Male Factors and Early Pregnancy Loss

Project 2E-107

Report prepared for the Co-operative Research Centre for an Internationally Competitive Pork Industry

By

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August, 2011

Established and supported under
the Australian Government's
Cooperative Research Centres
Program

Executive Summary

Rationale

Artificial insemination (AI) is the major driver of genetic improvement in the pig industry. However, the genetic potential of AI is only fully realised when fertility is optimal. Here there is considerable potential for cost-effective improvement as indicated by the following:

- Although sows generally ovulate ample numbers of ova (~21), and the vast majority (90-98%) are fertilised, pig reproduction (eg PPSY) is below par (APL report 2002).
- The major "issue" here is early pregnancy (i.e. post fertilisation) loss (EPL).
- Despite this, EPL has been neglected, even though long acknowledged as a cause of significant (~40%; Pomeroy 1960) loss of potential piglets.
- Where recognised, it has usually been assumed to be a "female" problem (see APL 2002)
- Work, primarily with other species, associates male factors with much (40%+) of this loss (Chenoweth 2007). Here, males differ not only in fertilising capability, but also in embryonic loss, abortions, sperm numbers for optimal fertility and insemination time "window" for best results (McMillan et al 1975; Maxwell et al 1992; Hill et al 1994; Saacke et al 2000; Flowers 2002).

However, although male factors have long been recognised as a significant cause of EPL (eg Pomeroy 1960; Mattei et al 1994; Chenoweth 2007), it is only recently that advancing knowledge and technology have allowed insight into specific causes and effects (Perreault et al 2003). Full exploitation of these advances has yet to occur in the livestock (eg pig) industries. Thus, this project provided an opportunity to apply modern andrological technologies to better understand the causes of male-related losses in the Australian pig industry and provide insights to their management.

Methods

(Terminology and Definitions can be found in Appendix 2)

Data were analysed on 8961 inseminations of sows and gilts with 318 single sire semen samples (199 sires of 8 sirelines) in terms of genetic and environmental effects on outcomes (total born, born live, stillborn, mummified, time to outcome and return type). Semen evaluations included aspects of sperm motility, morphology and DNA integrity. In addition IVF studies were performed using 36 semen samples. Two "side studies" were also conducted as follows; a) A comparison of methods (Acridine Orange, Aniline Blue and DiffQuik) to assess sperm chromatin status and integrity, and b) A study of the effects of extended storage time (4d @ 15 or 20^{0}C) of semen characteristics, compared with results of inseminations at 1,2 and 3 days post collection (a total of 143 ejaculates from 76 boars were used and mating data from 535 inseminations performed across 4 seasons).

Outcomes

(note spreadsheet summarising overall results in Appendix 3)

Genetic and Environmental Effects on Mating Outcomes.

Both Sire and Dam lines influenced (P<0.01) a number of mating outcomes including total pigs born, born alive, stillborn and mummified. Dam line also influenced (P<0.01) return to oestrus category. Season and month influenced (P<0.01) both total pigs born and mummified, while season also influenced (P<0.01) total pigs born. Dam parity influenced (P<0.01) total born, born alive and stillborn.

Semen Traits and Mating Outcomes

Semen arrival temperature influenced both Total Born (P<0.05) and Born alive (P<0.01). Percent normal sperm (%N), distal droplets (DD), total motility (TMOT) and VCL all influenced Total Born (P<0.01) and Born Alive (P<0.05), while percent abnormal sperm heads (HD) influenced (P<0.05) Born Alive. Re sow returns, a number of semen traits (VCL, PIA, ACRO, Clump, Bacteria scores) influenced return "type" (all P<0.05) whereas HD and ACRO were associated (both P<0.01) with return time.

Semen Traits and IVF Outcomes

One way ANOVA revealed significant interactions (P<0.01 for all) between % fertilised oocytes, % degenerated oocytes, % morulas, % blastocysts and the individual semen parameters: TMOT, %N, PD,

DD, LD, % intact DNA (DiffQuik stain), HD, MID, TAILS, semen pH, number of accessory sperm bound to the zona pellucida and also ovary temperature on arrival.

Tests for Sperm DNA Integrity.

Although all 3 tests (AO, AB, DQ) were associated with a number of other tests of semen quality, only DQ influenced (P<0.05) a breeding outcome (stillbirths), with AO showing a tendency (P<0.1) for this same trait.

Semen Storage Time (SST) Effects.

In this experiment, fertility outcomes were not affected by SST (1,2, and 3d) at 17°C, although litter size was influenced (P<0.05) by the storage time X season interaction. In addition, stillbirths were associated with damline X SST and parity X SST interactions (both P<0.01). Storage of extended semen at 15 and 20^{0}C for 4 days did result in decreased motility traits and increased pH, which have been shown to influence mating outcomes including Total, Live and Still Born as well as sow return time.

Ubiquitin

The choice of ubquitin (UBQ) as the target protein for investigation was validated by its strong relationships with a number of important sperm traits (eg motility, CONC, LD, %N and MID). Although retained cytoplasmic droplets were not significantly associated with UBQ, this is considered due to their confounding with midpiece defects. Further analysis is necessary to directly relate these findings to mating outcomes.

Relevance and Applications

Not surprisingly, this project reaffirms that genetics and environment significantly influence pig mating outcomes, both individually and in combination. Both dam lines and sire lines varied considerably in outcomes, as well as in their interactions with each other, season and, in sows, parity.

Perhaps also not surprising are the findings in this project re. effects of a number of semen/sperm traits on mating outcomes. Of particular note are those traits associated with semen handling and/or processing, and which can be relatively easily modified. These include shipping temperature and the presence of bacteria and clumping, all of which can be part of the same story (as sperm concentration was relatively uniform, it did not affect most estimates). An important finding here is that adverse effects can be associated with relatively small changes in semen handling protocols.

Similarly, evidence is presented for negative associations between mating outcomes and some sperm characteristics that hitherto have been considered either benign or difficult to pin down. These include distal cytoplasmic droplets, PIA, acrosome abnormalities and live-dead estimation. Other semen/sperm characteristics which have been long regarded as significant, eg sperm motility and morphology have had their importance reaffirmed. In addition, for the first time, attention is drawn to the implications of overt bacteria and sperm clumping when detected in extended boar semen.

In this study, sperm DNA integrity was an important consideration due to its direct relationship with early pregnancy loss (Chenoweth 2007; Didion et al 2009). For this assessment, conventional approaches utilize fluorescent procedures which are complicated and require specialized equipment. In this study, we show that a relatively simple, quick and widely used clinical-laboratory technique (i.e DiffQuik staining) can be used with basic microscopy to give results which are at least equivalent.

