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Oral Communications

Session 1

OC 1

Freezing–thawing induces alterations of the nucleoprotein–DNA binding through the breaking of disulfide bonds in boar sperm

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Previous reports proved that freezing–thawing alters the main nucleoprotein structure in boar sperm. The main aim of this work was to gain insight in the mechanisms, by which freezing–thawing induces such alteration. For this purpose, the alteration of freezing–thawing on the two main molecular mechanisms that modulate the protein–DNA linking, protein tyrosine phosphorylation and protein–DNA disulfide bounds was investigated. Protein tyrosine phosphorylation was analysed through Western blot analyses in samples previously subjected to immune-precipitation. Furthermore, the putative changes on disulfide bounds were studied through analysis of free cysteine radical levels. Freezing–thawing did not have any significant effect on the tyrosine phosphorylation levels of both protamine 1 and histone H1. However, thawed samples showed a significant ($p < 0.05$) increase in the free cysteine radical content (from 3.1 ± 0.5 nmol/μg protein in fresh samples to 6.7 ± 0.8 nmol/μg protein). In summary, our results suggest that freezing–thawing induces significant alterations in the nucleoprotein structure of boar sperm head by mechanism(s) linked with the rupture of disulfide bonds among the DNA. These mechanisms seem to be unspecific, affecting both the protamine–DNA unions and the histones–DNA bonds in a similar way. This work has been supported by Grant AGL2008-01792GAN, Ministerio de Ciencia e Innovación, Spain.

OC 2

Cryopreservation of boar semen does not modify the regulation of GSK-3 mediated sperm motility

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Sperm cryopreservation affects the intracellular events involved in sperm functions. Glycogen synthase kinase-3 (GSK-3) is a serine/threonine kinase, which enzyme activity is regulated by serine phosphorylation. In fresh porcine semen a significant positive correlation among several velocity parameters and GSK3α serine phosphorylation levels exists. Moreover, inhibition of GSK-3 activity by alsterpaullone leads to a significant increase in all sperm velocity parameters and coefficients. The aim of this study was to determine if cryopreserved boar semen might be more sensitive to inhibitor of GSK-3 activity. In total 18 ejaculates from six Iberian boars of proven motility and morphology were processed using the straw freezing procedure. After thawing, semen samples were incubated at 37°C for 30 and 90 min in presence or absence of 30 μM alsterpaullone. We evaluated motility parameters and the percentage of total motile spermatozoa by a computer-aided sperm analysis system. Viability (SYBR-14/PI), acrosomal status (FITC-PNA/PI), membrane fluidity (M-540/YoPro-1) and mitochondrial membrane potential status (JC-1) were evaluated by flow cytometry. In general, alsterpaullone addition caused an increase of all motility parameters (except for ALH) and coefficients and the percentage of motile spermatozoa. With regard to flow cytometry results, alsterpaullone treatment significantly reduced the percentage of spermatozoa with high mitochondrial membrane potential at 90 min incubation. These results demonstrate that frozen–thawed boar sperm can still regulate their motility through GSK-3 signalling pathway. Supported by JUEX PRI09A077 & GR10156, MICINN AGL2010-15188.

OC 3

EGTA-containing thawing extender improves the fertilization ability of cryopreserved boar sperm

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Cryo-capacitation during thawing process decreases the sperm motility and fertilization ability of frozen–thawed boar sperm. We reported that the addition of 10% (v/v) seminal plasma to thawing solution suppressed the cryo-capacitation, which resulted in a high success rate of artificial insemination (AI) (Okazaki et al., 2009, Theriogenology, 71, 491–498). To clear the positive effects of seminal plasma during thawing process, we focused on the increase of intercellular Ca^{2+} in sperm. The sperm-rich fraction was collected weekly from each boar using the gloved-hand technique. The seminal plasma was removed just after collection by centrifugation and was then frozen as described in our previous study (Okazaki et al., 2009). When the frozen–thawed sperm were incubated with Fluo-3/AM to determine the level of intercellular Ca^{2+}, the level of Ca^{2+} was increased in a time dependent manner, and the capacitation judged by tyrosine phosphorylation of sperm protein by Western blotting (Shimada et al., 2008 Development, 135, 2001–2011), was also induced in post-thawed sperm. The addition of EGTA to thawing solution significantly suppressed both the increase of Ca^{2+} level and the induction of cryo-capacitation. Moreover, the treatment increased the *in vitro* fertilization rate similar to IVF with sperm and in presence of seminal plasma. Furthermore we froze semen from Landrace, Large White, Duroc and Berkshire boars to examine whether the novel thawing extender is suitable for commercial use for different breeds. The motility and morphology of frozen–thawed sperm were not significantly different among the four breeds. Moreover, when the frozen–thawed sperm was used for AI (total 302 sows), the novel EGTA-containing extender

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significantly improved the implantation rate and litter size of sows as compared to AI without EGTA (implantation rate; 81% vs. 42%, litter size; 10.6 vs. 8.3), and the rates were similar to those of fresh semen AI. From these results, we concluded that EGTA-containing extender is a beneficial tool for AI with frozen–thawed boar semen.

OC 4

Negative effect of glycerol on the peri-nuclear theca of boar sperm

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Glycerol is the permeable cryoprotectant most commonly used for sperm cryopreservation. However, it has a detrimental effect on the cytoskeletal proteins. It is well known that the porcine sperm is highly sensible to glycerol toxicity. Therefore, freezing extenders contain $<$ 4% glycerol. Recent studies have shown important alterations of the peri-nuclear theca sub-structure (sPT) of boar sperm. The aim of this study was to evaluate the effect of two glycerol concentrations on the peri-nuclear theca (PT). Six ejaculates of three boars were used. In order to expose the PT, sperm samples were incubated with Brij 36-T detergent to remove the plasma and acrosomal membranes. In groups 1 and 2 samples were supplemented with 2% and 4% glycerol. A third group contained no glycerol (control group), and the fourth and fifth groups were supplemented with glycerol and with protease inhibitors (PI). All groups were incubated for 10 and 30 min. Thereafter, samples were fixed and processed for negative staining and evaluated by transmission electron microscopy (TEM). Before treatment the PTs appearance was preserved intact, showing a belt with a continuous and repeated pattern of chain links or frets, just below the equatorial region of sperm head. After 10 min of exposure to treatment, in the samples added with 2% and 4% glycerol, the PTs integrity was significantly altered ($p < 0.05$), loosing the continuity of the fret pattern in 72% and 70% respectively. However, when samples were maintained up to 30 min, the loss was 74.7% and 72%, respectively. Groups with PI treatment resulted in 71.3% and 74% loss of chain link continuity. The present study shows for the first time the adverse effects that glycerol exerts on morphological integrity of sperm PT, which could be one of the elements that negatively inducing low fertility of cryopreserved semen, since PT not only protects the paternal genome against the action of decondensing agents, but also participates dynamically during different fertilizing processes.

OC 5

Field fertility of frozen boar sperm: a retrospective report spanning 2007 to 2010 comprising over 2000 AI services

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Worldwide, $>$ 90% of sows are inseminated with fresh semen. Less than 1% are bred using frozen semen (Wagner 2000, Proc 14th Int Congress on Anim Reprod, Vol 2). Albeit, frozen semen is still leveraged as an effective technology for the transfer of genes between breeding pyramids and also to reliably provide semen for planned matings. Little information exists on the long-term use of frozen boar semen at the commercial level. Our objective is to highlight the successful application of frozen semen over the course of a 4-year period comprising over 2000 AI services. The frozen semen sourced from a boar stud in Manitoba Canada. All AIs occurred on a single 1800 sow farm in Indiana, USA. The sperm-rich fraction was collected and only those collections having $>$ 80% motility and $<$ 15% abnormal sperm were further processed. Semen was prepared for cryopreservation using Androhep® CryoGuard™, packaged in 0.5 ml French straws (average 500 million total sperm per straw) and frozen using a programmable freezer (IceCube™). For each frozen ejaculate, a post-thaw quality check was performed. Ninety-eight per cent of the ejaculates that were frozen showed at least a 50% post-thaw motility and were approved for shipment. For AI, eight straws were thawed and diluted with 60 ml of extender pre-warmed to 26°C. Within 2–5 min of thawing, the sows or gilts were inseminated via intra-cervical deposition using a standard AI pipette. Sows and gilts were inseminated three times PM/AM/PM and AM/PM/AM, respectively. Of 2074 recorded services, 1527 (73.6%) of the females farrowed a litter. The mean ($\pm$SD) total number piglets born (minimum and maximum), was 11.8 $\pm$ 1.1 (1 and 23). Comparatively, fresh AI fertility records on the same farm over a shorter time period demonstrated 1113 out of 1326 females farrowing a litter (83.9%) and 12.4 $\pm$ 1.7 mean ($\pm$SD) total number of piglets born ($p < 0.05$). We observed a progressive improvement of fertility over time (from 50% to 55% farrowing rate up to 80–85% in 2 years) due to adoptive procedures associated with an introduced technology. In summary, good fertility is possible with frozen semen and it has application as a reproductive management tool.

OC 6

A simple post-thawing quality threshold can improve *in vivo* fertility of frozen boar semen

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Despite regular technical improvements, extensive use of frozen boar semen is limited by its low and variable quality compared to fresh semen, and by the lack of predictors of *in-vivo* fertility. A trial was undertaken to evaluate the relationships between post-thawed quality and fertility after on-farm inseminations. Semen was collected at 4–6 occasions, on 10 Large White boars from the AI Center of the INRA-UEICP. A total of 50 fresh ejaculates were evaluated for microscopic subjective sperm motility (score 0–5), percentage mobile sperms and concentration. Irrespective of quality, ejaculates were frozen in 0.5 ml straws (800×10^6 total sperms/straw). Quality (microscopic evaluation of abnormalities, eosin–nigrosin test of viability) was checked on one straw per ejaculate at 10 and 120 min post-thawing. A total of 54 weaned Large White sows were cervically inseminated with frozen–thawed semen (five straws, 75 ml BTS), 12 and 24 h after detection of standing estrus, on parities 2–5. Relationships between semen quality and reproductive outcomes were finally investigated on 161 inseminated estruses. On these unselected ejaculates, pre-freezing mobility (85.8 $\pm$ 2.8%, range 80–90) and motility scores (3.7 $\pm$ 0.3, range 3–4) were in accordance with usual minimum quality criteria, but did not predict reproduction results with frozen semen. Pre and

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post-thawed quality criteria did not correlate, and reproductive performances were independent from sperm evaluations performed at 10 min. At 120 min, post-thawed mobility (49 ± 10%, range 20–70), and normal live sperms (16.7 ± 9.9%, range 1–38) were better related to conception rate ($p = 0.11$ and $p = 0.04$ respectively), live born ($p = 0.01$ and $p = 0.05$) and total born ($p = 0.02$ and $p = 0.10$). Inseminations with frozen semen having < 10% normal live sperms at 120 min (n = 50 vs. n = 111) had significantly lower conception rates (68% vs. 84%, $p = 0.026$), with fewer live born (8.7 vs. 10.5, $p = 0.006$) and total born (9.5 vs. 11.5, $p = 0.007$). After confirmation on additional data, this threshold value may be of practical interest for low-cost identification of high quality frozen boar semen.

Session 2

OC 7

Changes of sperm motility, membrane integrity and lipid peroxidation during liquid storage of boar semen

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The aim of this study was to examine the dynamics of three sperm parameters: sperm motility, membrane integrity and lipid peroxidation during 7 days of semen storage. Totally 162 ejaculates collected from 16 boars of three breeds (Large White, Landrace and Pietrain × Duroc) were used. Boars were 7 months to 7 years old. After collection semen was diluted with BTS extender and stored for 7 days at 17°C. Sperm examination was performed on days 1, 4 and 7 of storage. Percentage of motile spermatozoa was estimated under a phase contrast microscope with warm stage. Sperm membrane integrity was examined flow cytometrically using double labelling with propidium iodide and SYBR-14 and percentage of live, moribund and dead sperm was calculated. Lipid peroxidation (as indicator of oxidative damages) was assessed by induced photon emission (luminescence) measurement. Integral parameter (counts/integration time) and Peak max parameter (height of highest peak) were calculated. Mean sperm motility ranged from 60.0% (±1.6) at 1 day to 53.1% at 4 day and 48.8% (±1.6) at 7 day with statistically significant differences (ANOVA, $p < 0.01$) between 1 and 4 day. No differences for mean motility were seen between 4 and 7 days of storage. There were no differences in sperm membrane integrity during storage period. Percentage of live spermatozoa ranged from 81.7% (±1.4) at 1 day to 78.3% (±1.4) at 7 day while moribund spermatozoa: from 9.4% (±0.7) to 11.1% (±0.7) and dead ones: from 8.9% (±0.9) to 10.5% (±1.0) respectively. Oxidative damages measured by photon emission increased significantly during whole storage period. The Integral parameter changed from 15.0 (±0.9) at day 1 to 19.0 (±0.9) at day 4 (ANOVA, $p < 0.01$) and 21.9 (±0.9) at day 7 (4 vs. 7 day, ANOVA, $p < 0.05$). Significant difference was found also for Peak max parameter between day 1 and 7 of storage: 10.8 (±1.6) vs. 19.2 (±1.6) respectively (ANOVA, $p < 0.01$). In summary the significant decrease in sperm motility was observed only in the first 4 days of semen storage while oxidative damages have grown progressively during all 7 days. Surprisingly the sperm membrane integrity was kept on the same level during whole storage period

OC 8

Effects of AMP-activated kinase inhibitor compound C in the quality of extended boar semen after long-term storage at 17°C

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AMP-activated protein kinase (AMPK) acts as a sensor that detects the cell energy state and consequently regulates energetic metabolism. Semen storage for long-term at 17°C is accompanied by intense metabolic and energetic changes in the spermatozoa. Therefore, our objective was to study AMPK expression in boar spermatozoa and the effect of the AMPK specific inhibitor "Compound C" (CC) in extended semen after long-term storage at 17°C. Identification of AMPK and its phosphorylated form (catalytically active) were analysed by Western blotting. Sperm motility parameters were evaluated by computer assisted ISAS® program and germ cell viability, plasma membrane fluidity (PMF), acrosome reaction (AR), and mitochondrial membrane potential (MMP) by flow cytometry in sperm samples from boar ejaculates or from extended semen at days 1, 2, 4, 7 and 10. Our results show that AMPK is expressed in boar spermatozoa at high level and that the storage of semen at 17°C significantly decreases AMPK phosphorylation (activity). AMPK inhibition with CC (30 μM) in spermatozoa significantly increases PMF and AR after 4, 7, and 10 days of semen storage. However, AMPK inhibition has no observable effects neither on sperm viability nor MMP. Moreover, AMPK inhibition during semen storage decreases sperm velocity characteristics and the percentage of hyperactivated spermatozoa. However, a more lineal movement of spermatozoa is stimulated. Our results suggest that AMPK is involved in the regulation of sperm function during storage of extended boar semen at 17°C. Supported by JUEX PRI09A077 & GR10156 and by MICINN AGL2010-15188.

OC 9

Incidence of sperm with cytoplasmic droplets in boar ejaculates is associated with reduced responsiveness to bicarbonate *in vitro*

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The aim was to test whether the ability of spermatozoa in diluted boar semen samples being responsive to bicarbonate under capacitating conditions *in vitro* is linked to other sperm parameters. Therefore, 78 semen samples diluted in Beltsville Thawing Solution (BTS) from 13 different boar studs were examined at 24 h of storage for motility (CASA) and morphology. The integrity of plasma and acrosome membrane was flow cytometrically assessed with propidium iodide (PI) and FITC-PNA. Kinetics of changes in cytoplasmic Ca^{2+}-concentration in the plasma membrane intact sperm population were monitored with PI and Fluo-3/AM within 60 min incubation at 38°C in two variants of Tyrode's medium, containing either bicarbonate and calcium (medium A; at 5% CO_2) or only calcium (medium B). Specific response to

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bicarbonate (R_{60Bic}) was defined as the difference in the decline of % PI- and Fluo-3 negative sperm within 60 min of incubation in medium A and B ($R_{60Bic} = \Delta_{A3-A60} - \Delta_{B3-B60}$). Despite considerable variability, there were neither significant correlations between the specific response to bicarbonate (range: 3.4–69.3%) and the percentage of total or progressive motile sperm (70.9–98.4% and 42.4–96.7%, respectively), nor with the percentage of PI- and FITC-PNA-negative sperm (39.8–98.3%; $p > 0.05$). However, a negative correlation existed between the specific response to bicarbonate (R_{60Bic}) and the number of morphological abnormal sperm per sample (1.5–54.0%; $r = -0.72$; $p < 0.001$). Sperm with cytoplasmic droplets (0.5–37.0% per sample) accounted for the most frequent observed defect (47.3%) of all abnormal sperm. Correlation of R_{60Bic} was negative with respect to this specific defect ($r = -0.68$; $p < 0.001$). In conclusion, semen samples with higher incidence of sperm with cytoplasmic droplets show a reduced responsiveness to bicarbonate, whereas other sperm parameters are not related to the bicarbonate response. As retained cytoplasmic droplets are considered as a sign of immaturity, incomplete sperm maturation seems to be associated with impaired membrane function affecting the signalling cascade of the capacitation process.

