters in pig uteri and conceptuses

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Glucose present in the intrauterine environment can be metabolized, activate cell signaling pathways or be converted to a "storage" form. Total recoverable glucose in uterine fluid of pregnant, but not cyclic pigs increases from Day 12 after onset of estrus in concert with conceptus elongation (Bazer *et al*. 1991). Transport of glucose into the ovine uterus and its uptake by conceptuses involves sodium-dependent and facilitative glucose transporters (Gao *et al*. 2009). Glucose can activate FRAP1/mTOR "nutrient sensing" pathway in which protein kinases activate p70S6 through phosphorylation to increase translation of 5'TOP mRNAs (terminal oligopyrimidine tract) (Wen *et al*. 2005). Activated FRAP1 also regulates differentiation of trophectoderm (Tr) via Ras transformation by phosphorylating eukaryotic initiation factor 4E binding protein 1 (eIF4EBP1), a translational repressor of CAP-dependent translation (De Benedetti & Rhoads 1990). Select nutrients that stimulate FRAP1 activity in Tr include glucose, arginine (Arg), leucine (Leu) and glutamine (Gln) which may increase expression of *IGF2, ODC* and *NOS* mRNAs (Nielsen *et al*. 1995; Kimball *et al*. 1999; Martin & Sutherland 2001) which are required for conceptus development, differentiation and implantation through effects on production of NO (Kaliman *et al*. 1999) and polyamines (Van Winkle & Campione 1983). FRAP1 null mice die shortly after implantation due to impaired cell proliferation and hypertrophy in both the embryonic disc and Tr (Murakami *et al*. 2004). There are 14 isoforms of facilitative glucose transporters and 6 sodium-dependent glucose transporters. Of these, *SLC2A1, SLC5A1* and *SLC5A11* mRNAs are most abundant in endometria and SLC2A3 is uniquely expressed by ovine conceptus Tr and endoderm (Gao *et al*. 2009). The objective of this study with sexually mature gilts was to identify effects of pregnancy, long-term treatment of ovariectomized gilts with progesterone (P4) and estradiol-induced pseudopregnancy (PP) on changes in amounts of select nutrients (glucose, Arg, Leu and Gln) in uterine fluid and expression of glucose transporters in endometria and conceptuses.

Experiment 1 determined effects of day of the estrous cycle and pregnancy on total recoverable glucose, Arg, Leu and Gln in uterine flushings from gilts on Days 5, 9, 12 and 15 of the estrous cycle (Cy) and Days 9, 10, 12, 13, 14 and 15 of pregnancy (Px). Total recoverable glucose, Arg, Leu and Gln increased ($P < 0.05$) with day in Cy and Px gilts, but only Arg increased more in Px than Cy ewes (day x pregnancy status; $P < 0.05$) between Days 12 and 15.

Experiment 2 determined recoverable amounts of selected nutrients in uterine flushings of gilts ovariectomized on Day 12 and treated daily with either corn oil (OVX-CO; n = 4) or 200 mg progesterone (OVX-P4;n = 5) to Day 39 and hysterectomized on Day 40. Values (mean ± SEM; nmol) were greater for OVX-P4 than OVX-CO gilts for glucose (4,955 ± 2,534 vs 726 ± 133), Arg (207,112 ± 160,979 vs 7,409 ± 2,877) and Leu (248,255 ± 178,599 vs 13,983 ± 5,225), but differences were not significant due to high variability and small sample size.

Experiment 3 determined amounts of selected nutrients in uterine flushings of gilts on Day 90 of pseudopregnancy (PP) induced by treatment with 5 mg/day estradiol benzoate on Days

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11 to 15 after onset of estrus. The flushings (109±24 ml) contained significant amounts (nmol) of glucose (14,007±3,946), Arg (9,051±1,959), Gln (4,949±1,449) and Leu (2,455±771).

Results from examination of uterine and conceptus tissues by in situ hybridization for expression of Facilitative Glucose Transporters *SLC2A1, SLC2A2, SLC2A3*and *SLC2A4* mRNAs indicated that: 1) *SLC2A3* mRNA was weak or not detectable in conceptus and uterine tissues; 2) *SLC2A1* mRNA was expressed by all cell types in conceptuses and abundant in uterine luminal (LE) epithelium of Cy, Px, PP and OVX-P4 gilts; 3) *SLC2A4* mRNA was moderately abundant in LE of Px, but not Cy gilts between Days 13 and 25 of Px and by LE and GE of OVX-P4 gilts, but not detectable in LE or GE of PP gilts; 4) *SLC2A2* mRNA was most abundant in conceptuses from Days 12 to 40 of Px, decreased to Day 50 and then increased and was maintained specifically in placental areolae and apical regions of interdigitating endometrial folds to Day 80 of Px. *SL2CA2* was expressed in uterine LE of Px and PP gilts and LE and GE of OVX-P4 gilts.

Results indicated that: 1) glucose and Arg in particular, but also Leu and Gln, increase in uterine fluids of Cy and Px gilts; 2) these select nutrients are abundant in uterine flushings of PP and OVX-P4 gilts; and 3) temporal and cell specific changes occur in expression of specific glucose transporters in the uterus and conceptus. These select nutrients likely stimulate FRAP1 cell signaling in trophectoderm cells of conceptuses to influence proliferation, migration, attachment and gene expression necessary for conceptus development and survival in pigs.

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