Some other techniques used in these trials (eg CASA motility, IVF and proteomics) are not suitable for routine industry applications. However, their role was primarily to confirm or validate other tests which are more industry-friendly. Thus CASA estimates confirmed the importance of good sperm motility, and its accurate assessment, to most mating, as well as all IVF, outcomes. In turn, IVF has provided a valuable tool to emphasize the significant relationships which exist between sperm motility and morphology characteristics and fertility traits.

The data for piglets born alive, still born or mummified sheds light on potential male effects on pregnancy outcomes. Here aspects of sperm motility and morphology as well as LD affected both piglets born alive or stillborn (P<0.01 or 0.05). Of particular interest is the relationship between sperm chromatin assessments and these parameters; i.e. AO with born alive and still born (both P<0.05) and DQ with still births (P<0.01).

This would appear to confirm our initial hypotheses that:

a) significant male effects occur post fertilization
b) these are associated with sperm DNA/chromatin abnormalities

It would be tempting to assume that sperm DNA/chromatin damage is largely due to an imbalance of reactive oxygen species (as shown in other species), and that managerial techniques (environmental mitigation, feeding of anti-oxidants) could minimize such damage. However more work is needed to confirm (or not) this hypothesis.

The Bottom Line

Maintaining an effective sperm dose (ESD) is a key target for boar studs. This necessitates accurate and precise methods of assessing different semen traits to ensure that the final product is consistently reliable. It also implies assessment methods that are rapid and relatively simple, and which embrace those traits which are most important for fertility. This study adds to our understanding of which traits are most important, emphasizes the importance of appropriate QA in lab procedures and adds several options to assessment procedures which are feasible for routine industry use. Logical progression of these studies should result in two key objectives, viz

a) mitigating stress related infertility with targeted strategies such as anti-oxidants (both in animals and semen)

b) establishing an effective and consistent sperm dose which optimizes the transmission of improved genetics.

Recommendations

A number of recommendations will arise from this study and work is in hand to include these in a manual of good laboratory practices.

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1. Introduction

There is increasing emphasis on fertility traits with most boars being housed in dedicated boar stud facilities where the emphasis is on sperm production for artificial insemination (A.I.) doses (Safransky, 2008). Although fertilization rates in pigs are generally high, a major source of loss occurs in early pregnancy (Diskin and Morris, 2008). A significant portion of this loss is attributable to male effects (Chenoweth 2007), with particular emphasis on sperm DNA integrity. Advances in knowledge and technology now permit us to detect biomarkers for these effects in semen, while presenting opportunities to minimize their adverse effects.

Many studies have been conducted on boar semen, ranging from environmental and genetic influences to those associated with handling and processing, and including functional tests and their relationship with fertility (eg Wolf and Smithal, 2009). Whereas semen assessment has usually included traits such as sperm motility, concentration and morphology, these can now be complemented by new technologies which include proteomics, flurochromes, IVF and improved microscopy. Having access to such technologies provided part of the impetus for the current project. Another strong impetus was strong and generous collaboration provided by PIC Gong Grong in which extended boar semen used for A.I. being concurrently assessed within the Andrology Laboratory at CSU. This, in combination with a comprehensive databank of mating outcomes and genetic relationships, provided a unique opportunity for advancing our knowledge re male effects on fertility traits. In turn, this knowledge should prove useful in terms of improving pig reproduction and production.

2. Methodology

2.2. Sow fertility data and sperm traits

(Note definitions and terminology in Appendix 1)

Semen traits assessed included: motility by CASA (total, progressive and rapid); velocity by CASA (VAP, VSL, VCL, BCF), concentration and clump score (CASA), pH, temperature, chromatin condensation by acridine orange, membrane integrity by eosin/nigrosin stain, morphology by DIC microscopy x1000 under oil immersion (% normal, percent intact acrosomes, % cytoplasmic droplets (proximal and distal); abnormal heads, midpieces, tails, acrosomes and detached heads and bacterial score.

Data is available on 318 single sire semen samples representing 199 sires (Large White and Duroc) of 8 sirelines used for 8961 inseminations of sows and gilts. Of these sires, 109 boars were tested once, 36 boars were tested twice at different times and 21 boars were analysed three or more times. The databank includes total pigs born, number of piglets born alive and number of stillborn piglets (mummified piglets were largely ignored due to small numbers).

Statistical Analyses:

Unless otherwise stated a linear mixed model using restricted maximum likelihood were used to analyse the data using ASReml-R (Butler, Cullis, Gilmour & Gogel 2007). The model can symbolically be written as:

response _ mean + Parity + DamLine + SireLine + InseminationSeason +

Parity:DamLine + Parity:SireLine + Parity:InseminationSeason + DamLine:SireLine + DamLine:InseminationSeason + SireLine:InseminationSeason + Parity:DamLine:InseminationSeason + Parity:SireLine:InseminationSeason + *SireID* +*DamID* + *Inseminator*

Notation: Terms fitted in the model as random are italicised; all other terms are fitted as fixed terms.

For stillborn piglets, the modelling approach for this count data is a Poisson generalized linear model (GLM) with a logarithmic link function. The full model contained main effects of PARITY, DAMLINE, INSEMINATIONSEASON and SIRELINE, also all the two way interactions of these main effects. The GLM model was implemented using R (R Development Core Team 2010).

The model can be symbolically written as:

STILL _ PARITY + DAMLINE + INSEMINATIONSEASON + SIRELINE +

PARITY:DAMLINE + PARITY:INSEMINATIONSEASON + PARITY:SIRELINE +

DAMLINE:INSEMINATIONSEASON + DAMLINE:SIRELINE + INSEMINATIONSEASON:SIRELINE

The reduced model for the Total Number of piglets can symbolically be written as:

response _ mean + Parity + DamLine + InseminationSeason + SireLine +

DamLine:SireLine + DamLine:InseminationSeason + Parity:InseminationSeason +

SireID + *DamID* + *Inseminator*

For the statistical analyses of semen parameters and interactions with mating outcomes, the linear mixed model can symbolically be written as:

response _ mean + SemenTrait + Parity + DamLine + SireLine +

InseminationSeason + Parity:DamLine + Parity:SireLine +

Parity:InseminationSeason + DamLine:SireLine + DamLine:InseminationSeason +

SireLine:InseminationSeason + Parity:DamLine:InseminationSeason +

Parity:SireLine:InseminationSeason + *Inseminator*

Notation: Terms fitted in the model as random are italicised; all other terms are fitted as fixed terms.

2.3. Chromatin condensation tests

To determine a most appropriate method for detecting chromatin damage in sperm heads, extended chilled, semen (366 doses from different boars/ejaculates) were obtained (1 -3d of collection) from a pig breeding organization via normal shipping protocols. Semen was assessed as follows: % "Live/dead" (LD) and sperm morphology (%N; %Hd, %PD, %DD) using DIC (1000X). In addition, sperm (n=131) were stained with acridine orange (AO), aniline blue (AB) and diff quick (DQ) using established techniques.