OC 10

Expression analysis of porcine aromatase (CYP19) as a specific target gene in testis

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Cytochrome P450 aromatase is the key enzyme in estrogen biosynthesis, encoded by the *CYP19* gene. However, little is know about the *CYP19* roles in boar spermatogenesis. Therefore, the aim of this work was to investigate the mRNA and protein expression of CYP19 in boar reproductive tissues from boars with different sperm quality. For mRNA and protein expression study, a total of six boars were divided into two groups with Group I (G-I) and Group II (G-II), where G-I is characterized for a relatively better sperm quality. For the expression study between reproductive and non-reproductive tissues by semi-quantitative PCR study, mRNA from all six boars was pooled together according to the tissues. On the other hand, mRNA and protein expression study in different reproductive tissues from two divergent groups of animals were performed by semi-quantitative PCR, qRT-PCR and western blot, respectively. Due to the limitations of fresh samples from G-I and G-II boars, different fresh testis from a healthy breeding boar was collected after slaughtering for protein localization by immunofluorescence. The remarkable *CYP19* mRNA expression was detected only in testis. The mRNA expression of *CYP19* was not detectable in other reproductive tissue (epididymis and accessory glands) and non-reproductive tissue (brain, liver and muscle) by semi-quantitative PCR. When mRNA expression in reproductive tissues from G-I and G-II boars was compared by semi-quantitative PCR, the *CYP19* was detectable in testis for G-I and G-II boars. However, mRNA and protein expression were not differentially regulated between G-I and G-II boars ($p > 0.05$). The CYP19 protein was detected in testis from G-I and G-II boars. This protein expression result of western blot appeared to be consistent with results of the qRT-PCR. The CYP19 protein localization observed a strong staining in the cytoplasm of Leydig cells in testis. The mRNA and protein expression study of the CYP19 imply that it might have a role in spermatogenesis in pigs, which is specifically expressed in boar testis. However, association study and more functional study in boars with extreme divergent phenotypes are required.

OC 11

Characteristics of boar semen stored at different temperatures

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Purpose: To evaluate storage and temperature effects on extended boar semen characteristics and fertility outcomes.
Methods: Extended semen (143 ejaculates/76 boars) were examined within 1 day of collection and after 4 days storage at 20.4 ± 0.1°C (RT) or 14.7 ± 0.2°C (CT). Fertility data (total, live, mummified, stillborn pigs) was derived from 535 inseminations (sow parity range 1–8) using same-batch semen (41/76 boars) stored at 17°C for 0–4 days at stud. Evaluations included CASA (IVOS) sperm motility (total, progressive, rapid, VCL, VAP, VSL, BCF), sperm clump score (0–3), semen pH and temperature. Data were analysed by one-way ANOVA, GLM (stillbirths) and linear regressions (total born and born live) plus chi-square analysis for sow returns and farrowings. Inseminations were performed across four seasons.
Results: Initial (receiving) values were: concentration $49.3 \pm 1.4 \times 10^6$/ml; temperature 18.7 ± 0.2°C; clump score 1.4 ± 0.05; pH 7.45 ± 0.02; motilities: total 57.7 ± 2.4%; progressive 29.3 ± 1.6%; rapid 43.9 ± 2.3%; VCL 154.4 ± 2.8; VAP 75.5 ± 1.6; VSL 43.5 ± 0.9; BCF 33.8 ± 0.3. Both RT and CT storage for 4 days resulted in a rise in pH (7.7 ± 0.02; $p < 0.01$) and similar ($p < 0.01$) declines in a number of motility parameters (total, progressive, rapid and BCF) as well as for VSL ($p < 0.05$). For CT, declines also occurred in VCL and VAP (both $p < 0.01$). Clump score was not influenced by storage time or temperature. The proportion of sows farrowing vs. returns was similar for semen stored for 1, 2 or 3+ days. However, storage time and season interacted with litter size ($p < 0.05$), as did storage time, dam line, and parity with % stillborn (both $p < 0.01$). Total sperm motility positively influenced % live pigs and both total and progressive motility negatively influenced % stillborn (all $p < 0.05$).
Conclusion: Storage of extended semen for 4 days at approximately 15 or 20°C resulted in similar decreases in CASA sperm motility assessments. Overall fertility was not influenced by storage time (0–4 days at 17°C). Despite this, sperm motility measures (total and progressive) influenced % live pigs and % stillborn and storage time interacted with both season and sow factors re. litter size and % stillborn. This research was made possible by financial assistance from Pork CRC Australia.

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OC 12

Post-epididymal maturation of boar spermatozoa

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Sperm fertility is progressively acquired during maturation in the genital tract. It has recently been demonstrated that hypotonic resistance of porcine ejaculates is positively correlated with its *in vivo* fertility (Druart et al., 2009, Reproduction, 137, 205–13). Hypotonic resistance of ejaculated spermatozoa is lower than that of epididymal spermatozoa (EpidSPZ) most probably due to biochemical interactions of seminal plasma (SP) components with spermatozoa. The aim of the present study was to analyse the effect of SP on hypotonic resistance of boar EpidSPZ, and to identify by mass spectrometry those SP proteins that interact with spermatozoa. For this purpose, SP was collected from several boars with known fertility and stored. EpidSPZ (n = 3 boars) were recovered from cauda epididymis by micro-perfusion and then incubated in the presence of saline or SP for 10 min at 37°C before being assessed for hypotonic resistance by flow cytometry, and analysed by Intact Cells Matrix Mass Spectrometry (ICM-MS) that allows to achieve direct protein profiling. Our results showed that hypotonic resistance of three populations of EpidSPZ displayed similar profiles, in relation to SP, in which they had been incubated. Thus, two SP that induced the lowest, and two other that induced the best hypotonic resistance to EpidSPZ were selected (SP low and SP high, respectively). EpidSPZ incubated with SP high or SP low displayed different ICM-MS profiles, some molecular species being increased and other decreased (or absent) between the two SP groups. Identification of these peptides/proteins demonstrated that several sperm adhesines at least are involved. In conclusion, our results demonstrate that hypotonic resistance induced by SP to EpidSpz is related to their ICM-MS profiles, and that this approach may be useful to identify novel biomarkers of fertility in the boar.

Session 3

OC 13

Actin cytoskeleton and tyrosine phosphorylation in sex-sorted boar spermatozoa

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Sex sorting procedure is believed to induce changes in sperm that could be responsible for reducing the overall quality of sexed semen. The aim of this work was to verify by indirect immunofluorescence technique whether the process is responsible for capacitation-related changes in actin cytoskeleton polymerization and in protein tyrosine phosphorylation (PTP). We studied the distribution of separate sperm subpopulations through immune-reactive pattern in fresh (F) capacitated (Cap) and acrosome reacted (AR) sperm and compared these results with those observed in sexed spermatozoa. As for actin, three different patterns (A, B and C) were observed. A was mainly observed in F (A 91.8 ± 0.7%; B 5.8 ± 0.5%; C 2.4 ± 0.2%, mean ± SEM), B in Cap (A 21.3 ± 6.3%; B 70.4 ± 5.1%; C 8.3 ± 1.2%) and C in AR (A 5.1 ± 0.8%; B 24.5 ± 2.3%; C 70.5 ± 3.8%) sperm. In sexed sperm, the most expressed pattern was A (61.4 ± 8.6%), while B and C were 28.9 ± 8.6% and 5.8 ± 1%, respectively. No significant differences were observed between B and C patterns in sexed and F sperm, while A pattern was significantly different ($p < 0.05$) from that observed in F, Cap and AR sperm. As for PTP, we identified three patterns a, b and c. "a" pattern was typical of F cells (a 88.2 ± 3.7%; b 9.2 ± 3.1%; c 2.6 ± 1%), "b" was typical of Cap sperm (a 19.4 ± 5.4%; b 68.9 ± 5.8%; c 11.7 ± 0.4%) and "c" was mainly present in AR sperm (a 2.9 ± 0.7%; b 17.8 ± 2.7%; c 79.6 ± 2.1%). In sexed sperm (a 80.3 ± 3.3%; b 8.3 ± 3.3% and c 11.5 ± 2.8%) no significant differences in a and b patterns were found when compared with F sperm, while c was significantly higher ($p < 0.05$). We suggest that sex sorting procedure induces some changes in actin cytoskeleton polymerization that could represent a partial capacitative activation, while this kind of activation seems to be lower for PTP. Sexing increases acrosome reacted cells possibly due to mechanical damage rather to a direct stimulation of acrosome reaction. The authors thank Società Italiana Produttori Sementi.

OC 14

Age-related changes in quality and fertility of porcine semen

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The objective of this study was to investigate the influence of boar age on quality traits and fertility of liquid-stored semen. Boars were allocated into three groups. Each group consisted of 5–7 boars aged 7–10 months (young), 18–33 months (mature) or 51–61 months (old). Whole ejaculates of $\geq 0.2 \times 10^9$ sperm/ml and 70% sperm progressive motility were collected by the "gloved hand" technique, extended with a commercial medium to 0.03×10^9 sperm/ml, packaged in 100-ml insemination doses, stored at 17–18°C and used within 12–24 h for artificial insemination (AI) of 2062 multiparous sows in post-weaning standing estrus. Aliquots of diluted semen were assessed for incidence of sperm chromatin instability (SCI; acridine orange staining), proportion of live morphologically normal spermatozoa (LMNS; eosin stain exclusion assay) and head morphometry of LMNS. Data were analysed using two-way ANOVA (boar × boar age), Duncan multiple range test, χ^2 analysis and Pearson correlation coefficient. The results show that the boar was the main source of variation for all experimental end-points. Young boars had higher percentages of SCI and lower proportions of LMNS than those of the mature ($p < 0.05$) and old ($p < 0.001$) ones, respectively. Sperm head dimensions (length and width) of young and old boars were higher ($p < 0.03$–0.001) than those of mature boars. Farrowing rate of young boars (65%) was significantly lower ($p < 0.001$; $\chi^2 = 30$–61) than those of the mature (87.2%) and old (84.7%) ones. The effect of boar age on live litter size was non-significant. The relationship between sperm head dimensions and boar fertility was also non-significant. In conclusion, boar age contributes to the success of AI with preserved semen.

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OC 15

Membrane lipid peroxidation in boar spermatozoa subjected to different handlings

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The membranes of boar spermatozoa are rich in polyunsaturated fatty acids and, therefore, susceptible to lipid peroxidation (LPO) damage. This study aimed to measure LPO, and its relationship with sperm motility and viability, in boar spermatozoa subjected to liquid storage, cryopreservation and sex sorting with subsequent cryopreservation. Lipid peroxidation was assessed by the indirect measurement of the end point generation of malondialdehyde (MDA), using the BIOXYTECH® MDA-586 Assay Kit (OxisResearch, Burlingame, CA, USA). Sperm motility was assessed using a CASA system and viability, in terms of plasma membrane integrity, by cytometry (LIVE/DEAD® Sperm Viability kit). Eighteen sperm rich fractions (SRFs) were collected from six fertile hybrid boars (three SRFs/boar), extended (1:1, v/v) in BTS and transferred to the laboratory. At the laboratory, the extended SRFs were split into three aliquots to be subjected to three different handlings: (1) liquid storage at 17°C during 72 h after re-dilution in BTS to 3×10^7 sperm/ml; (2) cryopreservation at 1000×10^6 sperm/ml in standard 0.5 and 0.25 ml straw protocols; and (3) sex-sorted and cryopreservation at 20×10^6 sperm/ml in a standard 0.25 ml straw protocol. Sperm handling influences MDA generation ($p < 0.01$). In the liquid stored samples, the MDA concentration was influenced ($p < 0.01$) by storage time (from 0 to 72 h), increasing over time (from 17.7 ± 0.9 to 29.2 ± 1.2 μmol/30 × 10^6 cells). Cryopreservation increased MDA generation ($p < 0.01$), without differences among straw size (16.8 ± 0.7; 27.1 ± 0.7 and 26.9 ± 1.4 μmol/30 × 10^6 cells for fresh and thawed samples frozen in 0.5 and 0.25 ml straws, respectively). Sex-sorted and subsequent cryopreservation also influenced the MDA generation ($p < 0.01$). The levels of MDA measured after BTS dilution, staining with Hoechst 33342, sorting and at 30 min after thawing were 15.3 ± 0.9, 15.4 ± 1.1, 7.1 ± 0.9 and 22.4 ± 0.9 μmol/30 × 10^6 cells, respectively. MDA generation was negatively correlated ($p < 0.01$) with sperm motility and viability, showing an overall Spearman's rank correlation coefficients of −0.66 and −0.65, respectively. Supported by MICINN (AGL2008-04127/GAN), Seneca Foundation of Murcia (GERM04543/07) and Sexing Technologies (Texas, USA).

OC 16

Relationship between numbers of inseminated motile boar sperm cells and reproductive performance of sows

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The objectives of this study were to analyse the relationship between the numbers of motile sperm cells per insemination dose and sow reproductive performance and to assess if there are differences between boar lines for this relationship. Commercial AI doses originating from 12 boar lines (TOPIGS breeding company, Vught, The Netherlands) were produced from 2006 to 2010 at 15 Dutch AI stations (Pig AI Netherlands, Deventer, the Netherlands). Semen cell concentration and semen motility were assessed for each ejaculate by CASA (Computer Assisted Semen Analysis) using the UltiMate system (UltiMate™; Hamilton Thorne BioSciences, Boston, MA, USA). The number of inseminated motile cells per ejaculate ranged between 1.2 and 4 billion. Each ejaculate produced was linked with corresponding inseminations, which enabled analysis of the relationship of semen quality with reproductive sow performance (i.e. farrowing rate and litter size) of 440,000 inseminations. Only inseminations originating from one ejaculate were included in the dataset. The relationship between reproductive performance and number of inseminated motile sperm cells for the various boar lines was analysed after adjustment for AI station, age of the boar, age of the insemination dose and inter-ejaculation days by the general linear models procedure of SAS (SAS 9.2 Institute Inc., Cary, NC, USA). Farrowing rate was not related to number of inseminated motile cells between 1.2 and 4 billion, except for two boar lines ($p = 0.007$). For a Landrace line, farrowing rate increased with 4.2% with an increase in number of inseminated motile cells from 1.2 to 4 billion, whereas for a Piétrain line, farrowing rate decreased by 12.3% within this concentration range. Overall, litter size was not related to number of inseminated motile cells ($p = 0.19$) and also no differences between boar lines were found for this relationship. In conclusion, in a Dutch commercial pig AI setting reproductive sow performance is not compromised by lowering the number of motile sperm cells per insemination dose to 1.2 billion for all boar lines, except one Landrace line.

OC 17

The effect of prostaglandin addition to low number of frozen–thawed boar sperm on fertility following PG600® induced estrus

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Frozen–thawed boar sperm (FTS) can aid dispersion of highly valued genes where distance and transportation limit liquid semen use. Boar sperm damaged during freezing require more sperm use and precision AI timing to achieve acceptable fertility. Hormone addition to low quality semen has been shown to improve fertility with AI. This experiment was performed to test the effect of hormone addition (prostaglandin, PGF) to low doses of FTS on measures of fertility. Semen from nine boars was frozen in 0.5 ml straws at 1.4×10^9 sperm/ml. The breeding experiment was performed in replicates using Matrix™ synchronized gilts (n = 101) and then induced into estrus with PG600®, while parity 1–3 sows (n = 26) were weaned and then induced into estrus using PG600®. Following PG600, females were checked twice daily and at onset of estrus, assigned to treatment by parity to receive 0.5, 1, or 2 billion motile FTS with 0 or 5 mg (1.0 ml) of PGF added into each AI dose at 24 and 36 h after onset of estrus. Time of ovulation was estimated by ultrasound at 6 h intervals. Final pregnancy rate and litter size were determined at slaughter for gilts at d 50 of gestation and for sows at day 114. Data were analysed using the MIXED and GENMOD procedures of SAS for the effects of treatment, parity, replicate, boar, and estrus to ovulation. Measures for sperm quality for FTS were 50% motile, 58% live, and 99% acrosome intact. Estrus occurred at 96 h after PG600 and ovulation at 41 h following onset of estrus. Final pregnancy rate was affected by dose ($p < 0.02$) but not by PGF with 2×10^9 FTS (76%) greater than either 0.5×10^9 (50%) or 1.0×10^9 (52%). Total number of healthy fetuses was also affected by dose ($p < 0.02$) but not by PGF with 2×10^9

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producing more pigs (9.5) than 1×10^9 (8.7) or 0.5×10^9 (5.8). Although PGF did not increase pregnancy or litter size or interact with dose, measures were numerically greater with PGF. Factors such as boar, estrus to ovulation interval, ovary status at slaughter and parity also influenced pregnancy rate ($p < 0.05$) but had no effect on litter size. The results of this study indicate that insemination using 2×10^9 motile FTS used in an AM/PM schedule can result in acceptable fertility but with no measurable benefit for hormone addition.

OC 18

Differences in the lipid fingerprinting of boar and bull sperm detected by MALDI-TOF MS

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Lipids are known to play a crucial role in sperm membrane physico-chemical behaviour during cryopreservation procedures. Matrix-assisted laser desorption–ionization mass spectrometry (MALDI-MS) involving semen lipid extraction has shown to provide characteristic lipid ‘‘fingerprints’’ of semen from various species. In this work, we show the differences in the lipid profile of boar compared to bull sperm by MALDI-MS. Boar spermatozoa-rich fraction was collected using the gloved hand method and bull semen was collected using artificial vagina method. After twice centrifugation (1783.6 ***g***, 20 min at 4°C) the sperm sediment was carefully re-suspended with 20 ml 0.9% NaCl and the supernatant discarded. The final pellet was stored at −80°C. Lipids were extracted according to the Bligh and Dyer procedure. A Synapt HDMS mass spectrometer (Waters Corp., Milford, MA, USA) equipped with a MALDI source was used. All spectra were collected for 45 s in the positive ion mode at the mass range of *m*/*z* 700–1200. The volume of 1 μl of the semen pellet was spotted in the target plate and allowed to dry. Afterwards, 1 μl of 2,5-dihydroxybenzoic acid (DHB) was added as matrix. Data were processed using the software MassLynx 4.1 (Waters Corp.). Lipid spectra showed $[M + Na]^+$ or $[M + H]^+$ ions characteristic of sphingomyelins (SM), phosphorcholines (PC) and plasmalogens (phospholipids with one vinyl ether bond), which are lipid species more resistant to damaging effects of singlet oxygen and known to occur in sperm cells, especially in ruminants. Our initial data showed striking differences between bull and boar lipid profiles. Most intense ions in bull lipid profile were 753.6 (SM 18:0 + Na^+), 812.6 (1-pamytenyl-2-docosahexaenoyl-GPC + Na^+) and 828.6 (PC 16:1/22:5 + Na^+), while in boar sperm, 757.6 (still not attributed), 794.6 (1-pamityl-2-docosapentaenoyl-GPC + H^+) and 816.6 (1-pamityl-2-docosapentaenoyl-GPC + Na^+) predominated. Other plasmalogens such as 1-palmitenyl-2-docosahexanoyl-GPC (*m*/*z* 790.6) and1-palmityl-2-docosaheaenoyl-GPC were also detected in both species. We conclude that semen lipid fingerprinting by MALDI-MS allows fast and sensitive detection of membrane structural lipids differences between bulls and boars and this information can contribute understanding the membrane composition relationship to successful sperm cryopreservation. Project funded by CAPES and FAPEMIG.

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Poster Abstracts

I. Semen Freezing Technology

P1

Effect of boar semen quality parameters on post-thaw sperm motility

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The objective of this study was to investigate the effect of semen quality parameters comprehensive of seminal plasma components on post-thaw sperm motility in boars. Forty-nine fertile hybrid boars were used for this study. Semen volume, sperm motility, sperm concentration and morphologically abnormal spermatozoa were assessed in fresh native semen. The concentration of mineral components (Ca, Zn, Mg, K, Na), free amino acid (Tau, Asp, Thr, Ser, Glu, Gly, Ala, Val, Met, Ile, Leu, Tyr, Phe and Lys) and activity of the enzyme aspartate aminotransferase (AST) was analyzed in seminal plasma. The semen was cryopreserved using the straw freezing procedure described by Westendorf et al. (1975) and modified by Minitüb. Straws were thawed in a water bath at 38°C for 40 s and extended in a semen dilution ratio of 1 + 2 in Androhep (38°C). Post-thaw sperm motility was immediately evaluated and post-thaw activity of the enzyme AST was analysed too. Average volume of sperm was 223.57 ± 75.20 ml, sperm concentration $433.58 \pm 169.90 \times 10^3/mm^3$, morphologically abnormal spermatozoa 16.37 ± 14.94%, motility of fresh semen 76.94 ± 8.65%, post-thaw sperm motility 24.88 ± 13.81%, activity of the enzyme AST of fresh semen 283.36 ± 169.03 $mU/10^9$ and post-thaw semen 689.76 ± 565.48 $mU/10^9$ spermatozoa. Significant differences between fresh and post-thaw sperm were found in both sperm motility and activity of the enzyme AST ($p < 0.001$). The significant relationship of semen quality parameters to post-thaw sperm motility was observed. Post-thaw sperm motility correlated with fresh sperm motility ($r = 0.55$, $p < 0.001$), sperm concentration ($r = 0.26$, $p < 0.05$), concentration of K ($r = 0.43$, $p < 0.01$), Glu ($r = 0.36$, $p < 0.01$) and Gly ($r = 0.32$, $p < 0.05$) in seminal plasma and negatively correlated with post-thaw activity of the enzyme AST ($r = -0.54$, $p < 0.001$), concentration of Na ($r = -0.39$, $p < 0.01$), Lys ($r = -0.30$, $p < 0.05$) and Met ($r = -0.30$, $p < 0.05$) in seminal plasma. In conclusion, correlations between some basic porcine semen quality parameters as well as seminal plasma components and post-thaw sperm motility were approved in this study. Supported by Ministry of Agriculture of the Czech Republic (NAZV QH71284).

P2

Boar spermatozoa survive freezing after Single Layer Centrifugation with Androcoll-P

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Single Layer Centrifugation (SLC) with Androcoll™-P selects boar spermatozoa with good motility, normal morphology and intact membranes from the rest of the ejaculate (van Wienen et al., 2010, Veterinary Science, doi: 10.5402/2011/548385).

Objective: To determine whether SLC can be scaled-up into 200 ml tubes for potential use in the cryopreservation of boar semen.
Methods: Ejaculates (n = 3) from four boars aged 3–4 years, (Swedish Landrace, Swedish Yorkshire and two Norwegian Landrace) were collected using the gloved-hand technique and were immediately extended 1:1 (v/v) in warm (35°C) Beltsville Thawing Solution (BTS). Sperm concentration, measured using the Nucleocounter SP-100 (Chemometec, Denmark), was adjusted to 100×10^6 spermatozoa/ml. Extended semen (15 ml) was layered on top of 15 ml Androcoll-P Large in 50 ml tubes (SLC-15) and 60 ml extended semen was layered on top of 50 ml Androcoll-P (S) in 200 ml centrifuge tubes (SLC-60). After centrifugation at 300 ***g*** for 20 min, the sperm pellets were resuspended in BTS containing bSA (1.25 mg/ml) and sperm quality (motility, morphology, viability) was determined. Mean values were compared with ANOVA. Another sperm sample, processed by SLC-15 and SLC-60, was frozen by a modified simplified method (Saravia et al., 2010, Animal Reproduction Science, 117, 279–287).
Results: Means (±SD) were not significantly different in the fresh samples: SLC-15 progressive motility 87 ± 11%, normal morphology 94 ± 5%, intact membranes 94 ± 3%; SLC-50 progressive motility 83 ± 11%, normal morphology 87 ± 3%, intact membranes 95 ± 3%. After freezing, progressive motilities were 35% and 54% for SLC-15 and SLC-60 respectively.
Conclusion: SLC with Androcoll-P can be scaled up into 200 ml tubes without loss of sperm quality in fresh sperm samples. Selected spermatozoa from SLC-60 may survive cryopreservation better than SLC-15 because of a higher sperm concentration in the sperm pellet. It may be practical to cryopreserve boar spermatozoa after selection using SLC-60. Thanks to Annika Rikberg and Karin Selin-Wretling for the morphological evaluation. Funded by the Swedish Farmers' Foundation and Jordbruksverket, Sweden, and Erasmus.

P3

Sublethal stress treatment of boar semen before cryopreservation enhances cryotolerance – solutions for treatment protocols

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Former studies report about enhanced cryosurvival of boar spermatozoa if sperm was treated with sublethal hydrostatic pressure (HP) stress before freezing (Huang et al., 2009, Anim Reprod Sci 112, 136–149). The present experiment aimed to optimize boar semen cryopreservation protocol by investigating the placement of the HP treatment into various points of semen preparation. Semen was collected from eight boars. Sperm rich fraction was extended with commercial extender at 35°C (BT). Diluted semen was cooled to room temperature (RT) then centrifuged at 2400 ***g*** for 3 min. Pellets were then re-extended with Ext II. (lactose + egg yolk) and Ext. III. (Ext II. + glycerol and Equex Paste), giving a final concentration of 6% glycerol and 1×10^9 spermatozoa/ml. Semen was loaded to 0.5 ml straws, sealed and were placed into a cooling cabinet at 15°C for 1 h. Samples were further cooled to 5°C for 2 h. Straws were then placed 4 cm above liquid nitrogen for 20 min followed by plunging and storing. Extended semen was treated with 40 MPa HP for 80 min in plastic syringes closed by plastic cap. Treatment was executed either (1) after the first dilution

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at BT or (2) at RT, (3) after adding Ext. II., or (4) after Ext. III. Samples were split before the HP treatment. Control groups were frozen without HP treatment. Straws were thawed at 37°C for 30 s, extended with Ext. I. in a 1:15 ratio and incubated at 37°C for 20 min before evaluation by CASA (SpermVision, Minitube) counting ˜1000 spermatozoa per sample. Procedures were repeated for three times. Chi square test was used to determine statistical significance ($p \leq 0.05$). Each HP treatment improved sperm cryo-survival, however the treatment's place in the course of sperm preparation had an effect to the outcome. The highest effect was seen at protocols 2 and 3 (total and progressive motility ± SD: control: 38 ± 12% and 19 ± 9%; protocol 2: 50 ± 14% and 27 ± 15%; protocol 3: 50 ± 15% and 23 ± 10%).

Conclusion: Results confirmed the positive effect of HP treatment. The proper treatment should be executed at room temperature before adding the cryo-protective agent. The study was supported by KMOP-1.1.1.-08/1-2008-0065.

P4

Individual boar variations in frozen–thawed semen supplemented with different OEP concentrations

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It has been confirmed that Orvus Es Paste (OEP) exerts maximum effects on post-thaw boar sperm motility and acrosome integrity (Pursel et al., 1978, J Anim Sci, 47, 198–202). The active component of OEP is sodium dodecyl sulfate (SDS), a water-soluble anionic detergent, which could probably modify the structure of egg yolk components. Freezing of boar semen in an extender containing lipoprotein fraction of ostrich egg yolk (LPFo) exerts protective effects on post-thaw sperm function (Fraser and Strzezek, 2007, Theriogenology, 68, 248–257). This study investigated the male-to-male variations in sperm characteristics of frozen–thawed boar semen supplemented with different OEP concentrations. Fifteen ejaculates, collected from three Polish Large White boars (five ejaculates from each boar), were frozen in a lactose-LPFo-glycerol extender without OEP (group I) or supplemented with 0.25% OEP (group II) and 0.50% (group III). Post-thaw sperm assessments included motility, mitochondrial function (JC-1/PI), plasma membrane integrity, PMI (SYBR-14/PI) and normal acrosomal ridges (NAR). Boar variability and the different OEP concentrations had a significant effect ($p < 0.001$) on the analyzed post-thaw sperm characteristics. In all boars semen frozen in the absence of OEP exhibited reduced post-thaw sperm characteristics. Marked differences in post-thaw sperm motility between group II and III were not evident in all boars. The means (±SD) for post-thaw sperm motility ranged from 20.5 ± 3.4% to 29.5 ± 3.5% for group II and 29.0 ± 2.5% to 40.0 ± 2.9% for group III. A similar trend was shown for the percentage of spermatozoa with functional mitochondria, ranging from 26.9 ± 5.6 to 37.9 ± 3.5 for group II and 38.1 ± 5.9 to 50.8 ± 4.2 for group III. In all boars post-thaw sperm PMI and NAR were better maintained in group III. However, individual differences among boars in post-thaw sperm PMI and NAR were more marked in group II. It appears that, even though OEP augments the cryo-protective effects of LPFo, more studies are warranted to design individual cryopreservation protocol for boar semen.

P5

Tacrine can significantly improve motility of frozen–thawed boar spermatozoa

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The objective of this study was to investigate the feasibility of application of tacrine, one of anti-apoptosis reagents, to cryopreservation of boar semen. Boar semen was diluted (two-step approach) with the improved freezing extenders (1.10% glucose, 1.48% citric acid, 2.42% Tris, 0.02% *N*-acetylcysteine, 20.00% egg yolk, 6.00% glycerol) supplemented with 0, 0.05, 0.10 and 0.15 mM tacrine, and then subpackaged in 0.25 ml straws before equilibrating for 4 h at 5°C. The straws, frozen by a programmable freezer (the cooling rate was −6°C/min from +5 to −120°C, holding for 1 min at +5 and −120°C, respectively) were thawed (70°C, 5 s) after being plunged into liquid nitrogen for at least 5 min. Motion parameters of frozen–thawed spermatozoa were evaluated by means of a computer assisted sperm assessment (CASA). The significances of the differences in those derived data (n = 5) were analyzed by variance analysis. Tacrine supplementation did not significantly enhance ($p > 0.05$) the motility of extended/equilibrated spermatozoa or path velocity (VAP), progressive velocity (VSL), and track velocity (VCL) of frozen–thawed spermatozoa, but addition of this drug can obviously increase the motility (the control, 38.40 ± 4.22%; 0.05 mM tacrine, 45.20 ± 5.17%, $p < 0.05$; 0.10 mM tacrine, 51.20 ± 4.60%, $p < 0.01$; 0.15 mM tacrine, 47.80 ± 2.17%, $p < 0.01$) of frozen–thawed spermatozoa. In addition, there were not any significant differences ($p > 0.05$) among the three treatments with regard to the motion parameters of post-thawing spermatozoa. In short, the application of tacrine to cryopreservation of boar semen can significantly or very evidently improve motility of frozen–thawed boar spermatozoa, thus implicates its potential commercial application of freezing pig semen

P6

Effect of different types of sugar in the freezing extender on the qualities of cryopreserved boar semen

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The objectives of the present study were to determine the effect of different types of sugar used in the freezing extender on the qualities of cryopreserved boar semen. Sixteen ejaculates were collected from four Yorkshire boars. Semen with a subjective motility of ≥ 70% and viability of ≥ 80% was divided into four groups. Each group was supplemented with different types of sugar (i.e. lactose, fructose, glucose and sorbitol) in the freezing extender. The semen was frozen by conventional liquid nitrogen vapor method and was thawed at 50°C for 12 s.

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The post-thawed sperm subjective motility, sperm viability, acrosome integrity and functional plasma membrane integrity were determined after thawing. All of sperm parameters (arcsine transformation) were analyzed by using General Linear Mixed Model (MIXED) procedure of the SAS. It was found that all sperm parameters decreased after freezing and thawing process in treatment groups ($p < 0.05$). The lactose (33.8%) and fructose (34.4%) based freezing extender yielded a higher percentage of motility after thawing than glucose (30.3%) and sorbitol (21.2%) based freezing extenders ($p < 0.05$). The post-thawed sperm viability and acrosome integrity in the lactose (38.3% and 47.4%) and fructose (40.2% and 46.1%) groups were higher ($p < 0.05$) than sorbitol group (28.4% and 35.6%). Moreover, the sperm functional plasma membrane integrity was higher in the lactose, fructose and glucose than the sorbitol groups ($p < 0.05$). It could be concluded that the freezing extenders containing lactose or fructose yield a significantly higher post-thawed semen qualities than the extenders containing glucose or sorbitol. As a result, either lactose or fructose is recommended to be used in the extender for the cryopreservation of boar semen.

P7

Freezing procedure can result in more serious apoptosis of boar spermatozoa than bull spermatozoa

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The objective of this study was to determine the differences of apoptosis between boar and bull spermatozoa at different stages of cryopreservation protocols. Boar/bull spermatozoa from fresh, equilibrated and frozen–thawed semen were evaluated for apoptosis using mitochondrial membrane potential (MMP) detection kit (JC-1) under a fluorescent microscope. This experiment was conducted with five replicates and the significances of data differences were analyzed either using Student's *t*-test or variance analysis. The percentage of apoptosis of either bull or boar spermatozoa showed no difference ($p > 0.05$) between fresh semen (bull 27.56 ± 14.35; boar 44.87 ± 9.73) and equilibrated semen (bull 36.59 ± 23.66; boar 55.36 ± 7.94). However, the percentage of apoptosis with frozen–thawed bull and boar spermatozoa was significantly increased ($p < 0.01$, bull 63.82 ± 13.11; boar 90.94 ± 6.52). Frozen–thawed boar spermatozoa showed significantly higher rate ($p < 0.01$) of apoptosis than bull spermatozoa. In conclusion, the negative influence of the freezing procedure on boar and bull spermatozoa is not mainly affected by the pre-freezing procedures, but more by the freezing and thawing procedures. Moreover, it confirms that cryopreservation seems to have significantly more negative effects on boar spermatozoa than on bull spermatozoa. The results of this study reveal the reason why the commercial use of frozen boar semen lags far behind frozen bull semen.

P8

The heat-shock protein family: are all members good predictors of the ejaculate freezability in boars?

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Some chaperones have fundamental roles in the survival of boar sperm (Volpe et al., 2008, Reprod Dom Anim, 43, 385–92). Among them, the 90 kDa HSP90AA1 plays a putative function as cryoprotectant and has been described as a freezability predictor in this specie (Casas et al., 2010, Theriogenology, 74, 940–50). In the present assay we tested the potentiality of another chaperone, the 60 kDa HSP60, in freezability prediction through comparing its expression in boar sperm between good freezability ejaculates (GFEs, membrane integrity and progressive motility over 40% after thawing) and poor freezability ejaculates (PFEs). Samples coming from three GFEs and three PFEs diluted in BTS (1:2; v:v) were centrifuged twice at 600 ***g*** for 10 min in PBS. Pellets were re-suspended for 30 min in lysis buffer at 4°C (SDS with protease inhibitors). The homogenized suspensions were centrifuged at 15,000 ***g*** for 15 min, and 5 μg of protein per sample were loaded onto a SDS-PAGE to be then transferred to a PVDF membrane. Incubations with the HSP60 monoclonal antibody were performed during 1 h in BSA blocking solution at 1:1000 (v:v). The immunoreaction was finally developed with a horseradish peroxidase conjugated anti-mouse at 1:5000 (v:v). The experiment was performed for triplicate and alpha-tubulin was used as internal standard. From the final results we discarded the initial hypothesis since there were no apparent changes in the 60 kDa bands between GFEs and PFEs. From this study, we conclude that the expression of HSP60 is not a useful marker to predict the freezability of boar ejaculates by itself, despite this role has yet been demonstrated in other chaperones.

P9

Boar sperm from good freezability ejaculates maintain inner Cu/ZnSOD levels for up to six hours after thawing

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Frozen–thawed boar sperm from good freezability ejaculates with membrane integrity and progressive motility equal or over 40%, displays lower expression of the copper/zinc intracellular superoxide dismutase (Cu/ZnSOD) than sperm from poor freezability ejaculates, indicating the oxidative effects of cryopreservation to be less acute in the first group (Casas et al., 2010, Theriogenology, 74, 940–50). The purpose of the present study was to test if the Cu/ZnSOD is maintained at the same expression level along the thawing process in the good freezability group. Two frozen straws coming from each of the three different Piétrain good freezability ejaculates were thawed in Beltsville thawing solution (1:3; v:v). Aliquots were taken at 30 min, 2 and 6 h after thawing and they were centrifuged twice at 15,000 ***g*** for 10 min in phosphate buffer. Pellets were re-suspended in 500 μl sodium dodecyl sulphate lysis buffer to recover super-

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natants after 30 min at 5°C. Twenty micrograms of protein per sample were used to run a polyacrylamide gel electrophoresis. Western blotting was sequentially performed on a nitrocellulose membrane, which was later submerged for 30 min in 3% bovine serum albumin/Tween20 blocking solution and incubated overnight at 5°C with a Cu/ZnSOD antibody in blocking solution (1:1000; v:v). The immunoreaction was tested for 1 h at ambient temperature using a horseradish peroxidase conjugated antibody and was developed with a chemi-luminiscent substrate. The experiment was performed for quadruplicate and alpha-tubulin was used as internal standard. No apparent changes were observed in 15 kDa Cu/ZnSOD bands in any of the three good freezability ejaculates at the three time points indicated. It can be concluded that during at least 6 h post-thawing there is no increase in the antioxidant activity mediated by Cu/ZnSOD in boar sperm from good freezability ejaculates.