Briefly, semen smears were air dried and fixed in methanol (DQ fixative, also used for AB staining) for 30 minutes. For DQ, slides were then stained first in red and then in blue dye (Provet, Australia)for 30 minutes each time, rinsed with distilled water and air dried again. Fixed AB slides were stained for 5 minutes in 5% AB stain (5% aqueous aniline blue; ProSciTech, Australia made with 4% acetic acid, pH 3.4) for 5 minutes following fixation, rinsed and air dried. Assessment involves counting dark blue sperm heads (damaged histones) vs. non-stained or pale blue sperm heads (intact histones) at x400 magnification. For DQ stained sperm, heads with condensed double strand DNA appear to be pale blue whilst sperm with damaged DNA stain reddish purple.

Slides to be stained with AO were first heated for 10 minutes to 60°C, placed in acid buffer (0.15 M NaCl, 0.01% Triton X-100, 0.08 N HCl; pH 2) for 30 seconds and placed in 100%, 70% and 50% alcohol for 2 minutes each. This was followed by a rinse in distilled water for 5 minutes and then a rinse in phosphate buffered saline (PBS; MP Biomedicals, Australia), pH 7.4 for 2 minutes, staining in AO solution (0.1 g AO in 100 ml dH20 stock solution diluted 1:9 in PBS; ProSciTech, Australia) for 15 minutes in the dark and two more rinses in PBS before being air dried and coverslip mounted with Depex mounting medium (Ajax Fine Chemicals, Australia). AO slides were assessed for % red sperm heads (damaged i.e. single strand DNA) and % green sperm heads (intact double strand DNA) with a

fluorescent microscope x1000 magnification whilst AB and DQ slides were read with bright field microscopy at 400x magnification.

Fertility data (total born, % live born, % stillborn and % mummified) from single-sire matings (n=249) using the same ejaculates was compared with semen assessments. Data was subjected to two-tailed student's T test and one way ANOVA.

2.4. Sows failing to farrow

Data was also analysed for sows/gilts which failed to farrow due to return to oestrus (regular and irregular returns) or abortion. Sows of mixed age and parity (n=252) were inseminated with single sire AI semen doses twice during their oestrous period. Semen was then subjected to the same tests as described above using CASA and DIC microscopy.

Return categories were classified as: early return (0-18d, n=7); regular (19-23d, n=69); foetal skeletal calcification stage (24-35d, n=67), irregular (36-45d, n=23), late (>46d, n=83). Statistical analysis used linear mixed models, as below:

trait ~ DamLine + Parity + InseminationSeason + SireLine + *InseminatorID* + *SireID*

Here, all terms were fixed terms except for Sire and Inseminator IDs which were random. The effect of dam line, sire line, parity and insemination season on return type were investigated using ordinal logistic regression; a method also used to re-analyse semen values. The data was also analysed using the amended AIC variable selection method, the final model was:

ReturnType~PIA+Acro+VCL+Clump+Bacteria.

A proportional odds model was used to determine the odds of moving from one category to another.

Return categories were classed as: abort (anytime, n=3); early return (0-18d, n=7); regular (19-23d, n=69); foetal skeletal calcification stage (24-35d, n=67), irregular (36-45d, n=23), late (>46d, n=83).

A linear mixed model using restricted maximum likelihood was also used to analyse the data using ASReml-R (Butler, Cullis, Gilmour & Gogel 2007). The model can symbolically be written as:

response _ mean + covariates + Parity + DamLine + SireLine + InseminationSeason + Parity:DamLine + Parity:SireLine +*SireID* + *Inseminator*

Notation: Terms fitted in the model as random are italicised; all other terms are fitted as fixed terms.

Another type of statistical analysis was performed for the response RETURNTYPE, which is classified as an ordinal data type. To analyse this data an ordinal logistic regression is required. The based model fitted to this data can be symbolically written as:

RETURNTYPE SIRELINE + PARITY + DAMLINE + INSEMINATIONSEASON

The data was analysed in R (R Development Core Team 2010).

Please note that mummified piglets were excluded from most statistical analyses due to the small number of records. Out of 6681 sow insemination records, 6521 sows did not have any mummified piglets. Likewise, abortion records were dropped from analyses due to less than 1% of sows aborting their litters during the study period.

2.5. Effects of Storage and temperature

This experiment involved keeping 20 ml semen aliquots in a sealed esky at either room temperature (20°C) or with a cold freezer pack that was replaced daily (15°C) for 4 days. Semen was then tested in terms of CASA parameters (total, progressive and rapid motility;

straight line, curvilinear and average velocities; clump score) as well as semen pH. The semen of the same batch that was kept at the boar stud and used for inseminations (parity 1-8) after 1,2 or 3 days storage at the industry recommended temperature of 17°C was then checked against mating records to determine which factors were linked to conception failure (i.e. females returning to oestrus), litter size, pigs born alive and stillbirths. A total of 143 ejaculates from 76 boars were used for this experiments and mating data was checked from 535 inseminations performed across 4 seasons.

2.6. In vitro fertilisation

A total of 4 extended ejaculates were used each week for the IVF trial. This trial continued for 14 weeks with 4 different semen samples tested weekly i.e. 56 semen samples from 44 boars in total (12 boars were tested more than once). Ovaries were obtained immediately from slaughtered sows (usually between 60-100 ovaries obtained each time) at Sinclair Meats Abattoir in Benalla, Victoria and transported back to CSU in a heated esky to keep ovaries warm at >26°C. Upon arrival at CSU, ovaries were washed in pre-warmed purified water and then placed in warm 0.9% saline whilst awaiting follicle aspirations. Cystic ovaries and other unsuitable ovaries were discarded. Follicles between 3-6 mm in diameter were aspirated using a vacuum pump and 19G winged infusion needles (Provet, Australia). The follicular fluid supernatant was removed before the cellular pellet was resuspended in oocyte search medium. Suitable oocytes were then selected using a stereo microscope at 50x magnification and placed in *in-vitro* maturation media. Following a media change the next day into in vitro maturation medium 2, oocytes were cultured for another 24 hours at 5% CO_2, 38.5°C and 96% humidity. Oocytes denuded of cumulus were then placed in IVF media to equilibrate for 1 hr before sperm was added. All media recipes were obtained from Dr Bart Gadella at Utrecht University, The Netherlands. Sperm was prepared for fertilisation by twice washing and centrifugation at 700xg for 4 minutes. Fertilisation was done using an adjusted concentration of 1 million sperm/ml at a rate of 1000 sperm/oocyte. The next day, presumptive zygotes were transferred into embryo culture media 24 hours post-IVF. Embryos were scored for cleavage rates, fertilisation rates and accessory sperm attached to the zona pellucida 3 days post-IVF and for blastocyst formation at 8 days post-IVF.