P10

Role of glutathione and procaine to stabilize the protamine/DNA complex after freezing and thawing of boar spermatozoa

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Damages on DNA integrity in cryopreserved boar sperm are linked to changes in protamine/DNA interaction rather than DNA fragmentation. The stability of protamine/DNA complex largely depends on the disulphide bonds, which are mediated by cysteine residues. Against this background, the aim of the present study was to assess the effects of different concentrations of glutathione and/or procaine (0.5, 1, and 2 mM) on the number of cysteine-free residues as well as on the motility and survival of boar frozen/thawed spermatozoa. For these purpose, both antioxidative agents were added to LEY and LEYGO cryopreservation extenders during a standard freezing/thawing procedure of six separate ejaculates. Thirty minutes after thawing, the amount of cysteine-free residues was determined spectrophotometrically by using 2,2′-dipiridile reagent at a wavelength of 343 nM. Sperm viability was assessed using SYBR14/PI staining, while sperm motility was determined by means of a computer assisted sperm analysis (CASA). Data were transformed to arc sin $\sqrt{x}$ when necessary and then analyzed using one-way analysis of variance followed by the Scheffé test. The level of significance was set at $p < 0.05$. Percentages of cysteine-free residues were significantly lower in groups with glutathione 2 mM, procaine 1 mM and procaine 2 mM than in the controls. On the other hand, the percentages of viable spermatozoa were significantly higher in four treatments (mean ± SEM; glutathione 1 mM: 57.63 ± 2.12%, glutathione 2 mM: 59.82 ± 2.09%, and procaine 1 mM: 55.38 ± 2.28%, and glutathione 2 mM + procaine 2 mM: 58.13 ± 2.15%) than in the controls (46.27 ± 2.04%). No significant differences were observed when comparing the motility parameters among treatments (e.g. PMOT, control: 52.79 ± 2.59% vs. glutathione 2 mM: 54.08 ± 2.62%). We can conclude that the addition of glutathione and procaine to LEY and LEYGO extenders at the previously mentioned concentrations seem to protect the DNA/protamine interaction against of cryopreservation damages. Further research is being focused on a putative role of these antioxidants in other physiological processes like ROS generation, which are altered during cryopreservation.

P11

DNA fragmentation after boar sperm preservation by heat-drying or freezing without cryoprotectant

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Sperm storage either in a dry state or by freezing in a household freezer is discussed for threatened animal species in emergency cases. Although after such procedures spermatozoa are immotile, assisted fertilization techniques as intracytoplasmic sperm injection (ICSI) might be operated. In the present study pig sperm were used as a model in order to investigate DNA fragmentation after heat-drying or after freezing at −20°C without cryoprotectant. Following semen collection and swim up, sperm concentrations were adjusted to $0.5–1 \times 10^5$ cells/ml. Aliquots of sperm suspension (50 µl, respectively) were heat-dried at 50, 56 or 90°C each for 45 or 60 min, or at 120°C for 20 min, and were stored at 4°C. Further sperm samples were frozen and stored at −20°C without cryoprotectant (100 µl/sample). Preserved specimens were analysed on the day of preservation or were kept for 3 months before investigation. Fresh sperm were used as a control group. All samples were analysed for DNA fragmentation index (DFI) by using the diagnostic kit Halomax Sui® (Halotech DNA SL, Madrid, Spain), and stained with Propidium iodide combined with anti-fading agent (≥ 300 cells/well; 800× magnification, fluorescence microscope). After 3 months of preservation the DFI of samples dried at 50, 56, or 90°C reached 23.2%, 48.3%, or 56.9% after drying for 45 min vs. 62.7%, 82.4%, and 89.8% after drying for 60 min, respectively. Heat-drying of spermatozoa at 120°C for 20 min resulted in a DFI of 20.0%. However, DNA damage was lower when the sperm DNA assessment procedure was done on the day of preservation (DFI of 17.9%, 12.5%, 8.4%, and 8.1% for the four different temperatures, respectively). In semen samples stored at −20°C without cryoprotectant for 12 h or 3 months, a DFI of 15.1% vs. 64.3% was observed. Only 6.1% of fresh spermatozoa showed DNA damage. In conclusion, this study has revealed that the sperm DNA fragmentation index is affected by the preservation method and temperature as well as the duration of storage. Regarding longer storage periods, best results were obtained after drying spermatozoa at 120°C for 20 min.

P12

Evaluation of Piau swine breed semen submitted to three freezing protocols

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The objective was to assess physical and morphological aspects of raw semen and the effect of three semen cryopreservation protocols on *in vivo* and *in vitro* post-thawing sperm viability in Piau swine breed. Twenty-two ejaculates were collected from five adult males. To check raw sperm viability semen was evaluated for concentration, sperm motility (MOT), sperm vigour (VIG), total sperm defects (TD), hyposmotic test (HO), supravital staining (SUP) and percentage of normal acrosomes

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(PNA). Post-thawing quality was evaluated by MOT, VIG, HO, SUP, PAN and thermo-resistance test (TTR) (37°C for 2 h). Freezing Protocols were: P1 – by Fürst et al. (2005, Braz J Vet Med 57, 599–607) with modified diluent media; P2 – above-mentioned method, employing a modified cooling curve, and P3 – by Ohata et al. (2001, Arch Fac Vet Sci 29, 123–9). To test the *in vivo* fertility of frozen/thawed semen, 23 intrauterine inseminations (post-cervical) were performed in 14 females using semen from P3. The mean sperm concentration was $34.8 \pm 13.5 \times 10^9$ sperm/ejaculate. The values for MOT, VIG, SUP, HO, TD, and PNA of raw semen were 83.4 ± 5.4%, 3.3 ± 0.5%, 82.6 ± 0.5%, 88.0 ± 5.5%, 7.0 ± 3.0% and 95.9 ± 2.7%, respectively. Post-thawing, the average recorded for MOT and VIG were 20.9 ± 12.4%, 29.5 ± 10.9% and 49.5 ± 12.1% and 2.5 ± 0.5%, 2.9 ± 0.4% and 3.4 ± 0.4%, respectively, for P1, P2, and P3, with a gradual decrease in these parameters over 2 h of TTR. P3 recorded higher mean values of SUP, PNA and HO (39.9 ± 10.4%, 8.8 ± 5.7% and 44.6 ± 14.4%, respectively) compared to P1 and P2. Four mature sows became pregnant. Physical and morphological aspects of raw semen showed a similar pattern to that of pigs from commercial lines. P3 had the best results for sperm *in vitro* viability tests, and an average of eight piglets born, considered suitable for cryopreserved semen. Project funded by CAPES and FAPEMIG.

P13

Influence of seasonality on the thawed boar semen under tropical conditions

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The aim of the study was to assess the influence of climate on the production of cortisol and the rates of freezing of boar semen at different seasons in south-eastern Brazil. We collected samples of faeces and semen of 10 boars in 2 months in winter and in summer. The data of temperature and humidity were obtained from data loggers installed in the shed housing of the animals. Faecal samples were collected, lyophilized and intended to check the levels of hormone metabolites of cortisol, which was measured using *Cortisol Coat*-A-*Count* radioimmunoassay kit. After ejaculate collection, an sample was taken for smear evaluation of sperm morphology and the rest of the ejaculate was diluted in Beltsville Thawing Solution extender at a ratio of 1:1 (v:v), then centrifuged at 600 ***g*** for 10 min, and after discarding the supernatant the pellet was re-suspended for amid freezing, based on a TRIS-Egg yolk extender supplemented with a cryo-protectant combination of glycerol (2%) and methyl-formamide (2%) (v:v). After the semen storage in 0.5 ml straws, they were chilled to 5°C over a period of 8 h. Thawing of straws was performed at 46°C for 20 s. The parameters of sperm kinetics were assessed by *CASA* (*Computer assisted Sperm Analyzer*) and plasma membrane integrity was tested by a dye combination of propidium iodide and carboxyfluorescein diacetate under a fluorescence microscope. During the experiment mean temperatures and relative humidity differed significantly ($p < 0.0001$) in the participating AI stations from 26°C and 70% in summer and 19°C and 49% in winter, respectively. Concentrations of cortisol metabolites in faeces did not differ between seasons ($p > 0.05$). Of the sperm parameters assessed by *CASA* after thawing, only the percentage of progressive motility showed significant differences ($p < 0.003$), the average being 30.6 and 23.8 between summer and winter. The analysis of sperm morphology of fresh semen and sperm membrane integrity of thawed spermatozoa showed no statistical difference ($p > 0.05$) between seasons. These results demonstrate that, despite great differences in climatic conditions found in tropical regions, the animals are adapted to such conditions.

II. Liquid Semen Preservation

P14

Effect of dilution temperature on boar sperm quality

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A two-step dilution of pig semen is commonly practiced in AI-centers worldwide. However, it has not been well investigated yet whether performing the second dilution at room temperature, instead of using a solution with exactly the same temperature as the semen, affects sperm quality. In a two-step dilution protocol, the effect of the temperature of the extender for the final dilution on boar sperm quality during liquid storage was investigated. Fifteen boars (12 Pietrain, 2 Large white, 1 Landrace) housed in a commercial artificial insemination (AI) centre and aging from 9 to 54 months (27.1 ± 13.2) were included. One ejaculate per boar was collected and further processed with a commercially available semen extender (Beltsville Thawing Solution, BTS). Each ejaculate was first diluted (1:1) at 30°C and the temperature of the dilution was measured. A final dilution to 30×10^6 sperm/ml (commercial dose concentration), with either pre-heated extender [29.3 ± 0.2°C, Group (G) A] or extender kept at room temperature (22.73 ± 0.6°C, GB), was performed. Subsequently, samples were transported to Ghent University in two different but identical isotherm boxes (one per group). A subsample from each semen dilution was investigated during three consecutive days (D) (D0–D2) while the remainder of the sample was stored at 17°C. The following parameters were studied at D0, D1 and D2: % of motile sperm (% M), % of progressive sperm (% P) [both by Computer Assisted Semen Analysis (CASA)] and the % of sperm with intact membrane (% IM) by eosin nigrosin staining. At D0 and D2, the % of sperm with intact acrosome (% IA) was studied by PSA staining. Repeated measures ANOVA from D0-2 were used for statistical analysis (SPSS v18). The average temperature of the 1:1 dilution was 29.4 ± 1.1°C. No significant differences were found between groups for % M (GA: 79.3 ± 9.0; GB: 81.1 ± 9.2; $p = 0.491$) or % P (GA: 56.5 ± 13.3, GB: 58.4 ± 13.8, $p = 0.817$). No differences were found for % IM [85.1 ± 10.7 (GA) and 84.5 ± 3.8 (GB); $p = 0.749$] and % IA [72.2 ± 9.4 (GA) and 68.3 ± 16.6 (GB); $p = 0.785$]. In conclusion, when a two-step dilution is performed, preheating the extender for the second dilution to match the semen temperature, did not improve sperm quality compared to a dilution at a moderate room temperature.

P15

Effect of semen processing procedures on quality of cooled boar spermatozoa

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The aim of this experiment was to evaluate the effects of semen holding medium and semen centrifugation method on post-cooling sperm motility and velocity, incidence of sperm

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agglutination, sperm chromatin instability (SCI) and proportion of live morphologically normal spermatozoa (LMNS). Whole semen ejaculates (n = 10) of $\geq 0.25 \times 10^9$ sperm/ml and 80% sperm motility were collected from five boars, split-diluted (1:1) with Beltsville Thawing Solution or Tolerance medium (Nidacon, Sweden) and incubated at 18°C for 3 h. Diluted semen was then subjected either to the standard centrifugation regime (800 ***g*** for 10 min) or to a cushioned centrifugation (1000 ***g*** for 20 min) over three sources of cushion media: Maxifreeze (IMV, France), CushionFluid (Minitub, Germany) and PorciCushion (Nidacon). The volumes of cushioned medium were 0.04 and 0.5 ml/10 ml diluted semen. Sperm pellet was re-suspended in lactose-egg yolk extender to a concentration of 1.5×10^9 sperm/ml, cooled to 5°C over a 3-h period and evaluated for sperm traits. Data were analysed using multi-factorial ANOVA (holding medium × centrifugation regime × cushion source and volume) and Duncan multiple range test. Tolerance medium reduced the incidences of sperm agglutination and SCI ($p < 0.02$) after the standard centrifugation regime. Cushioned centrifugation improved sperm motility, velocity and recovery rate of LMNS ($p < 0.05$–0.001). Spermatozoa treated with PorciCushion had higher ($p < 0.01$) values of sperm motility and velocity than those treated with Maxifreeze and CushionFluid. The effect of cushion volume on sperm traits was non-significant. In conclusion, semen holding medium and centrifugation regime influence quality of cooled spermatozoa.

P16

Cooling of concentrated spermatozoa for boar semen transport: effects on sperm viability and sow fertility

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The possibility of storing and transporting sperm doses at 5°C in special containers with controlled cooling rate will be of great interest to preserve boar semen for longer periods and will make distribution of semen more efficient. Recently, we developed a special container for boar semen transportation with controlled cooling rate, but able to transport only 12 doses (Roner et al., 2006, Arq Bras Med Vet Zootec, 58, 78–85). Thus, these experiments aimed to optimize the transport of boar semen cooled at concentrated form and re-extended within 17 h of storage at 5 or 17°C. In the experiment I, 89 ejaculates from 11 boars (Duroc and crossbreed Duroc × Pietran) were extended with glycine egg yolk (GEY; Foote, 2002, Reprod Dom Anim, 37, 61–3) or MR-A®, to contain 30 (control), 60 or 90×10^6 sperm/ml and cooled at 5 or 17°C, respectively, in a special container. In the experiment II, 24 ejaculates from five Landrace boars were extended in GEY or MR-A®, to contain 30 or 120×10^6 sperm/ml, and cooled at 5 or 17°C, respectively, in a special container. Doses cooled with 60, 90 or 120×10^6 sperm/ml were re-extended, after 17 h of storage, to 30×10^6 sperm/ml in the same extenders described above, and kept in the container at 5 or 17°C, until use. Data were examined by ANOVA using the General Linear Models procedure of the SAS statistical package, version 5.0, with a significance level of 95%. The motility was estimated during 48 h of storage and inseminations (n = 68, Exp. I; n = 46, Exp. II) were carried out up to 34 h after re-dilution. Sperm survival was good over a 48 h of storage (≥ 65%, 3) at either 5 or 17°C. The conception rate (Exp. I: 91.2%; Exp. II: 91.3%), farrowing rate (Exp. I: 90.1%; Exp. II: 91.3%), and total number of born alive (Exp. I: 11.1; Exp. II: 11.9) did not differ among treatments ($p > 0.05$). These results demonstrated that cooled semen at high concentration prior to subsequent re-dilution could maintain sperm fertility, reduces semen storage costs and makes AI doses distribution more efficient.

P17

Resistance of liquid preserved boar spermatozoa to temporarily chilling

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Among mammalian species boar spermatozoa are especially sensitive towards cold-shock. During transport in winter, temperature of liquid preserved boar semen may drop temporarily below the critical threshold of 15°C before semen become re-warmed to regular storage temperature of 17°C. This transient chilling shortly after dilution is supposed to impair sperm quality, especially when long-term stored is used. The aim of this study was to develop strategies to enhance the resistance of diluted boar sperm to temporarily chilling. This involved a holding time of freshly diluted semen at 25°C and the use of a potentially cold-shock protective extender. In Experiment 1, six ejaculates of different boars diluted in isothermic Androstar Plus® were treated in three groups before storage at 17°C for 7 days: A: cooling to 17°C (control); B: cooling to 10°C for 12 h and re-warming to 17°C; C: holding at 25°C for 24 h, cooling to 10°C for 12 h and re-warming to 17°C. In Experiment 2, six ejaculates were split-sample diluted in isothermic BTS and Androstar Plus® and either cooled to 17°C (Control) or hold at 25°C for 24 h, cooled to 10°C for 12 h and re-warmed to 17°C before storage for 7 days. CASA-motility and acrosome integrity were evaluated at the day of dilution (d 0), d 3 and d 7. In Experiment 1, at d 3 acrosome integrity was significantly higher ($p < 0.01$) in Group C vs. Group B samples, whereas motility did not differ. At d 7, Group A (88.2%) and Group C (87.6%) showed higher motility ($p < 0.01$) compared to Group B (79.0%); acrosome integrity was higher for Group C (85.9%) compared to Group B (80.7%) ($p < 0.01$). In Experiment 2, differences between groups were significant at d 7 ($p < 0.05$): semen diluted in Androstar Plus® showed higher motility and acrosome integrity compared to samples diluted in BTS regardless of the presence of a holding time before cooling. In conclusion, holding of freshly diluted semen at 25°C for 24 h increases the resistance of spermatozoa to subsequent temporarily chilling. This effect becomes especially prominent when semen is long-termed stored at conventional storage temperature after chilling. A potential cold-shock protecting effect of the Androstar Plus® extender was confirmed.

P18

Changes in responsiveness to capacitating stimuli in chilled boar spermatozoa *in vitro*

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Chilling-injury may result in cell death or sub-lethal damage in diluted boar spermatozoa. Essential cell functions such as the regulated response to capacitation stimuli may be impaired. In this study, the responsiveness of chilled boar spermatozoa to

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bicarbonate was monitored using Ca^{2+}-influx. Seven ejaculates of different boars were diluted split-sample in Beltsville Thawing Solution (BTS) or Androstar Plus® (APlus). After 90 min at room temperature (RT) and after 24 h of storage at 17, 10 or 5°C, CASA-motility and membrane integrity [propidium iodide (PI) and FITC-PNA] were assessed. Percoll-washed sperm were incubated at 38°C in Tyrode's medium under capacitating conditions or in a control medium (w/o bicarbonate and Ca^{2+}). Changes of Ca^{2+}-influx were assessed by flow cytometry in 20 min intervals. Compared to storage at 17°C, motility and membrane integrity were lower in sperm stored in BTS at 10 and 5°C ($p < 0.05$); these parameters did not differ between 10 and 17°C in APlus-samples. At 3 min of incubation in Tyrode's medium, samples stored in BTS at 10 or 5°C showed a larger population of Ca^{2+}-pos./PI-neg. cells compared to 17°C ($p < 0.05$). Storage temperature has not affected the percentage of Ca^{2+}-pos./PI-neg. at 60 min in samples stored in BTS for 24 h. However, this percentage was significantly higher for semen stored in APlus at 5°C. Response to capacitating conditions (changes in % of stable Ca^{2+}-neg./PI-neg. cells between 3 and 60 min of incubation: Δ_{3-60}) declined significantly for BTS-samples from 65.0% (RT) to 50.6% (10°C) and 46.2% (5°C; $p < 0.05$). In contrast, in the control medium it increased from 4.2% (RT) to 11.6% (10°C) and 20.0% (5°C; $p < 0.05$). Samples diluted in APlus showed a similar trend under capacitating conditions, but the response in the control medium was significantly lower (10°C: 6.7%; 5°C: 9.4%) than in BTS. In conclusion, chilling induces an initial rapid response to capacitation stimuli in a population of destabilizing sperm but it seems to reduce the response to capacitation stimulus over time. Extenders can mediate chilling-induced response indicating different protective effects on sperm function under fertilizing conditions *in vitro*.

P19

Supplementation of refrigerating boar semen extenders with antioxidants

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Boar semen is extremely vulnerable to cold shock and it is sensitive to peroxidation due to the high content of unsaturated fatty acids in the plasma membrane. Antioxidants exert a protective effect on the plasma membrane of frozen boar sperm. Therefore, the present study was performed to evaluate the effect of different refrigerating extenders containing antioxidants (cysteine, ascorbic acid, vitamin A and C) on boar semen quality. Semen collected from 15 boars was refrigerated in BTS, BT, BTA1, BTA2, BTA3, BTA4, BTC1, BHT, BHC, BTA8, BGC, BC, BTT, BTC2, BTC3, BTA14, BTA15, BTA16, BGT, BTC4, BTA19, BTA20, BFT1, BFT2, BFT3, BFT4, BST1, BST2, BST3 and BST4. Sperm samples were taken to assess total motility, viability, acrosome integrity and response to hypo-osmotic swelling test (HOST) from 1 to 13 day. Compared to the other groups the results showed an increase in acrosome integrity in presence of cysteine included in BTC2 and BTC3, as well as in BTS. The addition of ascorbic acid with extenders such as BTC1, BHT and BHC provided no beneficial effect on spermatozoa, the concentration of this acid was very low. At higher concentrations, ascorbic acid decreased ph. Conversely, acrosome status and motility was improved by BTA2 and BT3. These extenders contained vitamin E and C. Antioxidants-enriched freezing extender improved refrigerated boar semen quality, showing a significant beneficial effect on acrosome integrity and motility for vitamin E and C, or just on acrosome for cysteine. Further studies are needed to define the protection mechanism of these antioxidants.

P20

Influence of ejaculate portion and preservation method on the *in vitro* characteristics of boar spermatozoa

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Spermatozoa present in the first collectable 10–15 ml of the sperm-rich fraction of the boar ejaculate (portion 1, P1) have shown a higher viability during handling, cooling and cryopreservation, compared with spermatozoa in the rest of the ejaculate (portion 2, P2), probably due to different composition of the surrounding seminal plasma. In the present study, we investigated whether these ejaculate portions (P1 and P2) were able to differently influence sperm viability during cooling at 5 or 17°C. Two portions of boar ejaculate – P1 and P2 – were collected weekly from five mature boars and split in four treatments: T1 – spermatozoa from P1 extended in MR-A® and cooled at 17°C; T2 – spermatozoa from P1 extended in glycine-egg yolk (GGO) and cooled at 5°C; T3 – spermatozoa from P2 extended in MR-A® and cooled at 17°C; and T4 – spermatozoa from P2 extended in GGO and cooled at 5°C. All doses were kept in a special container, previously described (Roner et al., 2006, Arq Bras Med Vet Zootec, 58, 78–85). Spermatozoa motility, vigour, and morphological characteristics were evaluated up to 72 h of storage. All treatments kept an acceptable motility at first 24 h of storage (54.5%, 64.5%, 66.0% and 74.5% for T1, T2, T3 and T4, respectively). The T4 kept a similar motility (70%; $p > 0.05$) over storage time (72 h) while the others treatments showed a decrease in motility (15.0%, 30.0% and 45.0% for T1, T2 and T3), mainly after 24 h of storage. Moreover, all treatments showed morphological abnormalities at 72 h of storage but within the acceptable thresholds (21.7%, 19.0%, 22.9% and 19.0% for T1, T2, T3 and T4). However, the spermatozoa extended in GGO and cooled at 5°C showed higher ($p < 0.05$) acrosome damage compared to spermatozoa extended in MR-A® and cooled at 17°C (6.5% vs. 1.1%). Finally, the use of a special container for boar semen transport in combination with a well-defined portion of the ejaculate, e.g. P1, was proved efficient to maintain sperm viability. Further, this methodology will allow the development of new technologies concerning the transport of boar semen, while the rest of the ejaculate could be used in farm routine to produce conventional liquid semen doses.

P21

Influence of seminal plasma and extender on the quality of highly diluted boar semen

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Economic use of ejaculates from boars with high genetic value imposes high dilution rates, especially if the sperm concentration in neat semen is high. It is well known that excessive dilution leads to loss of motility and destabilization of sperm membranes presumably due to the reduction of protective seminal plasma components. We hypothesized firstly that the

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addition of seminal plasma to extender medium counteracts the dilution effect, and secondly that extenders differ in their ability to protect sperm against the dilution effect. Furthermore, potential interaction between seminal plasma and extender on highly diluted, stored semen was examined. Semen of six different boars was used in a blinded study. In Experiment 1, semen was split-sample diluted in BSA-free extenders Beltsville Thawing Solution (BTS) and Androstar Plus® to 2.5, 1 or 0.5×10^9 sperm/80 ml. CASA motility and acrosome integrity were evaluated until day 7 of storage. Motility was significantly lower in 0.5 and 1×10^9 doses compared to 2.5×10^9. This effect became more expressed with ongoing storage (d 0 vs. d 3 vs. d 5, $p < 0.05$). At d 3 and 5, sperm diluted to 0.5 and 1×10^9 and stored in Androstar Plus® showed higher motility and less acrosome defects compared to BTS ($p < 0.05$). In Experiment 2, the same extenders were used without (control) or with 10% or 20% seminal plasma from a pool. Semen was diluted to 1 or 0.5×10^9 sperm/80 ml. At d 5, samples with seminal plasma showed higher motility and less damaged acrosomes compared to control ($p < 0.05$). The seminal plasma effect was dose-dependent and more expressed in the 0.5×10^9 compared to 1×10^9 group ($p < 0.05$). Motility and acrosome integrity were higher in Androstar Plus® diluted semen compared to BTS–samples both in seminal plasma and control groups ($p < 0.05$). In conclusion, high dilution impairs quality of stored boar sperm, with an extent depending on the dilution rate and the extender. The dilution effect can be alleviated by supplemented use of seminal plasma and, in an additive effect, by protective extenders.

P22

The effect of a plant protein component of boar semen extender on apoptotic-like changes in the spermatozoa

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The study was undertaken to determine the effects of plant protein (PP) (Animal Farma BV, the Netherlands) supplementation on apoptotic-like changes in porcine spermatozoa and semen survival time during storage at 15°C in *Biosolwens Plus* (BP) (Biochefa, Sosnowiec, Poland) extender. The PP is a mixture of several plant proteins and soya lecithin. Three fluorescence methods were used to evaluate semen quality: an assay to assess the early changes in sperm membrane integrity using the fluorophore YO-PRO-1, an assay for phosphatidylserine translocation across the plasma membrane using Annexin-V-Fluos/PI and mitochondrial probes JC-1 to measure changes in mitochondrial membrane potential. The semen from five boars (crossbreds of Polish Landrace and Large White; 10 ejaculate/boar) were diluted to a final concentration of 2.5×10^9 sperm in 80 ml BP extender (control) and in 80 ml BP extender supplemented with 0.001 g PP/ml. Motility and fluorescence analysis were assessed on each day of the experiment until motility of sperm decreased to 30%. Significance of the differences between means ± SD was tested by Duncan's multiple range test. Mean survival time of the semen diluted with BP (6.7 days) and BP + PP extender (6.2 days) did not significantly differ. Significantly lower percentages were observed for apoptotic sperm detected by YO-PRO-1/PI assay (4.3 ± 1.5 vs. 8.3 ± 1.4), and early apoptosis (3.3 ± 1.5 vs. 8.5 ± 4.1) using Annexin V-Fluos/PI assay in extender supplemented with PP compared to control from the third day of storage. Moreover, on the day when sperm motility decreased to 30%, significantly higher percentage of live sperm were detected by these two assays in BP extender supplemented with PP (49.4 ± 3.4 and 47.4 ± 6.8) compared to control samples (32.4 ± 3.3 and 33.4 ± 7.2). JC-1 probe yielded no significant differences in the percentage of sperm with high and low mitochondrial membrane potential during storage time in extender supplemented with PP compared to control. In conclusion, addition of PP component to BP extender significantly reduced the percentage of sperm with apoptotic-like changes and maintained the percentage of live spermatozoa during storage.

P23

Improving boar semen quality by polyunsaturated fatty acids feed additive

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The objective of the present study was to determine after adding tuna oil and vitamins to basic diet whether such supplements improve lipid and fatty acid (FA) composition of sperm and sperm quality in boars having low to normal quality (~70% motility and viability). Seven boars were assigned to the study after they had undergone a previous test (eight ejaculates) indicating a low to normal motility status. They received the basic diet together with tuna oil and vitamins C and E for 16 weeks. Semen was collected into pre-warmed (37°C) containers using the glove-hand method once a week during this present study. Semen quality and lipid composition of sperm and seminal plasma were evaluated. Briefly, semen volume and motility were evaluated by conventional methods. We evaluated sperm morphology by William staining, sperm viability by eosin-nigrosin staining and sperm permeability by HOS test. The study was divided into two periods. Period 1 represented semen collected in the first 8 weeks, whereas Period 2 represented semen collected during the second 8 weeks. Semen was then centrifuged to separate sperm from seminal plasma and both were stored at −20°C until lipid analysis of by gas chromatography. Sperm parameters, and lipid composition in sperm plasma membrane were subjected to analysis of variance, which was performed by a MIXED-procedure. The sperm motility (83.8 ± 4.2% vs. 71.6 ± 9.2%) and viability (87.4 ± 5.9% vs. 71.6 ± 8.2%) increased significantly. Additionally, sperm output increased significantly during Period 2 compared to Period 1 ($p \leq 0.05$). Sperm from Period 2 had higher total lipid content, percent phospholipids and percent cholesterol than those from Period 1 ($p \leq 0.05$). Regarding fatty acid compositions, Period 2 had higher percentages of DHA and total *n*-3 fatty acids than those from Period 1 ($p \leq 0.05$). In conclusion, diet supplementation with a source of *n*-3 PUFA, which play an important role on sperm motility, viability and fluidity can improve these parameters in the boars with low to normal sperm quality.

P24

The effect of volume and sperm number on the quality of extended boar semen after long-term storage at 17°C

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Post-cervical insemination (PCI) in sows is a simple, effective and safe technique and allows a threefold reduction of spermatozoa to be inseminated. In Spain, PCI already

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accounts for 15–20% of productive sows. Most commonly, 1000×10^6 spermatozoa in a volume of 30 ml are inseminated. However, in most of the PCI studies sows have been inseminated few days after semen collection. In several situations long-term semen-extenders can be preferable. To analyse a long-term storage effect of low volume and sperm number, which could affect semen quality. In an experiment eight boar ejaculates were diluted in a long-term extender (Zoosperm®) containing 2500×10^6 useful spermatozoa. Extended semen from up to four boars was pooled in different combinations and each pool was split into three samples. Total sperm doses of approximately 2500, 1000 or 500×10^6 useful spermatozoa were packaged in plastic bottles in volumes of 80, 30 or 15 ml, respectively, transported to laboratory and preserved at 17°C for 7 days. On Days 1, 4 and 7, we evaluated motility parameters and the percentage of total static spermatozoa by a computer-aided sperm analysis system. Viability (SYBR-14/PI), acrosome status (FITC-PNA/PI), membrane fluidity (M-540/YoPro-1) and mitochondrial membrane potential status (JC-1) were evaluated by flow cytometry. With regard to flow cytometry results, only the percentage of spermatozoa showing a high mitochondrial membrane potential showed significant differences between groups, with the lowest values for 15 ml – 500×10^6 spermatozoa doses. On days 1, 4 and 7 the proportion of static spermatozoa was lowest for 80 ml – 2500×10^6 spermatozoa doses and highest for 15 ml – 500×10^6 spermatozoa doses. Throughout the entire storage period motility pattern were significantly different between groups but similar between 80 and 30 ml doses. Highest values of velocity were obtained for 80 ml doses. We conclude that volume and sperm number of semen doses can negatively affect sperm quality during long-term storage. Therefore good semen storage and transport conditions are crucial for keeping appropriate extended boar semen quality. Supported by JUEX PRI09A077 & GR10156, MICINN AGL2010-15188.

P25

Characteristic of acrosome integrity in boar semen extended in medium term and long term extenders and stored for 10 days at 18°C

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In many countries, including Thailand, there are numerous commercial boar semen extenders, which can be divided into: short term = keep diluted sperm for ≤ 3 days; medium term = keep diluted sperm for 3–5; and long term extenders = keep diluted sperm for 5–7 days). However, few studies have been conducted comparing these commercial extenders with regards to maintaining sperm acrosome integrity during storage. The aim of this study was to assess motility, viability and acrosome integrity of boar spermatozoa extended in Merck-III and VITASEM LD. Ejaculates from boars (n = 6) were collected and sub-samples were diluted (3×10^9 spermatozoa/ml) in the different extenders (groups A = Merck-III, B = VITASEM LD) and stored for 10 days at 18°C (using a refrigerator of Magapor®, Spain). On every second day (days 0, 2, 4, 6, 8, 10), semen samples were examined under fluorescent microscope for acrosome integrity (using FITC-PNA/EthD-1, and classified into intact acrosome and non-intact acrosome). Data were analysed by Student's *t*-test (SPSS version 18). On every second day after storage, there were numerically small, but statistically significances in characteristic of acrosome integrity between extenders. For instance, on days 6 (75.4 ± 8.5 vs. 66.0 ± 2.2), 8 (71.6 ± 10.6 vs. 67.5 ± 6.6) and 10 (70.4 ± 4.8 vs. 61.5 ± 6.2), the percentages of acrosome integrity was higher ($p < 0.001$) in group B than group A, respectively. As expected, after 4 days of storage, long-term extender demonstrated an ability to maintain sperm motility, viability and acrosome integrity that was dramatically different from medium term extender. It has been reported that acrosome integrity are an important parameter in boar semen quality. Conclusion, changes in acrosome integrity during storage was affected by the extender utilized, however long term extender maintained a high percentage of acrosome integrity through 10 days of storage. This indicates a superior fertilizing ability of spermatozoa extended with long-term extender over medium term extender. Pornchai Intertrade Ltd., (Distributor of Magapor products in Thailand) is thanked for providing VITASEM LD for this study.

P26

Apoptotic-like changes in the spermatozoa of fresh and stored boar semen and the quality of embryos produced *in vivo*

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The aim of this experiment was to study the relationship between apoptotic-like changes in boar semen and DNA fragmentation in porcine embryos. Three ejaculates from each of three boars were used in this study. After collection and separation of gel, each ejaculate was diluted in *Biosolwens Plus* extender. All ejaculates were stored at 15°C until Day 5. Semen, both fresh and stored (Day 5 of storage) was analysed to assess the early changes in sperm membrane integrity using the fluorophore YO-PRO-1 and used for insemination of superovulated gilts, which were used as embryo donors in this study. Thirty-six superovulated donor gilts were inseminated with fresh semen (four gilts per group for each of three boars) and 36 donors were inseminated with stored semen at Day 5 of preservation. All animals used in the study were crossbreeds of the Polish Landrace and Polish Large White. Embryos at the blastocyst stage from both groups were flushed on 5.5 days after insemination. After morphological evaluation the quality of the pre-implantation embryos was determined by DNA fragmentation (In Situ Cell Death Detection Fluorescein kit; Roche, Mannheim, Germany) and caspase-3 labelling (PhiPhiLux G_2D_2 kit; Calbiochem, San Diego, CA, USA). Significance of the differences between means ± SD was tested by Duncan's multiple range test. The percentage of apoptotic sperm was significantly lower in fresh semen (6.4 ± 2.2%) compared to stored semen (21.1 ± 6.7%). In total 505 and 434 of expanded blastocysts were collected from gilts inseminated with fresh and stored semen. The TUNEL-index in morphologically normal embryos obtained after insemination with stored semen was 12.2, which is significantly higher ($p < 0.01$) than the index of embryos derived from gilts inseminated with fresh semen (1.6). Moreover, the significantly highest number of embryos derived from gilts inseminated with stored semen had activity of caspase-3. In conclusion, the liquid preservation of boar spermatozoa causes apoptotic-like changes. The embryos obtained after insemination with stored semen consists of higher percentage of apoptotic sperm and showed higher susceptibility to DNA fragmentation and more of embryos had caspase-3 activity.