2.7. Ubiquitin in sperm

Ubiquitin was chosen as the protein of interest based on literature reviews and discussions with Dr Peter Sutovsky (U. Missouri). It is a chaperone protein which tags defective sperm. A total of 80 semen samples which had defective sperm (as determined by morphology, motility, membrane integrity or chromatin decondensation) and 25 semen samples which were considered to be superior in all parameters tested were used. Semen samples were washed in Percoll by using a 70%:35% Percoll (2 ml:4 ml) gradient with a 3 ml extended semen layer on top. Falcon tubes containing the Percoll and semen were centrifuged first for 10 minutes at 200xg followed by 20 minutes at 900xg. Supernatant was removed and the sperm pellet resuspended in 1 ml of Hepes/saline buffer for the final centrifugation at 600xg for 5 minutes. Sperm pellets were then adjusted for final concentration. SDS-PAGE followed by Western blotting was performed by standard methods and the blots blocked overnight at 4°C with cold fish skin gelatin (Sigma, Australia) containing Tween 20. Antibodies used to detect ubiquitin in sperm samples were a polyclonal anti-pig ubiquitin antibody (ADI-SPA-200-F; Sapphire Biosciences, Australia) and a polyclonal goat anti-rabbit IgG antibody conjugated to horseradish peroxide (ADI-SAB-300-J; Sapphire Biosciences, Australia). Quantification was done using the software program GelAnalyzer.

3. Outcomes

3.1. Sow Genetics and Insemination Season

Genetic analyses of sire line and dam lines showed the main effects of PARITY, DAMLINE and SIRELINE were all highly significant (all $P < 0.001$). The main effect of INSEMINATIONSEASON was also significant ($P = 0.034$). The significant interaction terms were DAMLINE:SIRELINE ($P < 0.001$), DAMLINE:INSEMINATIONSEASON (p-value = 0.014) and

DAMLINE:PARITY (P = 0.038) Of the random terms, both DAMSTIG and SIRESTIG were highly significant (P < 0.001). For example, crossing sire line 2 with dam line 2 produced a predicted value of 11.7 ± 0.2 piglets[f]. By comparison, crossing sire line 3 with dam line 2 produced a predicted value of 10 ± 0.3 piglets[abc] (P < 0.05). At extremes, sire line 15 over dam line 304 only produced a predicted 2 ± 3.1 piglets[abcef] whilst the most piglets (16.1 ± 2.9) were predicted for sire line 16 crossed over dam line 315[abcdef].

Litter size was also affected by season. Dam line 315 inseminated in autumn produced a predicted 11.6 ± 0.6 piglets[cde] but for a spring insemination, this increased to 12.1 ± 0.8 piglets[cdef]. Winter inseminations produced a predicted 12.3 ± 0.6 piglets [cde]whilst summer inseminations produced a predicted 10.9 ± 0.6 piglets[abcd]. Similar trends were observed for other dam lines but more work is required to analyse this in more detail.

Parity influences litter size also for each dam line. A trend of increasing litter size with greater parity number was observed with parity 1 being the least productive but again, further data analyses are required to determine significance. For some dam lines, litter sizes were greatest for parity 3-6, for others, parity 2-6 with some dam lines at parity 10 occasionally producing a larger litter compared with preceding parities.

3.2. *Sow fertility data and sperm traits*

The most significant semen traits affecting total pigs born were arrival temperature, % distal droplets, % normal morphology and % intact sperm membranes (i.e. "live/dead"). These same factors (excluding % distal droplets) also significantly influenced piglets born alive. Relationships were as shown in Table 1 below:

Table 1 - Relationships between semen traits and birth outcomes

Trait	Total Pigs Born	Total Born Alive	Number of Stillborn Piglets
Increased Arrival Temp 1^{0}C	Decrease 0.14	Decrease 0.24	n/s
Increase Distal Droplets by 1%	Decrease 0.04	n/s	n/s
Increase Membrane Intact Sperm 1%	Increase 0.03	Increase 0.04	Decrease 0.98
Increase % Normal Morphology 1%	Increase 0.01	Increase 0.02	n/s
Increase Beat Cross Frequency by 1	n/s	n/s	Increase 1.03
Increase Abnormal Midpieces by 1%	n/s	n/s	Increase 1.01
Increase VCL by 1%	n/s	Increase 0.00012	n/s

These results indicate that considerable progress can be made in pig reproduction by increased attention to quality control measures in semen processing and handling, which confirms an initial objective of this project. Several semen trait correlations are significant or highly significant as shown below in Table 2:

Table 2 - Correlations among semen/sperm traits

Parameter	% normal morphology	PIA	Distal droplet	Intact membranes	Total motile	VAP	VCL	BCF	pH
Tails	-0.4	-.26	0.02	-0.02	-.0.21	-0.18	-0.09	0.06	-.04
Distal droplets	-0.65	-.18	1	-0.19	-0.35	0.02	-0.17	-.62	-0.2
Loose heads	-0.47	-.34	0.24	-0.18	-0.05	-0.16	-0.1	0	-.04
Total motility	0.29	0.41	-0.35	0.4	1	0.7	0.84	0.31	0.62
VAP	0.05	0.26	0.02	0.48	0.7	1	0.86	-.08	0.42
VCL	0.18	0.28	-0.17	0.47	0.84	0.86	1	0.05	0.52
VSL	-0.1	0.16	0.18	0.33	0.23	0.68	0.29	-.16	0.1

Parameter	% normal morphology	PIA	Distal droplet	Intact membranes	Total motile	VAP	VCL	BCF	pH
BCF	0.22	0.34	-0.62	-0.06	0.31	-0.08	0.05	1	0.36
pH	0.03	0.52	-0.2	0.09	0.62	0.42	0.52	0.36	1
Clump	-0.1	-0.5	0.09	0.08	-0.16	0.01	0.05	-.41	-.43
Tempera-ture	-0.04	-.24	0.1	0.02	-0.47	-0.19	-0.34	-0.1	-.49
Concen-tration	0.19	0.21	-0.23	0.29	0.48	0.45	0.41	0.31	0.22
Acridine Orange	0.06	0.16	-0.09	0.04	0.15	0.18	0.19	0.14	0.07

Highly significant at P<0.01; Significant at P< 0.05; Trend P 0.05 to P0.10

For Legend of CASA velocity parameters, see Appendix

Table 3 shows the main effects of PARITY, DAMLINE and SIRELINE were all highly significant, having p-values < 0.001. The main effect of INSEMINATIONSEASON was also significant, having a p-value = 0.034. The significant interaction terms were DAMLINE:SIRELINE (p-value < 0.001), DAMLINE:INSEMINATIONSEASON (pvalue= 0.014) and DAMLINE:PARITY (p-value = 0.038) Of the random terms,both DAMSTIG and SIRESTIG were highly significant (p-values < 0.001).