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P27

Seasonal changes in boar sperm DNA fragmentation

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The aim of the study was to monitor seasonal changes in sperm chromatin damages (DNA fragmentation) in boars used for artificial insemination. Semen from 16 boars of three breeds (Large White, Landrace and Pietrain × Duroc), aged from 7 months to 7 years was collected month by month. Totally 142 ejaculates were collected. After collection semen was diluted with BTS extender and stored for 7 days at 17°C temperature. The SCSA (Sperm Chromatin Structure Assay) protocol and flow cytometry were used for DNA fragmentation examination. The ANOVA was used for statistical calculations. The DFI (DNA Fragmentation Index) was used to express percentage of spermatozoa with abnormal, fragmented DNA. The mean DFI of examined ejaculates was 2.58 (SE = 1.25). Significant differences ($p = 0.019$) were found between ejaculates collected in April–September and October–March periods. Mean DFI for these periods were 2.79 (SE = 0.19) and 2.29 (SE = 0.2) respectively. There were no significant general relationship between DFI and months of semen collection ($p = 0.056$). However it was found that there are significant differences in DFI level between some of months, i.e.: January (1.96, SE = 0.31) vs. May (2.74, SE = 0.27, $p = 0.047$), June (2.95, SE = 0.33, $p = 0.02$) and September (3.24, SE = 0.31, $p = 0.003$); March (1.90, SE = 0.35) vs. May (2.74, SE = 0.27, $p = 0.048$), June (2.95, SE = 0.33, $p = 0.02$) and September (3.24, SE = 0.31, $p = 0.004$); April (2.32, SE = 0.33) vs. September (3.24, SE = 0.31, $p = 0.04$); September (3.24, SE = 0.31) vs. December (1.15, SE = 0.87, $p = 0.02$). The highest DFI value observed during whole experiment was 6.39. There were no relationships between DFI and boars' age ($p = 0.68$) or breed ($p = 0.67$). To summarize: although mean sperm DNA fragmentation stayed on relatively low level, significant changes were observed during the whole year. It can be supposed that such changes are related to environmental temperature.

III. Semen Quality Assessment and Semen Preparation

P28

Introducing a novel CASA System for semen analysis

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Computer assisted semen analysis (CASA) is a powerful tool for evaluation of semen in andrology and animal breeding. Measurement and documentation of quantitative kinematic data of sperm motility is the main advantage over manual semen quality estimation. Commercially available CASA Systems evaluate sperm motility by using phase contrast or negative phase contrast microscopy, which however hampers sperm identification by species-specific problems. Advances employing fluorescent techniques improved sperm identification resulting in valid determination of sperm concentration (WHO laboratory manual, 5th edn, 2010) and can also be applied for sperm motility analysis. We developed a novel CASA-System, which is completely based on fluorescence techniques allowing sperm motility analysis together with other parameters of semen quality in a one step procedure. Records are analysed online with a recently developed software module tailored on our fluorescent analysis system. Sperm concentration and sperm motility is quantified resulting in the sperm count, VCL, VSL, VAP and ALH as primary results. From these data secondary parameters (e.g. linearity or wobble) are calculated, if necessary. Boar spermatozoa stained with fluorescent dye (Hoechst 33342) showed within minutes the same motility as unstained controls. Sperm counting was validated by comparative analysis using manual counting with a hemo cytometer and automatic analysis with a Mika Cell-Motion-Analyzer (negative phase contrast). The main advantages of a fluorescence-based analysis are obvious: (1) Sperm are specifically stained by the dye thus avoiding interferences with other particles of the semen sample, (2) Fluorescence staining improves sperm track analyses and the automatic detection of sperm aggregates, (3) Adding other specific dyes, multi-parametric semen quality analyses are possible in one step together with the determination of sperm motility.

P29

Effect of the frame rate and the number of frames per capture on boar sperm CASA analysis

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Previous studies reported that CASA settings could affect motility results recorded by CASA devices (Rijsselaere, 2003, Theriogenology, 60, 1553–68; Contri, 2010, Theriogenology, 74, 424–35). The aim of this study is to estimate the effect of different frame rates (25 and 60 Hz) and different number of frames per field (25 or 60 frames) on boar sperm CASA analysis. Semen from 46 boars was included in this study. Each ejaculate was diluted in a commercial extender to a concentration of 30–35 million spermatozoa per ml, and transported to our laboratory at 17°C within 30 h after collection. Diluted ejaculates were activated by incubation at 38.5°C for 20 min and then, evaluated by a CASA system (ISAS). Captures were made for each sample only in the first third of the sample inlet area of the chamber. Four different capture settings were used: (1) 25 Hz, 25 frames, (2) 25 Hz, 60 frames, (3) 60 Hz, 25 frames, (4) 60 Hz, 60 frames. The parameters considered in this study were total motility (TM, %), progressive motility (PM, %), average path velocity (VAP, μm/s), straight-line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), amplitude of lateral head displacement (ALH, μm), beat cross frequency (BCF, Hz), straightness (STR, %), and linearity (LIN, %). Statistical analyses of the kinematics parameters were performed using SPSS 15.0.1. A statistically significant effect ($p < 0.05$) of the capture settings was shown for all parameters. Velocity values increased with increasing frame rates, but decreased with increasing number of frames. The lowest TM and PM and the highest VCL, VSL, VAP and BCF were obtained when setup C (60 Hz, 25 frames) was used. The highest LIN, STR and ALH values were obtained when setup A (25 Hz, 25 frames) was used. In theory, using a frame rate of 60 Hz computes the position of sperm heads in a closer distance and trajectories are better defined compared to 25 Hz. In addition the number of frames per field acquired during the analysis affects the kinetic results. The reconstruction of the trajectory from 60 frames at 60 Hz is more accurate and differs from the reconstruction of the other setting values. Therefore, we recommend for CASA analysis of boar semen a routine frame rate of 60 Hz and 60 frames per field.

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P30

Leja-4 chambers toxic effect on boar sperm motility depends on manufacture date

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Toxic effects of Leja-4 counting chambers on sperm motility have been reported previously (Hansen, 2009, VIIIth International Conference on Pig Reproduction, Alberta, Canada, 213–51). The aim of this study was to estimate this effect depending on the date of manufacture of the different Leja-4 chamber batches. Semen from 30 boars routinely used for AI was included in this study. Each ejaculate was diluted in a commercial extender to a concentration of 30–35 million spermatozoa per ml, and transported to our laboratory at 17°C within 30 h of collection. Diluted ejaculates were activated by incubation at 38.5°C for 20 min and then, evaluated by a CASA system (SCOPUS 1.0; BIOPROYECT, Spain). At least five images were captured from each sample, only in the first third of the sample inlet area of the chamber. The chamber batches were classified regarding of the time passed since their manufacturing date: (Batch A) 2 months, (Batch B) 26 months, (Batch C) 32 months, (Batch D) 38 months. Total motility (% TM), progressive motility (% PM), sperm average path velocity (μm/s VAP) and linearity index (LIN) were analysed. Statistical analyses were performed using SPSS 15.0.1. A statistically significant effect ($p < 0.05$) of the chamber batch was seen for TM and VAP, but not for PM or LIN. Mean ± SD values were for TM: (A) 74.27 ± 10.91, (B) 65.23 ± 16.84, (C) 69.5 ± 19.0, (D) 68.4 ± 16.2 and for PM: (A) 45.5 ± 14.4, (B) 38.6 ± 11.3, (C) 44.2 ± 16.0, (D) 45.0 ± 13.1 and for VAP: (A) 68.5 ± 16.3, (B) 51.2 ± 18.9, (C) 58.9 ± 19.2, (D) 43.6 ± 12.7 and for LIN: (A) 40.4 ± 13.6, (B) 36.3 ± 10.0, (C) 39.6 ± 12.0, (D) 37.5 ± 8.8. As expected, the best motility results were achieved when sperm samples were evaluated in the 2-months old chambers, and the worst results were found for the 38-months old chambers. Interestingly, batches B and C, made < 6 months apart, but showed significant differences in VAP. Also, the batch C, older than B, showed better motility results. For batches B, C, and D the 12-month warranty period was expired. We have often observed that some laboratories used Leja chambers without performing any quality control. They used even (sometimes unknowingly) expired batches. Any information regarding expiry date appears on the Leja boxes. Efforts will be made to inform all Leja chamber users about the correct use of these slides.

P31

Boar sperm plasma membrane integrity assessed by eosin–nigrosine and SYBR14/PI: a comparison

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Eosin–nigrosine stain is still used to assess sperm plasma membrane integrity (viability) by many laboratories around the world due to its low cost and easy use. However, fluorescent stains have superseded it because they can be used with both fluorescent microscopy and flow cytometry. The objective of this work was to compare the measurement of sperm plasma membrane integrity analysed by Eosin-Nigrosine or SYBR14/PI labelling (fluorescent microscopy). Boar spermatozoa (n = 6, 30 ejaculates) were centrifuged and diluted in standard BF5 freezing medium without glycerol at room temperature. Only semen samples with at least 70% motile sperm after re-suspension in BF5 freezing medium and before freezing were used for the experiment. Sperm suspensions were slowly cooled over 3 h to 5°C, BF5 medium with glycerol (final concentration 1% v/v) was added at 6.5°C and diluted spermatozoa were then packaged in 0.5 ml plastic straws. Straws were further cooled to −5°C and were then immediately frozen over nitrogen vapour, plunged and stored in liquid nitrogen. Thawing was carried out after 30 days of cryopreservation, plunging the straws in a water bath at 39°C for 30 s, thawed spermatozoa were then poured in dry plastic tubes and one drop was diluted 1:10 (v/v) in BTS medium for sperm assessment (viability, motility). Viability of fresh spermatozoa stained by either Eosin-Nigrosine or SYBR14-PI was not different: 90% vs. 91% respectively. In contrast, viability of frozen–thawed spermatozoa was different: 66% vs. 35% ($p < 0.05$) respectively. Further details on sperm cryo-survival from this work have already been published (Garzon-Perez et al., 2010, CryoLetters, 31, 438–44). Similar findings have been observed in fowl and bull spermatozoa; explanation for the discrepancy in the output of both techniques may be related to the molecular weight of stain components, time of sperm exposition to stains and interference of glycerol and egg yolk (freezing media) with the stain. In conclusion, taking in consideration motility of frozen–thawed spermatozoa was about 45%, sperm viability assessed by SYBR14/PI seems to be more accurate. C Garzon-Perez was supported by the Mexican government (CONACYT). Project partially supported by Universidad Nacional Autonoma de Mexico (PAPPIT IN207009).

P32

Assessment of the fertilizing ability of trehalose/cryopreserved boar sperm by the Dual HOST/BBC assay

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Sperm function test help to evaluate sperm quality of frozen–thawed samples and allow predictions about the potential fertilizing ability of the tested samples. Furthermore, trehalose is a disaccharide, which maintains sperm integrity during cryopreservation. The aim in the present study was to evaluate the fertility of frozen–thawed boar sperms cryopreserved in two different media using the hypo-osmotic swelling test in combination with Blue Brilliant Coomassie stain (HOST/BBC test) for simultaneous evaluation of functional membrane and acrosome integrity. Sperm were placed into the designated freezing medium: T1 (20% egg yolk, 1% glycerol, 250 mM trehalose) or G3 (20% egg yolk, 3% glycerol, and 230.8 mM dextrose). At 15 days of cryopreservation, samples were thawed and they were evaluated by dual test HOST/BBC in addition to the thermo-resistance rapid test (TRT) and a homologous *in vitro* penetration (hIVP) assay, using *in vitro* matured pig oocytes. Results of the TRT showed that G3 sperm maintained the best type of movement ($p < 0.005$). However sperm from T1 maintained higher acrosomal integrity ($p < 0.005$). After counting the positive HOST/BBC sperm, the ones with intact acrosome but

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damaged plasma membrane were discarded. As a result no statistical difference was observed between groups (T1 20.0 ± 3.7% vs. G3 23.2 ± 4.2%). On the other hand, although the number of sperm per oocyte was slightly higher in T1, no significant difference was found in the percentage of oocyte penetration rates ($p > 0.05$) between both samples. According to the criteria proposed by Gadella et al. (1998), both presented intermediate percentages of oocyte penetration (62.6 ± 6.6% vs. 52.8 ± 5.3%). In conclusion, the dual HOST/BBC test is a rapid simple method that allows evaluating the fertilizing ability of frozen thawed sperm, could be applied as a routine technique in semen analysis. This test discarded damage cells, and then no differences were detected in the samples cryopreserved in either T1 or G3 media.

P33

Measurement of intracellular calcium dynamics with Fluo-4 AM in frozen–thawed boar sperm by flow cytometry

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Semen cryopreservation is an important tool for assisted reproduction, although the fertility of frozen–thawed spermatozoa is reduced, possibly due to precocious capacitation-like changes that are known to occur. The aim of our study was the evaluation of intracellular free calcium level changes in cryopreserved boar sperm using an improved Fluo-4 acetomethoxy ester (AM) (Invitrogen), combined with a viability assessment to remove dead cells from the analysis. Frozen–thawed semen from four different boars was divided into two parts. Control and washed with Porcipure™ (Nidacon) (300 ***g***, 10 min, washed with Porciwash™) with or without 1 μM calcium ionophore (A23187). Ejaculates were collected by the gloved-hand method. Semen was cryopreserved according to Eriksson (2000, Anim Reprod Sci, 63, 205–20), packaged in 0.25 ml straws, and thawed in water at 50°C for 12 s. Fluo-4 was used at 10 nM (adjusted for boar spermatozoa). Cellular viability was assessed with propidium iodide (PI) by flow cytometry (FACScalibur; Becton Dickinson). Fluo-4 and PI fluorescence was acquired using a 520/10 and a 620/20 nm filter, respectively. The samples were incubated from 15 to 150 min in the dark. Data were analysed with linear mixed-effects models, studying time, male and treatment (ionophore and washing) as effects. Incubation time did not affect the proportion of viable spermatozoa or viable spermatozoa with increasing calcium levels. Porcipure washing slightly decreased viability (60.1 ± 0.6% vs. 66.6 ± 0.9%, $p < 0.05$). Ionophore increased (17.3 ± 0.1% vs. 1.4 ± 0.7%; $p < 0.05$) Fluo-4 positive spermatozoa in control and washed cells. There were no significant differences in Fluo-4 positive spermatozoa among control and washed samples, irrespectively of its exposure to ionophore. There were significant differences between individuals ($p < 0.05$). In conclusion, Fluo-4 AM combined with PI may be used to detect intracellular calcium increase in boar spermatozoa. Porcipure washing did not seem to affect the effect of the ionophore, although there was a high variability among males regarding the response of spermatozoa to the treatments. Supported by DPI2009-08424.

P34

Incidence of sperm with cytoplasmic droplets in boar ejaculates is associated with reduced responsiveness to bicarbonate *in vitro*

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The aim was to test whether the ability of spermatozoa in diluted boar semen samples being responsive to bicarbonate under capacitating conditions *in vitro* is linked to other sperm parameters. Therefore, 78 semen samples diluted in Beltsville Thawing Solution (BTS) from 13 different boar studs were examined at 24 h of storage for motility (CASA) and morphology. The integrity of plasma and acrosome membrane was flow cytometrically assessed with propidium iodide (PI) and FITC-PNA. Kinetics of changes in cytoplasmic Ca^{2+}-concentration in the plasma membrane intact sperm population were monitored with PI and Fluo-3/AM within 60 min incubation at 38°C in two variants of Tyrode's medium, containing either bicarbonate and calcium (medium A; at 5% CO_2) or only calcium (medium B). Specific response to bicarbonate (R_{60Bic}) was defined as the difference in the decline of % PI- and Fluo-3 negative sperm within 60 min of incubation in medium A and B ($R_{60Bic} = \Delta_{A3\text{-}A60} - \Delta_{B3\text{-}B60}$). Despite considerable variability, there were neither significant correlations between the specific response to bicarbonate (range: 3.4–69.3%) and the percentage of total or progressive motile sperm (70.9–98.4% and 42.4–96.7%, respectively), nor with the percentage of PI- and FITC-PNA-negative sperm (39.8–98.3%; $p > 0.05$). However, a negative correlation existed between the specific response to bicarbonate (R_{60Bic}) and the number of morphological abnormal sperm per sample (1.5–54.0 %; $r = -0.72$; $p < 0.001$). Sperm with cytoplasmic droplets (0.5–37.0% per sample) accounted for the most frequent observed defect (47.3%) of all abnormal sperm. Correlation of R_{60Bic} was negative with respect to this specific defect ($r = -0.68$; $p < 0.001$). In conclusion, semen samples with higher incidence of sperm with cytoplasmic droplets show a reduced responsiveness to bicarbonate, whereas other sperm parameters are not related to the bicarbonate response. As retained cytoplasmic droplets are considered as a sign of immaturity, incomplete sperm maturation seems to be associated with impaired membrane function affecting the signalling cascade of the capacitation process.

P35

The relationship between sperm nuclear shape and boar fertility using Fourier harmonic analysis

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The objective of this study was to characterize sperm nuclear shape of boars with known fertility using Fourier harmonic analysis (FHA). Harmonic amplitudes 0–5 (HA0–HA5), derived from FHA were previously shown to be an accurate, objective and repeatable measure of sperm nuclear shape. Boars ($n = 108$) from five different farms and seven different genetic lines had an average of 110.2 ± 7.7 single sired matings and were assigned to either an acceptable ($n = 96$)

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or unacceptable fertility group (n = 12) based on a farrowing rate (FR) < 60%. The average FR was 82.01 ± 0.98% and 54.75 ± 0.88%, respectively. Semen was collected from each boar, analyzed for motility, fixed (2.9% Na citrate + 0.2% paraformaldehyde + 0.003 mg/ml BSA) and shipped to Wisconsin, USA to be analyzed. Cells were stained with Hoechst-33342 for 30 min at 39°C, washed and then dried on a microscope slide. Cells were imaged using a Nikon phase contrast microscope configured for epifluorescence and a Nikon camera. Phase and Hoechst images were analyzed with an Image J program written for FHA. The data revealed that fertility groups differed in sperm nuclear shape (p < 0.05) and that only HA2 (1.136 ± 0.005 vs. 1.098 ± 0.011 μM) and HA4 (0.227 ± 0.003 vs. 0.202 ± 0.008 μM) were significant between the acceptable and unacceptable groups, respectively (p < 0.05). Diagnostic statistics determined the best discriminate model for predicting the fertility group membership of a particular boar and evaluated values for true positive (TP, classifying an acceptable boar correctly and true negative (TN, classifying an unacceptable boar correctly). The model with the fewest parameters and highest TP + TN value included HA0-HA5 values for mean, log variance and skewness. This model was highly significant (p = 0.035) and correctly identified all 12 unacceptable boars. The data revealed that there are differences in sperm nuclear shape between boars with acceptable and unacceptable fertility that supports previous data in the boar. However, a new set of boars needs to test the current model to determine if the model can be used as fertility predictor.

P36

Single layer centrifugation with Androcoll-P can be scaled-up into 500 ml tubes

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Single layer centrifugation has been used to select boar spermatozoa with the best motility, morphology and survival from the rest of the ejaculate (Morrell et al., 2009, J Reprod Dev, 55, 547–52). The SLC method was scaled-up for use in 100 ml tubes, processing 25 ml extended semen per tube (SLC-25) (van Wienen et al., 2010, Veterinary Science, doi: 10.5402/2011/548385) and has subsequently been used for 200 ml tubes (SLC-60) (van Wienen et al., unpublished data).

Objective: To determine whether SLC with Androcoll-P can be scaled-up further into 500 ml tubes (SLC-150).

Methods: Aliquots (150 ml) of boar semen extended to 100×10^6 spermatozoa/ml were prepared by (1) SLC on top of 150 ml Androcoll-P (Large) in a 500-ml tube (SLC-150); and (2) by simple centrifugation (sperm "washing"). After centrifugation at 300 ***g*** for 20 min the supernatant was removed and the sperm pellets were resuspended in Beltsville Thawing Solution (BTS) supplemented with bovine serum albumin (5 mg/ml). In another experiment, aliquots of semen were prepared by SLC-150 or by SLC-15 [150 Androcoll-P (S) and 15 mL Androcoll-P Large, respectively]. Sperm motility in uncentrifuged control samples and in the SLC-selected samples was measured by computer assisted motility analysis using the Cell Motion Analyzer, both on the day of preparation and after 7 days.

Results: Although centrifugation without a colloid (sperm washing) was detrimental to boar sperm survival, the SLC-selected spermatozoa showed higher motility than uncentrifuged controls (wash 61%, SLC-150 82%, control 68%), both immediately and after storage for 7 days at 16–18°C (wash 55%, SLC 75%, control 70%). The yield from SLC-150 was 80%. In the second experiment, sperm motility was also better in the SLC-selected samples than in the control (time 0: SLC-15 92%, SLC-150 87%, control 66%; 7 days: SLC-15 69%, SLC-150 91%, control 64%).

Conclusion: SLC-150 with Androcoll-P selects the most motile spermatozoa from boar semen and can aid sperm survival during storage, particularly where Androcoll-P (S) is used. Funded by Jordbruksverket, Jönköping, Sweden.

P37

Fertility of boar spermatozoa prepared by single layer centrifugation in *in vitro* fertilization (IVF)

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Single Layer Centrifugation (SLC) through Androcoll-P selects good quality boar sperm. However, *in vitro* fertilization (IVF) with these spermatozoa resulted in polyspermic zygotes and few blastocysts. In the present study, the ability of SLC-prepared spermatozoa to fertilize *in vitro* matured oocytes with subsequent development to the blastocyst stage was assessed using lower oocyte:sperm ratios than previously. Spermatozoa selected by SLC or by standard centrifugation (control group) were re-suspended in IVF medium. Gametes were co-incubated for 24 h at oocyte:sperm ratios of 1:100 or 1:300 for SLC (8 or 24×10^3 cells/ml, respectively) and at 1:100 for the control. Presumptive zygotes were then transferred to culture medium. Cleavage rate was assessed on day 2, with non-cleaved oocytes being stained with Hoechst 33342 to assess sperm-zona binding and polyspermy. The embryos were cultured up to day 7 when blastocysts were stained to count the number of nuclei. The following parameters were recorded: number of spermatozoa binding to the zona, polyspermy, cleavage rate, development to blastocyst, and number of nuclei in the blastocysts. Statistical analysis was done using one-way ANOVA. Data are shown as mean ± - SEM. The control and SLC 1:100 did not differ in any of the parameters listed. For SLC 1:300, the number of spermatozoa bound to the zona was higher (p < 0.001) (5.4 ± 0.4, 5.9 ± 1.3, 20.4 ± 2.5 for control, SLC 1:100 or 1:300, respectively) although polyspermy was not significantly different between groups (34.3 ± 8.2%, 40.9 ± 12.0%, 56.2 ± 8.5% for control, SLC 1:100 and 1:300 respectively). Cleavage rate was reduced for SLC 1:300 (53.6 ± 6.0%, 54.6 ± 4.0%, 31.6 ± 5.0% for control, SLC 1:100 and 1:300; p < 0.01), as was the number of embryos developing to the blastocyst stage (17.9 ± 4.9%, 16.6 ± 2.8%, 5.3 ± 1.6% control, SLC 1:100 and 1:300; p < 0.05). However, embryos reaching the blastocyst stage were similar in quality and nuclei counts in all groups. To conclude, sperm function was not altered by Androcoll-P although apparently very low sperm concentrations are needed to avoid polyspermy. Thus, SLC can be used to select high quality spermatozoa for IVF with low sperm numbers. Funded by Formas and SLF (V0750239).

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IV Artificial Insemination

P38

Recommended procedures of insemination of sows and their probable consequences

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In 2005 in 24 European countries 60.74% of 15,071,000 sows were in 2.38% of herds holding more than 200 sows. Average number of sows per herd was 542.5 sows. In such situation French experts advised to transfer gravid sows and gilts to the farrowing room on Monday and to wean sows on Thursday after the suckling period, which at the time lasted 27.2 days. For weaning on Thursday morning and detection with the boar twice daily it has been recommended to inseminate weaned sows, when estrus starts on Monday or Tuesday, 24 h after the detection the first time, 36 h after the detection the second time and the third time 48 h after the detection, if the sow is still in estrus. If estrus starts on Wednesday or Thursday the first AI was recommended to be 12 h after the detection, the second AI 24 h after the detection and the third AI 36 h after the detection, when the sow is still in estrus. In the case that estrus starts on Friday, the first AI is recommendable to be executed on the detection and, if the sow is still in estrus, the third AI should be performed 24 h after the detection. Since it has been scientifically reported, from the sows weaned on Thursday the percentages of sows found in the heat could be 0.67% on Sunday, 6.69% on Monday, 43.91% on Tuesday, 20.64% on Wednesday, 5.39% on Thursday, 2.10% on Friday, 1.16% on Saturday and 0.62% on next Sunday. It was found out earlier on that from the start of heat till the beginning of the period of lust passed 24 h. From the start of period of lust till the beginning of the ovulation passes 24 h. The ovulation lasts 18 h, the period of lust 58 h and the heat 90 h. Eggs can be fertilized during 5 h. Concerning just mentioned data it was found out that after weaning on Thursday from Monday till Friday, working according to the recommendations of French experts, 22.31% of sows would be inseminated without reason, 21.42% too late, 32.45% at the end of the period of lust, 28.13% in advance and 28.13% favourably. The use of 78% more dozes of the boar's semen than it is biologically based is probably only one consequence of implementing of broadly accepted recommendations.

P39

Fertility of sows after AI with extended semen from two portions of boar ejaculate, stored at 5 or 17°C

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Spermatozoa present in the first collectable 10–15 ml of the sperm-rich fraction of the boar ejaculate (portion 1, P1) have shown a higher viability during handling, cooling and cryopreservation, compared with spermatozoa in the rest of the ejaculate (portion 2, P2). However, since fertility of sows inseminated with spermatozoa from P1 or P2 have not been compared, the follow-up experiment was designed in order to seek eventual differences in cooling survival and fertility between spermatozoa present in both portions. Two portions of boar ejaculate – P1 and P2 – were collected weekly from five mature boars and 40 DB25® sows were inseminated with 3×10^9 motile spermatozoa, from four treatments: T1 – spermatozoa from P1 extended in MR-A® and cooled at 17°C; T2 – spermatozoa from P1 extended in glycine-egg yolk (GGO) and cooled at 5°C; T3 – spermatozoa from P2 extended in MR-A® and cooled at 17°C; and T4 – spermatozoa from P2 extended in GGO and cooled at 5°C. All doses were kept in a special container, previously described (Roner et al., 2006, Arq Bras Med Vet Zootec, 58, 78–85). Spermatozoa motility, vigour, and morphological characteristics were evaluated over 72 h of storage. All treatments kept an acceptable motility at first 24 h of storage (54.5%, 64.5%, 66.0% and 74.5% for T1, T2, T3 and T4, respectively). The T4 kept a similar motility (70%; $p > 0.05$) over storage time (72 h) while others treatments showed a decrease in motility (15.0%, 30.0%, and 45.0% for T1, T2 and T3), mainly after 24 h of storage. Spermatozoa extended in GGO and cooled at 5°C showed higher ($p < 0.05$) acrosome damage compared to spermatozoa extended in MR-A® and cooled at 17°C (6.5% vs. 1.1%). The influence of treatments on the conception rates was not observed ($p > 0.05$) and they were 100% for T1, T2, and T3 and 80% for T4. The total number of piglets born alive was 16.30 ± 1.21, 12.80 ± 1.21, 14.90 ± 1.21 and 14.13 ± 1.35 for T1, T2, T3, and T4, respectively ($p > 0.05$). However, the total number of piglets born alive was superior for spermatozoa extended in MR-A® and kept at 17°C in comparison to spermatozoa extended in GGO and kept at 5°C (14.3 ± 0.8 vs. 11.8 ± 0.7, respectively; $p < 0.05$).

P40

Whey proteins in the commercial extender TRIXCell+ improve the performance of boar semen: prolonged storage capabilities and higher litter size

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The *in vivo* performance of swine semen diluted in a new liquid medium TRIXCell+ is presented. The new extender TRIXCell+ is based on TRIXCell, a commercial swine semen extender, combined with a particular mixture of proteins extracted from whey. *In vivo* trials covering 416,446 routine inseminations performed on 16 large commercial swine production farms during the years 2007 through 2009 showed that the farrowing rate obtained using semen diluted in TRIXCell+ and stored for 3–5 days is similar to that of semen stored for 1–2 days in Beltsville Thawing Solution (BTS). But the use of TRIXCell+ resulted in a consistently 0.6 higher litter size compared to BTS. The increase in profitability of an average professional AI center and of swine production farms by using TRIXCell+ with prolonged storage capabilities and increased litter size is discussed.

P41

Implications of dose volume and sperm concentration in pig post-cervical artificial insemination

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In the last 5 years different artificial insemination techniques for pigs have achieved great importance. Main reason for these techniques is the reduced number of spermatozoa

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required for insemination and such systems have contributed to improve economics and daily management in several pig farms. Moreover, it enables efficient use of boars with the best genetics. Most of the recent studies on porcine AI only account for the sperm concentration applied, neglecting the volume of the seminal dose. In that respect, some authors have referred already to the importance of the volume inseminated in field conditions (Sumransap, 2007, Reproduction in Domestic Animals, 42, 113–7). The objective of this study assessed the effect of two different sperm concentrations in two different volumes applied for artificial insemination under field conditions. A total of 153 Landrace × Large White crossbreeding weaned sows were inseminated. Only sows from first to forth farrowing were used (no gilts). The study was conducted for a total of 4 weeks. The first insemination was performed at 24 h post-estrus detection while the second insemination was applied at 24 h after the first insemination. Artificial inseminations were performed employing a double deposition catheter (Magaplus DD®). Sow populations were randomly distributed into four groups each week, depending on the type of seminal dose used ($\times 10^6$ sperm/ml: 1500/50, 1500/30, 750/50 and 750/30). Ejaculates of two fertility-tested boars were employed in heterospermic conditions. Fertility was checked at 25 days by ultrasonography. Total litter sizes were counted (both dead and live piglets were included). Under our test conditions, we observed significant differences in pregnancy rate (%) related to the volume inseminated (30 vs. 50 ml, $p < 0.001$). However, there were no significant differences between both groups with different sperm concentrations of 1500 vs. 750 million spermatozoa ($p = 0.482$). In addition, no volume based or sperm concentration related differences were observed for litter size ($p = 0.186$; $p = 0.997$). These results suggest that under the conditions of our study (using Magaplus DD® catheter), it is possible to use either 750 or 1500 million spermatozoa per dose with no significant detrimental effect in pregnancy rate or prolificacy performance when 50 ml are inseminated. However, a slight reduction was observed when 30 ml seminal doses were employed. We conclude that the dose volume appear to be restrictive for pregnancy rates when inseminations are performed with 750 or 1500 million spermatozoa. Therefore, these results may attract attention to dose volumes used in pig artificial insemination under field conditions.

P42

Seminal plasma and spermatozoa modulate gene expression in the porcine uterus

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Central reproductive features like ovulation and implantation are influenced by the maternal immune system. This study aimed to investigate whether such processes might be initiated by exposure of the uterus to inseminate components. Using a custom-made microarray the expression of 384 immune-relevant genes in the porcine endometrium after insemination was examined. Synchronized German landrace (GL) sows were inseminated (five sows/group) either with spermatozoa (S) + seminal plasma (SP), S + PBS, epididymal sperm + PBS, SP alone, PBS alone or left without insemination. The doses contained fresh semen from a fertile GL boar washed in PBS and extended to 3×10^9 sperm in 100 ml of the appropriate diluent or diluent alone. Endometrial samples were collected 2 h after AI and stored at −196°C until mRNA extraction and analysis with a Geniom Biochip. Group comparisons showed the differential regulation of 31 genes ($p < 0.05$). Neither SP alone nor Sperm in PBS changed the gene expression compared to the negative control. However, the combination of sperm and SP lead to a significant down-regulation of 14 genes and the up-regulation of PPARα, a transcription factor modifying cytokine expression. The network functions mainly associated with the regulated genes were determined to be cell-to-cell signaling, inflammatory response, cellular movement and hematological system development and function. In conclusion, inseminate components influence the initial uterine immune response and thus might also shape the following reproductive events.

V Bacterial Contamination of Boar Semen

P43

Bacterial and fungal burden in fresh and diluted boar semen

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Regardless of the aseptic conditions in which the semen is collected, the presence of the bacterial flora in the seminal material cannot be contested. A trial has been conducted on one hundred ejaculates, collected from 20 different boars ($n = 20$), in two farms. A micro-bacterial examination was performed immediately after the collection of the material, both quantitative and qualitative, and also 12 h later after the collection. In the raw semen, an average value of 41×10^3 CFU/ml was registered. In the first farm, the variations determined were between 22.4×10^3 and 118.2×10^3 CFU/ml, with a average value of 65.4×10^3 CFU/ml. The second farm registered a average value of 99.33×10^3 CFU/ml. From the 14 types of bacteria identified, the most frequent were: *Escherichia coli* with 81%, *Staphylococcus aureus* and *S. Hyicus* with 72%, *Pseudomonas aeruginosa* 63%, *Streptococcus* spp. 45%, *Proteus* spp. 36%. Also identified, but with a lower frequency (27%), were *Yersinia enterocolitica* and *Y. ruckeri*, *Pantoea* spp., *Shiqella* spp., *Seratia marcescens*, *Tatumella* spp. After the dilution of the seminal material, the total number of germs decreased to 0.354×10^3 CFU/ml (0.120×10^3–0.588×10^3). The extenders used contained anti-bacterial substances, but not any fungicide. A number of nine types of fungi were observed in the seminal material, *Geotrichum* spp. 72%, *Penicillium* spp. 63%, *Aspergillus* spp. 63% being the most frequent, but the semen also contained in variable percentage *Mucor* spp. 45%, *Fusarium* spp. 36%, C*ladosporium* spp. 36%, *Alternaria* spp. and *Acremoniu* spp. 18%. A highly important fact noticed 12 h after the dilution was the presence of yeast such as *Candida parapsilosis* 91%, and *C. sake* 92% in 90% of the determinations made. Only 8% of the semen samples examined 12 h post-dilution were sterile, while 92% of the samples contained germs, in 74% of the cases, an association flora being present. Semen preservation at +17°C for 5–7 days provides bacteria and especially yeasts favorable conditions for their biochemical and metabolically processes and as a consequence negative effects on of semen quality and fecundity may appear.

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P44

Bacterial contamination of boar semen and its effect of sperm quality parameters during conservation

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Bacterial contamination of boar semen is frequent and has many sources. Moreover, it can be also due to the developed resistance against some antibiotics incorporated in semen extender. Basically, this study was designed to check bacterial contamination of commercial porcine semen in Spain, and to determine the effect of bacteria infection on sperm quality parameters during *in vitro* storage. Bacterial content in semen samples was assessed. Boar semen diluted with long term conservation extender and free of antibiotics was divided in aliquots and infected with 10^6 CFU/ml of pure isolated and identified strains: *Klebsiella oxytoca*, *Burkholderia cepacia*, *Proteus mirabilis*, *Serratia marcescens*, *Myroides* spp., *Morganella morganii*, *Providencia rettgeri*, *Achromobacter xylosoxidans*, *E. coli* and *Pantoea* spp. Samples were analysed for sperm motility, viability, agglutination, HOST and ORT test after 1, 4 and 7 days of storage at 17°C. Results of total commercial semen samples showed 26.24% of bacterial contamination, from which 18.9% were Gram+ and 81.08% Gram−. In samples of *in vitro* infected sperm with pure strains, motility of sperm with *Burkholderia cepacia* showed a slight decrease (10%) after 24 h of conservation, while the rest of samples maintained motility over 80%. After 4 days, sperm motility was significantly reduced in all samples except semen with *Pantoea* spp., *Myroides* spp., *Achromobacter xylosoxidans* and *Proteus mirabilis* ($p = 0.0064$). Agglutination did not exhibit differences between samples after 24 h of storage, however, this parameter clearly increased after 4 days ($p = 0.034$). Furthermore, functional integrity of sperm membrane was altered significantly since 24 h of storage. ORT parameter was also significantly altered after 4 days with some strains. Nevertheless, sperm viability parameter was not affected significantly and maintained over 85% in all samples during storage. It was concluded that bacterial infection reduced sperm quality during storage, probably, by different mechanisms developed by each bacteria such as agglutination which inhibits motility, action on sperm membrane and ions channels reducing its functionality, without alteration of viability.