Table 3 - TOTAL BORN reduced model (only significant variables)

Influence	F Inc	Pr
Dam Parity	8.53	0.000
Dam Line	5.02	0.000
Season	4.83	0.034
Sire Line	3.31	0.000
Dam Line: Season	1.55	0.014
Dam Line: Sire Line	1.80	0.000
Season: Parity	1.54	0.040
Dam Line: parity	1.26	0.038
Sire Line: parity	0.78	NS
Sire Line: Season	0.87	NS
Season: Parity: Sire Line	1.15	NS
Season: Parity: Dam Line	0.94	NS

Highly significant; P<0.01 and significant; P<0.05

Table 4 - Semen trait effects on total number of piglets born

Trait	Df	Den Df	F.inc	Pr
Temperature	1.0	858.0	10.49	0.226
Distal droplets	1.0	858.0	10.49	0.001
Loose heads	1.0	858.0	0.84	0.359
% normal morphology	1.0	105.5	9.8	0.002
Intact membranes	1.0	858.0	0.99	0.32
Beat Cross Frequency ('head wobble')	1.0	840.0	1.99	0.159
Total motility	1.0	858.0	7.04	0.008
VCL (curvilinear velocity)	1.0	840.0	7.56	0.006
Bacteria	1.0	704.0	1.12	0.291
Proximal droplet	1.0	858.0	1.00	0.318
Acridine orange (AO)	1.0	858.0	6.57	0.011
pH	1.0	814.0	0.43	0.512
Concentration	1.0	858.0	1.12	0.289

Trait	Df	Den Df	F.inc	Pr
VAP (average velocity)	1.0	840.0	5.85	0.016
Percent intact acrosomes	1.0	858.0	0.03	0.861
% abnormal heads	1.0	858.0	5.80	0.016
% abnormal tails	1.0	858.0	0.00	0.983
VSL (straight line velocity)	1.0	840.0	0.88	0.350
Clump	1.0	814.0	1.14	0.287
% abnormal mid pieces	1.0	858.0	4.61	0.032
% abnormal acrosomes	1.0	858.0	0.50	0.478

The covariates of Distal droplets, % Normal morphology, Total motility, VCL, AO, VAP, % Abnormal heads and % Abnormal midpieces were all individually significant either at the 1% or 5% significance levels.

Three of these co-variates were significant when tested in the final model. They are DD, % Normal morphology and % Abnormal head with p values of 0.009, 0.033 and 0.041 respectively. The coefficients for those covariates were -0.04020, 0.01551 and -0.04495.

These results can be interpreted as follows:

- As the % sperm with a distal cytoplasmic droplet increases by 1%, the total number of piglets born increases by -0.04. The range of sperm with a distal cytoplasmic droplets (0 – 44%). Sires with the maximum % sperm with a distal cytoplasmic droplet have on average 1.8 less piglets born than the sires with the minimum % sperm with a distal cytoplasmic droplet.

- As the % sperm with normal morphology increases by 1% the total number of piglets born increases by 0.016. The range of sperm % Normal morphology is (7-94). Sires with the maximum % Normal morphology have on average 1.3 more piglets born than the sires with the minimum % Normal morphology.

- As the % of abnormal heads increases by 1% the total number of piglets born increases by -0.045. The range of sperm % of abnormal heads is (0 - 35). Sires with the maximum % of abnormal heads have on average 1.6 less piglets born than the sires with the minimum % of abnormal heads.

Genetic and Environmental Effects on Piglets Born Alive

Table 5 - Piglets Born Alive - ANOVA 1 (all variables)

Influence	F Inc	Pr
Dam Parity	6.37	0.000
Dam Line	5.59	0.000
Insemination Season	3.33	0.019
Sire Line	3.77	0.002
Dam Line: Season	1.69	0.012
Dam Line: Sire Line	1.48	0.059
Season: Parity	1.44	0.071
Dam Line: parity	1.28	0.061
Sire Line: parity	0.92	0.626
Sire Line: Season	1.09	0.354
Season: Parity: Sire Line	1.20	0.099
Season: Parity: Dam Line	0.93	0.674

Dam parity, dam line and sire line are all highly significant influences (P<0.01) on the number of piglets born alive. Insemination season and the interaction between dam line and season were significant at the 5% significance level.

Table 6 - Sperm traits and their influence on the number of piglets born alive

Sperm traits	Df	denDF	F.inc	Pr
Temperature	1.0	254.2	0.03	0.863
Intact membranes	1.0	297.3	0.73	0.394
% normal morphology	1.0	112.4	9.51	0.003
VCL (curvilinear velocity)	1.0	174.4	5.11	0.025
Total motility	1.0	209.4	4.47	0.036
Loose (detached) heads	1.0	257.6	0.02	0.882
Concentration	1.0	302.2	1.18	0.278
Bacteria	1.0	86.8	0.56	0.458
Proximal droplets	1.0	197.5	1.00	0.318
Distal droplets	1.0	90.0	5.36	0.023
pH	1.0	233.7	0.08	0.782
Percent Intact Acrosomes	1.0	340.1	0.03	0.855
VAP (mean velocity)	1.0	227.9	4.16	0.043
Acridine orange	1.0	162.8	3.42	0.066
Beat cross frequency ('wobble')	1.0	117.5	0.15	0.697
% abnormal heads	1.0	241.9	4.40	0.037
% abnormal mid pieces	1.0	140.2	2.44	0.121
Clumps	1.0	261.0	1.70	0.193
% abnormal tails	1.0	154.2	0.02	0.876
% abnormal acrosomes	1.0	277.1	0.02	0.894
VSL (straight linear velocity)	1.0	172.2	1.17	0.280
Damline	10.0		22.71	
%N (DiffQuick)	1.0	58.7	0.93	0.339

The covariates of % NORMAL ($P<0.01$), Distal Droplets, VCL, TMOTILE, % abnormal Head and VAP (all $P<0.05$) were all significant.

However, only one of the covariates was significant when all were included in the final model. This was % Normal morphology ($P = 0.002$). The coefficient for % Normal morphology (0.025) indicates that as the percentage of sperm with normal morphology increases by 1% the number of piglets born alive increases by 0.025. The range of sperm % Normal morphology is (7 - 94). Sires with the maximum % Normal morphology have on average 2.2 more live piglets than the sires with the minimum % Normal morphology.

Table 7 - Deviance table for stillborn piglets

	Df	Deviance	Residual Df	Residual Dev.	P(>IChil)
Null			6680	11420.44	0.000
Acridine Orange	1	5.08	892	1540.92	0.0242
Parity	9	29.70	883	1511.22	0.0005
Dam line	13	26.94	863	1459.63	0.0127
Insemination Season	3	4.99	860	1454.64	0.1727
Sire Line	7	24.66	876	1486.57	0.0009
Parity: Sire line	47	92.10	813	1362.54	0.0001
Parity: Dam line	49	92.09	764	1270.45	0.0002
Parity: Insemination season	20	59.67	744	1210.78	0.000

	Df	Deviance	Residual Df	Residual Dev.	P(>lChil)
Sire line: Insemination season	20	47.04	709	1098.77	0.0006
Dam line: Insemination season	20	35.47	689	1063.30	0.0177
Sireline: damline	15	64.97	729	1145.81	0.0000

Acridine Orange was significant (P 0.024)

As the % sperm with intact double strand DNA as depicted with AO fluorescence increases by 1% the number of stillborn piglets born increases by a multiple of approximately 1.007; a relatively large magnitude effect.

None of the co-variates were found to be significant at the 5% level for mummified piglets.

Table 8 - Semen traits and stillborn piglets

	Df	Deviance	Resid. Df	Resid.Dev	P(>lChil)
Acridine orange	1	5.080	892	1540.92	0.024
BCF	1	4.863	874	1500.67	0.027
Temperature	1	3.922	892	1542.08	0.048
Proximal droplet	1	0.3288	892	1542.71	0.070
Total motile	1	1.894	892	1544.11	0.169
Distal droplet	1	1.704	892	1544.30	0.192
VCL	1	1.631	874	1503.90	0.202
VAP	1	1.613	874	1503.92	0.204
%Intact membranes	1	1.466	892	1544.54	0.226
%normal	1	1.262	892	1544.74	0.261
%abnormal heads	1	0.778	892	1545.22	0.378
%Intact Acrosomes	1	0.457	892	1545.54	0.499
VSL	1	0.447	874	1505.08	0.504
%Loose heads	1	0.375	892	1545.63	0.540
pH	1	0.342	848	1477.38	0.559
%abnormal midpieces	1	0.312	892	1545.62	0.577
%abnormal acrosomes	1	0.299	892	1545.70	0.584
%abnormal tails	1	0.253	892	1545.75	0.615
Clump	1	0.130	848	1477.59	0.718
Concentration	1	0.083	892	1545.92	0.774
Bacteria	1	0.001	737	1252.40	0.972

The covariates of ACRIDINEORANGE, BCF and TEMPERATURE were all individually found to be significant at the 5% significance level.

Table 9 - Summary of semen quality parameters and correlation coefficients between these semen traits and sow outcomes.

Covariate	Mean	Range	Total Born	Born Alive	Stillborn	Mummified
%Normal morphology	72.46	7-94	0.13	0.13	0.03	-0.00
%Intact acrosomes	88.84	54-99	0.01	0.01	-0.02	-0.01
% abnormal heads	7.71	0-35	-0.08	-0.07	-0.02	0.02
%abnormal acrosomes	0.66	0-12	-0.02	-0.02	-0.01	-0.04
%abnormal midpieces	8.83	0-83	-0.07	-0.07	-0.01	-0.02
%abnormal tails	2.16	0-33	-0.00	0.00	-0.01	0.05
%proximal droplets	2.42	0-19	-0.03	-0.05	0.04	-0.01
%distal droplets	4.25	0-44	-0.1	-0.10	-0.03	-0.03
%loose heads	1.16	0-9	-0.03	-0.03	-0.01	0.01
Bacteria	0.96	0-3	-0.04	-0.04	0.00	0.01
%intact membranes	78.04	42-99	0.03	0.05	-0.03	-0.03
Total Motility	69.09	1-97	0.09	0.08	0.03	-0.03
VAP	84.22	32-136	0.08	0.07	0.03	-0.00
VCL	166.04	71-266	0.09	0.08	0.03	-0.01
VSL	51.13	20-96	0.03	0.03	0.02	-0.01
BCF	33.21	6-41	0.05	0.03	0.05	0.03
pH	7.46	7-8	0.02	0.03	-0.01	0.00
Clump	1.13	0-3	-0.04	-0.03	-0.01	-0.04
Temperature	19.24	11-24	-0.04	-0.02	-0.05	0.01
Concentration	54.15	16 -122	0.03	0.04	-0.01	0.02
%intact DNA (diffquick stain)	88.03	36-98	-0.05	-0.07	0.01	0.10
%intact DNA (Aniline Blue)	92.43	79-98	-0.07	-0.09	0.02	0.06
%intact DNA Acridine orange)	90.23	26-100	0.08	0.07	0.05	-0.02

3.3. Sperm Chromatin Assessments

Acridine orange (AO) was significantly correlated with Aniline blue (AB) and Diff Quick (DQ; both $P<0.05$), and AB with DQ ($P<0.01$). None of the DNA procedures was significantly associated with %N or %L/D, and neither AO nor AB were significantly associated with %HD (all $P>0.05$). Both AB and DQ were significantly negatively associated ($P<0.01$) with proximal droplets (%PD) and DQ ($P<0.05$) with distal droplets (%DD). DQ was the only DNA

measure which was linked with fertility outcomes; total pigs born (P=0.05) and % stillborn pigs (P=0.005).

Means (±SDs) for sperm assessments are provided below in Table 10.

Table 10 - Means (±SDs) for sperm assessment traits

Trait	%AO	%DQ	%AB	%LD	%N	%Hd	%PD	%DD
Mean	90.13	93.77	92.95	78.77	68.12	8.81	2.18	3.44
SD	12.29	7.5	5.31	11.08	17.08	7.41	3.99	3.88
SEM	1.07	0.66	0.52	0.97	1.49	0.65	0.35	0.34
n	131	131	105	131	131	131	131	131

Legend: AO = acridine orange; DQ = DiffQuik; AB = Aniline Blue; LD = live/dead; N = normal sperm (all % "Normal"). Hd = sperm head abnormal; PD = proximal droplets; DD = distal droplets

Although AO, DQ and AB provided similar outcomes, these did not predict the sperm assessment parameters of %N and %Hd (although both AB and DQ were negatively associated with PD or DD or both). This may indicate that tests of sperm DNA status may complement routine semen assessments. DQ was the only DNA status measure to reflect a mating outcome, i.e. % stillbirths; a trait which could be influenced by sperm DNA integrity. (It should be noted that AO approached significance for this trait also). As the % of sperm with intact or condensed chromatin (DNA) as shown by Diffquik stain increases by 1% the number of stillborn piglets born decreases by a multiple of approximately 0.986, this equates to an approximate 1.4% decrease in stillborn piglets.

3.4. Sow return to oestrus after insemination

Return categories were classed as outlined in Table 11 below.

Table 11 - Return type classification

Return Type	Days to outcome	Number of records	Probability of being in each category
Regular	0-18d	586	0.0302
Late	19-23d	743	0.2148
Early	24-35d	89	0.2525
Calcification of foetal skeleton	36-45d	595	0.1109
Irregular	>46d	235	0.3916

Odds of moving from one category to the next:

1) Increases by a factor of 1.033 ($e^{0.0326}$) as Percent Intact Acrosomes (PIA) increases. PIA range was 54-99%
2) Increases by a factor of 1.396 ($e^{0.3335}$) as % defective acrosomes increases. Range was 0-12%
3) Increases by a factor of 1.004 ($e^{0.0044}$) as curvilinear velocity (VCL) increases. Range was 70.9-249.8
4) Decreases by a factor of 0.6244 ($e^{-0.4709}$) as clump increases. Range was 0-3
5) Decreases by a factor of 0.6794 ($e^{-0.3866}$) as bacteria increase. Range was 0-2.5

Dam line interacted with return type (P<0.05) while parity approached significance at 10% confidence interval. There were no significant interactions between return type and other semen parameters, sire line or insemination season.

Sperm traits significantly associated with Return Time were abnormal head % and abnormal acrosome % (P = 0.007 and 0.001 respectively) with predicted values as below.

Table 12 - Predicted return type values from linear mixed model

Return Time	Abnormal Head %	Abnormal acrosomes %
0-18d	11.65 ± 1.72^{b}	0.93 ± 0.45^{ab}
19-23d	6.79 ± 0.77^{a}	0.25 ± 0.18^{a}
24-35d	7.67 ± 0.80^{ab}	0.41 ± 0.18^{ac}
36-45d	7.27 ± 1.06^{ab}	1.12 ± 0.26^{bc}
>46d	8.32 ± 0.73^{ab}	0.94 ± 0.16^{b}

Different rankings within a column indicate significant differences (Tukey's multiple comparison at 5% significance level)

Dam line significantly (P< 0.05) influenced oestrus return time.
When semen traits were subject to ordinal logistic regression;% Intact Acrosomes, % Abnormal Acrosomes, Curvilinear Velocity, Clump and Bacteria Scores all influenced (P<0.05) sow return category.

Table 13 - ANOVA for the base model RETURNTYPE _ SIRELINE + PARITY + DAMLINE + INSEMINATIONSEASON

	LR Chisq	Df	Pr(>Chisq)
Parity	21.54	9	0.0105
Sire line	12.43	7	0.0872
Dam line	16.08	14	0.3084
Insemination season	2.33	3	0.5065

Only dam line is significant (P<0.01046) while the other variables are non-significant.

Table 14 - ANOVA for sperm traits and sow returns

Semen Parameter	Wald Statistic	Pr(Chisq)
Percent intact acrosomes	25.468	0.000
% defective acrosome	15.811	0.003
% Normal (determined by DiffQuick stain)	8.943	0.063
pH	7.782	0.100
Intact membranes	7.652	0.105
Acridine Orange	7.024	0.135
Proximal droplet	5.958	0.202
Loose heads	5.157	0.272
Beat Cross Frequency	4.985	0.289
% defective midpieces	4.948	0.293
Clumps	4.428	0.351
Aniline blue	3.943	0.414
Distal droplets	3.755	0.440
% Normal (DIC)	3.612	0.461
% abnormal tails	2.996	0.559
Concentration	2.888	0.577
Temperature	2.600	0.627
VSL	2.504	0.644
VCL	1.652	0.799
Tmotile	1.493	0.828
Bacteria	1.480	0.830

Semen Parameter	Wald Statistic	Pr(Chisq)
VAP	1.426	0.840
% abnormal heads	1.048	0.902

There are two semen quality traits that have a significant relationship with the return type. They are DQPIA, and ACRO, having p-values of < 0.001 and 0.003.

3.5. Storage Experiment (Effects of time and temperature on semen characteristics)

Table 15 below shows the initial sperm parameters and sperm parameters after 4 days of storage at 15ºC or 20ºC.

Table 15 - Effects of time and temperature on sperm traits.

Sample	tMot %	pMot %	rMot %	pH	VAP	VSL	VCL	BCF	Clump
Initial (18.7 ±0.2°C)	57.7 ±2.4	29.3± 1.6	43.9± 2.3	7.45± 0.02	75.5± 1.6	43.5± 0.9	154.4± 2.8	33.8±0 .3	1.4±0.05
Stored (15 ± 0.2°C)	44.7 ±2.5	20.8± 1.5	31.9± 2.2	7.7±0 .02	69.8± 2.04	39.6± 1.0	146.3± 3.8	31.7±0 .5	1.4±0.05
Stored at 20.4 ±0.1°C	53.8 ± 2.5	25.5 ±1.5	41.3± 2.3	7.7±0 .02	75.9± 2.1	42.1± 1.07	159.9± 3.8	30.5±0 .5	1.3±0.05

tMot=total motility; pMot=progressive motility; rMot=rapid motility; VAP=mean velocity; VSL=straight line velocity; VCL=curvilinear velocity; BCF=beat cross frequency ('head wobble').

1) Both storage at 20°C and 15°C for 4d resulted in a rise in pH (7.7±0.02; P<0.01).
2) Both resulted in declines (P<0.01) in a number of motility parameters (Total, Progressive, Rapid and BCF) as well as for VSL (P<0.05
3) For semen stored at 15°C, declines also occurred in VCL and VAP (both P<0.01).
4) Clump score was not influenced by storage time or temperature.

Mating outcomes:

i. There was no difference in sows farrowing vs. sows that returned to oestrus or aborted when inseminated with semen stored at 17°C for 1,2 or 3 days after collection.
ii. Litter size was affected by the interaction of storage time & insemination season (P<0.05)
iii. Stillbirths influenced by the interactions of dam line & semen storage time (SST) and parity & SST (P<0.01)
iv. Total motility positively influenced % live piglets born (P<0.05)
v. Total and progressive motility both had a negative effect on % stillborn piglets (P<0.05)

3.6. In Vitro Fertilisation

Analyses were conducted on IVF trials using 36 oocyte batches (i.e. 9 batches of oocytes divided over 4 tissue culture wells). Each well was fertilised with sperm from a different boar.

There were 2 significant correlations between semen parameters and IVF outcome vizs; %abnormal sperm tails with % unfertilised oocytes (r=0.4204, P=0.0107) and sperm DNA damage (r=-0.4329, P<0.05). Not surprisingly, ovary temperature on arrival at the lab (up to 3.45 hours after the first sow was slaughtered) was correlated with both %fertilised

oocytes) and %unfertilised oocytes (r=0.4329, P<0.05). The %degenerated oocytes at day 3 post-fertilisation was correlated with DNA damage as recorded with DiffQuick staining (P = 0.034). This reinforces the finding that sperm which have DNA damage (detected with AO) were significantly associated with stillbirths.

One way ANOVA revealed that there were significant interactions (P<0.01 for all) between the combination of %fertilised oocytes, %degenerated oocytes, %morulas, %blastocysts and the individual semen parameters: total motility, %normal morphology, %proximal droplet, %distal droplet, %intact membranes, %intact DNA (Diffquick stain), %abnormal heads, %abnormal midpieces, %abnormal tails, semen pH, accessory sperm bound to the zona pellucida and also ovary temperature on arrival.

3.7. Ubiquitin

The free ubiquitin assay performed showed highly significant (P<0.001 0r 0.01) relationships with a number of semen test parameters (Sperm motility, concentration, PIA and clumping) and significantly (P<0.05) with MID (%N approached significance at P=0.06).

4. Application of Research

The findings from these studies will be best applied in terms of improved QA, monitoring and tests applied in commercial boar AI centres. Such improvements should result in cost-effective increases in P/S/Y. Appropriate extension and education programs could be initiated to facilitate this process.

As shown in this study, sperm DNA damage is detectable with relatively simple and inexpensive tools. The finding that sperm DNA damage is associated with a number of loss categories in pig breeding provides incentives to find mitigating or remedial factors.

5. Conclusions

Fertility data

This project has shown that although dam line, parity and season contribute to embryonic loss or conception failure, the boar (as well as sire line) makes a significant contribution to reproductive losses. In particular, sperm morphology, motility, pH, membrane integrity and chromatin condensation are all linked to various degrees to litter size, pigs born alive and stillbirths as well as sows returning to oestrus (conception failure and embryonic loss). An encouraging aspect is that attention to QA (eg shipping temperatures) can produce benefits in terms of fertility.

Sperm Chromatin damage assessment

This work on chromatin damage detection methods further indicates that Diff Quick (DQ), a relatively inexpensive and rapid procedure, shows promise as a potential routine procedure for assessing the DNA status of boar sperm, although further work is indicated. DQ is a common stain used in veterinary practice for haematology and cytology. It therefore represents a useful and easily applied stain to use for both sperm morphology and sperm chromatin damage detection. In addition, DQ is also a useful stain for detecting bacterial contamination as bacterial colonies are readily stained by DQ.

Sow Returns

As acrosomes are necessary for successful sperm binding to the ovum (Waberski et al., 2006), it is logical that abnormal acrosomes would be linked with sow returns, although predictive values in this study are not high. Several sperm head abnormalities are similarly associated with failure of fertilization, although others have been linked with post-fertilization loss (Fatehi et al., 2006). In this study, semen factors (defective acrosomes and head defects) were linked with sow returns associated with both failure of fertilization and early pregnancy loss. Sperm factors affect the probability of sows returning to oestrus with early embryonic death also being more likely with increased

clump score and bacterial contamination. Dam line was also shown to be highly significant in sow returns suggesting a genetic disposition towards pregnancy failure.

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Semen storage experiment

Although qualified by the fact that only 1 extender was used in this study, the findings indicated the following;

a) Fertility was not affected by storage time (either 1,2 or 3d) at 17°C (at the boar stud)
b) In semen stored at either 15 or 20^{0}C for 4 days, decreases in sperm measures such as motility were associated with decreased % live and % stillborn pigs. This effect was more evident in semen stored at 150C.

In vitro fertilization trials

A critical finding was that sperm DNA damage was linked with oocyte degeneration. This experiment also demonstrated that ovary temperature was critical in oocyte preservation which then had an effect on fertilisation success. Further analyses are required to link the IVF data to in-vivo mating results as time constraints mean that sows inseminated with the same batches of semen used for IVF have not yet farrowed. It will be interesting to find out the association between IVF results and in-vivo fertilisation and these findings will be made available once these are made available by the boar stud. Although there were some significant correlations, a larger dataset would have been preferable for data analyses.

Sperm chaperone protein ubiquitin

At this stage, the conclusion is that the assay for free ubiquitin shows considerable promise as a biomarker for damaged boar sperm. However, results are not yet available for fertility data associated with these findings.

6. Limitations/Risks

Although these trials benefited from a number of aspects including access to complex technologies as well as detailed breeding data, there are limitations to interpretation of the results. These include the facts that genetics are limited to those of a particular pig breeding organization and that only 1 semen extender was represented. Despite these caveats, it is considered that the amount and type of data presented, and their interpretation, advances current knowledge in several relevant areas which include the identification of important biomarkers for sperm damage and the potential effects of such damage on early pregnancy loss in pigs.

A number of procedures used in these trials are not appropriate for routine industry usage, including IVF techniques and proteomics, for the reasons below.

In-vitro fertilisation (IVF) is a great research tool but limited in practical pig industry use due to, 1. high cost of setting up an IVF facility, 2. expense and labour requirements and 3. lack of local sources of pig ovaries. This latter consideration is exacerbated by the fact that gilt ovaries are not suitable for IVF.

Similarly with proteomic procedures. Despite the fact that the presence and concentration of ubiquitin in sperm can be used as a fertility biomarker in a research setting, the procedures involved are expensive, highly technical and time consuming. Ultimately, rapid, simple and inexpensive technologies will be developed for such tests. However, more research is needed to determine a) which proteins are most likely to be predictive of semen fertility, and b) how to detect them both qualitatively and quantitatively using quick and cheap assays.

Finally, as with any such large data set, there will be ongoing analyses to probe further insights. For example, genetic and genetic-environmental interactions will be studied for a number of traits.

7. Recommendations

The following recommendations are made based on the results of these studies:

1) Quality assurance should be reinforced and maintained in boar studs. This should include the use of best laboratory practices in determining boar semen motility and concentration, as well as in preventing contamination.
2) Greater awareness of the importance of appropriate (17-20^{0}C) temperature maintenance in shipped semen. This could be facilitated by improved designs of semen shipping containers.
3) More emphasis needs to placed upon the routine morphological monitoring of boar sperm. This should include awareness of the significance of defects such as retained cytoplasmic droplets.
4) Routine assessment of sperm DNA/Chromatin integrity should be encouraged, especially as simple and rapid techniques (eg DiffQuik staining) are now available.
5) Educational programs should be established to facilitate all of the above.

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