P45

Antibiotic effects on boar sperm motility and movement characteristics

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Presence of bacterial contamination in pig seminal doses is frequent in boar studs, and may have a negative effect on its durability and functionality, even using ejaculates with good spermatic quality. Use of antibiotics in semen extenders is required. However, the choice of appropriate antibiotics, doses and formulation to be used is very important. This study was designed in order to investigate the effects of 10 commonly used antibiotics on boar sperm motility and movement characteristics. Antibiotics: A1 and A2 (Aminoglycosides), A3 (Penicillin), A4 and A5 (Fluoroquinolones), A6 (Amphenicols), A7 and A8 (Aminoglycosides), A9 (Polypeptide) and A10 (Cephalosporin) were purchased from Sigma-Aldrich (identity of antibiotics is not revealed for commercial reasons). Boar net semen samples were diluted with Vitasem® extender without any antibiotics to reach 30 million esp/ml. Samples were incubated with five increased concentrations (0.3, 1, 2, 4 and 8 mg/ml) of each antibiotic. CASA system examination of diluted semen samples stored at 17°C for 1, 2 and 7 days was carried out to estimate the effects of antibiotics on the motility parameters of spermatozoa: VCL, VSL, VAP, LIN, and ALH. Remarkably, it was found that some antibiotics exhibit a dependent concentration and a positive relationship with sperm motility such as A2, and a negative relationship in case of A10 and A5 ($p < 0.001$). However, many antibiotics were non-toxic to sperm and showed good motility and viability (> 70%) during 7 days of sperm preservation. Aminoglycosides and Polypeptides did not show a concentration dependent for VCL, VSL and VAP parameters, while significant dose-dependent inhibition in percent rapid-moving spermatozoa was observed in case of fluoroquinolone and cephalosporin. In case of A8, VCL (37.66 ± 1.4 μm/s) and ALH (1.7 ± 0.08 μm) were lower in the first day of sperm conservation, and higher after 7 days (50.99 ± 8.2 μm/s) and (1.9 ± 0.2 μm) respectively. In addition, in case of A9, the value of VCL was maintained higher all the time during sperm conservation ($p = 0.006$). All data demonstrated that the choice of the correct concentration and combination of antibiotics could improve prevention of sperm contamination maintaining the capacity of semen functionality and fecundity.

P46

Bacterial contamination of boar semen and antibiotic sensitivities

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Bacterial contamination in extended porcine semen occurs frequently in artificial insemination centers, and may occur during whole semen processing. The majority of bacteria can present a detrimental effect on semen conservation, its fecundity and reproductive parameters. The choice of the appropriate antibiotic treatment included in semen extender is very important, in particular regarding semen toxicity. The aim of this study is to check the main bacterial species isolated from seminal doses, to assess the activity of antibiotics commonly used against these microorganisms and to verify possible resistances of bacteria. The study was carried out with commercial extended semen coming from more than 100 Boar studs located throughout the Spanish geography. Bacterial cultures from semen samples, isolation and identification were performed. Antibiograms for all isolated bacteria were carried out using 29 antibiotics. Statistical analysis was developed with relative frequency description for bacterial isolates (StatView 5.0 statistical package). Semen culture showed that 26.24% of examined samples were infected (more than 300 CFU/ml), of which 21.2% were Gram-positive and 78.7% Gram-negative. In total 113 species of bacteria were isolated and cloned. The most commonly found in extended semen samples were: *Proteus vulgaris* (3.5%), *Serratia marcescens* (6.19%), *Serratia liquefaciens* (12.39%), *Stenotrophomonas maltophilia* (7.96%), *Staphylococcu* spp. (9.4%), *Cedea*

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(4.4%), *Micrococcus* spp. (5.3%), *Morganella mornanii* (4.4%) and other species (*E. coli*, *Bordetella*, *Enterococcus*, *Klebsiella*, *Kurthia*, *Pseudomonas* etc.). Analysis of antibiograms of all isolated bacteria showed a higher sensitivity profile of Gram-positive bacteria to antibiotics (sensitivity to 18 of 29 total antibiotics), and lower of Gram-negative (sensitivity to 9 of 29 total antibiotics). Probably, this result is due to the low frequency of Gram-positive in total population of bacteria which contaminate boar semen. It is surprising to observe that sensitivity of Gram-negative bacteria exactly to nine antibiotics is similar in total isolated bacteria. This finding implies an important tool to select the good antibiotic for semen effective preventive treatment or biotherapy, against large spectrum of bacteria.

P47

Antibio-treatment of boar semen infected *in vitro* with bacteria: time and concentration dependence of antibiotic action

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Bacteriospermia in pig seminal doses occurs with some frequency in artificial insemination centers, and may have a negative effect on its durability, even using ejaculates with good sperm quality. Usually, the source of this contamination is the own boar. However, other sources can cause bacterial contamination. To overcome this problem it is essential to have a good hygiene and disinfection protocol. Furthermore, it is necessary to use extenders with an effective antibiotic combination. The aim of this study was to check the appropriate combination of antibiotics to control bacteria. Boar semen was collected into extender free of antibiotics. Samples were divided in aliquots and infected with 10^6 CFU/ml of two strains: *Serratia marcescens* (Gram-negative), and *Micrococcus* spp. (Gram-positive). Infected semen samples were diluted to reach 30 million sperm/ml and incubated with 4 increased concentrations of 10 antibiotics: A1 (beta lactam), A2 (cephalosporin), A3, A4, A5, A6, and A7 (aminoglycosides), A8 (polypeptide), A9 (macrolide), A10 (fluoroquinolone) (identity of antibiotics is not revealed for commercial reasons). Experimental concentrations were a multiple of the minimal inhibitory concentration (MIC) of each antibiotic (according to CLSI): 1×, 5×, 25× and 125× μg/ml. All samples were incubated at room temperature and cultivated for bacteria content assessment after 50 min and 24 h of treatment. The results showed a significant reduction in both bacteria content after 50 min of incubation with some antibiotic concentrations. Interestingly, *Serratia marcescens* and *Micrococcus* spp. content in culture after 50 min of treatment was < 1 CFU/ml with 25× MICs concentration of A1, A3 and A10 and 125× MIC concentration of A2. At lower concentration, content of *Serratia marcescens*, and *Micrococcus* spp. was reduced slightly after 50 min, but significantly to < 1 CFU/ml after 24 h. Only *Micrococcus* spp. content was reduced to < 1 CFU/ml with A5 (at 5× μg/ml of MIC), A6, A7, A8 and A9, and 125× (at 125× μg/ml of MIC respectively). This finding suggests a new possibility of antibiotic combination for bacteriospermia treatment in boars.

P48

Variation on PCV2 – DNA shedding in boars' continuous ejaculates and semen quality in PRRS (−) farms in Venezuela

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Reproductive form of PCV2 is a well know situation in pig farms. Vertical transmission of PCV2 has been well documented (Bermudez et al., 2008, Proc 20th IPVS, Pag. OR0161). Shedding of PCV2 in semen is known but not fully demonstrated (Opriessnig et al., 2008). Abortions, mummies, early embryonic death (EED), stillborn and weak newborn piglets are seen in PCV2 in gilts and sows (West et al., 1999, J Vet Diag Invest, 11, 530–2; Bermúdez et al., 2008; Pittman, 2008, Journal of Swine Health and Production). Sperm quality can be lower or borderline without testicular damage but cases of hydrocele and testicular degeneration accompanied with sperm agglutinations might occur. The main objective proposed was to evaluate the shedding of PCV2 genome in semen and its relationship to fertility in PRRS (−) farms. In total 20 boars were studied. They belonged to two different farms, 12 from farm A and 8 from farm B. All boars breeding soundness (BSE) was evaluated clinically and their ejaculates tested three times at least 1 week apart. A paired serum and seminal plasma samples from each boar was assessed by ELISA Ag-Capture (Symbiotech®). A centrifuged sperm pellet from each boar ejaculate was paraffin-embedded and blocks stained by IHC (ISU-VDL). In three replicates PCV2 DNA shedding assessed by rtPCR turned positive in five out of 12 boars (41%) to PCV2 with large variation in shedding rate. It is very revealing the fact that PCV2 semen shedding is markedly inconsistent by time (copies of DNA between 25 to 40 C_t to nil 1 month later). This shows the significance of vertical transmission of the disease in a time/PCV2 shedding frame fashion through boar semen. Farm B (four out of eight PCV2, 50%) showed positive boars with similar trend as seen in farm A. The IHC showed all boards from both farms to be negative to PCV2 MAb staining. In contrast, captured Elisa test on seminal plasma detected PCV2 Ag in farm A only in two boars and in one boar in farm B. With over 85% the BSE of boars indicated sufficient potential of fertility in all boars from both farms. However, in three out of five PCV2 boars in farm A primary abnormalities were above normal range and two out of four PCV2 boars on farm B had similar pattern. In addition, increased numbers of immature sperm (Spheroids) were found in those boars showing slightly increased numbers of leukocytes. In farm A in two consecutive years a hydrocele was misinterpreted as Orchitis and a few negative boars came up with testicular degeneration and large numbers of sperm agglutinations, and with a perivasculitis in the spermatic cord vessels of an euthanized boar. At the same time an increased in abortion rate was seen in the farm A including fetal fluids and heart homogenates with large amount of DNA (C_t) of PCV2 and histo-pathologically fetal lesions in the heart, kidneys, skin, spleen as well as nodes being the classical lesions of this virus. Our results confirm PCV2 shedding in boar semen and vertical transmission with testicular changes and abortions in farm A as well as abortions and PCV2 positive boars in both PRRS negative farms. Variation of the three different techniques used to detect PCV2 in semen and seminal plasma influenced in the results. A time shedding pattern of PCV2 in

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semen compared to the shedding rate was determined by rtPCR being more sensitive than the other tests. To our knowledge, appearance of lesions in boars is reported here the first time.

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Dicol®: new concept of bacterial contamination control in boar semen

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It is well known the frequent and inevitable contamination of porcine semen by bacteria due to several factors such as the developed resistance by bacteria to many antibiotics used in semen extender. Dicol® is a new medium containing a combination of bactericides acting against a large spectrum of Gram-negative and Gram-positive bacteria. It was designed for collection and preventive antibiotic treatment of porcine semen before final dilution with usual extender. The aim of this study is to demonstrate the effect of Dicol® on control of semen contamination *in vitro*. Boar semen was collected with 150 ml of Dicol® and divided immediately in 10 aliquots, which were infected with 10^6 CFU/ml of final concentration from pure isolated and cloned strains: *Klebsiella oxytoca*, *Proteus mirabilis*, *Serratia marcescens*, *Pseudomonas fluorescens*, *Morganella morganii*, *Providencia rettgeri*, *Achromobacter xylosoxidans*, *Escherichia coli*, *Delftia acidovorans* and *Pantoea* spp. Standard semen extender was used as control, and an extender free of antibiotics as negative control. All strains were checked for sensitivity to several antibiotics by antibiograms. Semen samples were incubated at room temperature, and assessed for bacteria content by culture after 10, 20, 25, 30, 40, 50 min and 24 h. Results showed a significant decrease of bacteria content in semen sample with Dicol® ($p < 0.0001$) and significant differences between strains. The maximum decrease (< 1 CFU/ml) was reached at 10 min of incubation with *Klebsiella oxytoca*, *Serratia marcescens* and *Delftia acidovoran*; at 20 min of incubation with *Proteus mirabilis* and *Providencia rettgeri*; at 30 min of incubation with *Pseudomonas fluorescens* and at 40 and 50 min of incubation with *Morganella morganii* and *Achromobacter xylosoxidans*, respectively. These last two strains showed multi-resistance to tested antibiotics. After 24 h, bacterial content was reduced drastically and reached < 1 CFU/ml in all samples with Dicol®. Moreover, control samples with standard extender exhibited a slight decrease of bacteria content and reached < 1 CFU/ml only after 24 h in case of *Klebsiella oxytoca* and *Pantoea* spp. We concluded that Dicol® is a new tool, which offers an interesting control of common bacteria in porcine semen.

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Decoupling dosing of antibiotics from degree of boar semen dilution: a necessity?

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Implementation of effective strategies for control and measurement for prevention of the development of antimicrobiotic resistance in boar studs depends on reliable data. The present systematic data report examination of the resistance situation in 10 facilities in Germany, Austria, and Switzerland. Semen samples of 133 boars were investigated by microbiological and spermatological tests. Live germ counts in native ejaculates fluctuated between 10^2 and 10^6 CFU/ml, with 50% of the samples in the medium ranges. In 26% of diluted ejaculates the live count was 10^2–10^5 CFU/ml after 48 h storage at 16°C. Thus, in three-quarters of the contaminated samples, live germ counts reached above 10^3 CFU/ml. A total of 25 different bacterial species and groups were found in native semen. Gram-negative bacteria represented up to 72%, and *Enterobacteriaceae* up to 44% of the total samples. The majority (53%) of the ejaculates contained coagulase-negative staphylococci and coryne-shaped rods. The contaminated and diluted ejaculates showed 16 different gentamicin-resistant bacterial species. Gram-negative bacteria amounted to 69% (*Enterobacteriaceae* 24%, pseudomonads 18%) and most frequently isolated Gram-positive bacteria were enterococci (18%). In three of the investigated boar studs no gentamicin-resistant bacteria were detected. A preliminary study of 14.620 diluted boar ejaculates demonstrated considerable variations in factually confirmable efficacious antibiotic concentrations per portion. It is obvious that a lower extender-to-semen ratio results in decreased concentration of antimicrobials in diluted semen tubes. One quarter of the analyzed samples revealed a reduction to 50% antibiotic output concentration. One of thousand diluted boar ejaculates showed no effective gentamicin concentration. Consequently, it becomes critical that dosage of antimicrobial additives be calculated independent of the degree of dilution. Thus, dosage of antibiotics has to be performed subsequent to the dilution. This will guarantee calculated quantities of antibiotics in each boar semen tube. Supported by FBF, Germany.

VI Genetic Aspects

P51

Improving fertility through reciprocal translocation identification in pigs

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Karyotyping for reciprocal translocations (RTs) was performed to understand the incidence of these chromosomal rearrangements in the North American PIC boar population. Reciprocal translocations, although rare, have serious implications on fertility and can cause up to a 50% reduction in litter size. Boars carrying an RT typically meet semen quality standards and have normal sexual behaviour; therefore, karyotyping boars using a fresh blood sample is the only way to determine if a translocation is present. One of the first papers describing an RT in a boar was published in the 1960s. Since that time, over 100 chromosomal rearrangements have been described in the pig. In 2009, Genus expanded their capacities to become the first lab in North America to offer a significant porcine karyotyping service. Blood samples from 2226 boars have been karyotyped across 12 different genetic lines. Boars averaged 24 weeks of age at the time of testing. Metaphase chromosome spreads were produced from each blood sample and images were analyzed using software that matches chromosome pairs based on size, the position of the centromere and banding patterns. Any boar that was found to have an RT was culled from production and their pedigree was investigated to identify any

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living relatives. Sows (n = 89) that were related to boars with identified RTs were also karyotyped. Results of the karyotyping revealed seven different translocation combinations t(1q-;2q+), t(6p-;7p+), t(4p+;9p-), t(7p-;15p+), t(5p-;17p+), two independent t(2q-;13q+) events and one double translocation t(7p-;15p+ and 5p-;17p+). Although researchers have been studying RTs since the 1960s, karyotyping has not been available on a significant scale in North America until recently. Even though RTs are rare, it is important to test for them given their detrimental impact on productivity and profitability for swine producers. Karyotyping has proven to be successful in identifying boars and sows carrying RTs and will help improve fertility in pork production systems.

P52

Effect of parity and lactational body weight loss on reproductive performance of a modern sow genotype

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This study investigated the effect of parity (PO) and body weight loss (BWL) during lactation on subsequent reproductive performance of a modern sow genotype (Camborough®23). Sows were weighed at post-farrowing and at weaning (n = 666). Sows were allocated into three classes of PO: PO1 (n = 254), PO2 (n = 184) and PO3-5 (n = 228) and into three classes of percentage of lactational BWL (≤ 0%, > 0 to ≤ 5% and > 5%). Lactation length averaged 21.2 ± 0.04 days being 10.1 ± 0.05 piglets weaned. After weaning, sows were monitored twice daily to check estrus onset and daily to check return to estrus after insemination. Litter data at next farrowing was recorded. Reproductive performance was evaluated as return to estrus rate, weaning to estrus interval (WEI) and litter size at next farrowing. At post-farrowing body weight averaged 185.3 ± 0.85; 217.1 ± 1.55 and 246.7 ± 1.8 kg for PO1, PO2 and PO3-5, respectively. Percentage of BWL averaged 1.6 ± 0.33, 1.2 ± 0.35 and 0.41 ± 0.31 for PO1, PO2 and PO3-5, respectively. The effect of BWL on return to estrus was more marked in PO1 than PO2 sows. Odd ratios to return to estrus were 9.0 and 8.9 for > 5% and > 0 to ≤ 5%, respectively, compared to ≤ 0% ($p < 0.01$). PO2 sows with > 0 to ≤ 5% of BWL had 4.9 times more chance to return to estrus ($p < 0.05$). BWL was not a risk factor to return to estrus in PO3-5 sows ($p > 0.05$). There was no effect of the interaction between parity class and weight loss ($p > 0.05$) on WEI and subsequent litter size. PO1 females showed longer WEI (4.6 ± 0.10, 4.1 ± 0.12 and 3.9 ± 0.11 days, respectively, $p < 0.05$) and smaller litter size at subsequent farrowing compared to PO2 and PO3-5 females (10.6 ± 0.25, 12.4 ± 0.28 and 12.6 ± 0.27, respectively; $p < 0.05$). BWL did not influence WEI (4.1 ± 0.14, 4.2 ± 0.10 and 4.4 ± 0.10 for > 5%, > 0 to ≤ 5% and ≤ 0% classes, respectively; $p > 0.05$). Regardless of PO, sows without BWL had higher subsequent litter size (11.6 ± 0.33, 11.5 ± 0.23 and 12.5 ± 0.23 for > 5%, > 0 to ≤ 5% and ≤ 0% classes, respectively; $p < 0.05$). The results demonstrate that BWL increases reproductive failure in young females and it affects subsequent litter size in all parities sows.

VII Sexed Boar Semen

P53

Sex-preselected piglets derived from surgical artificial insemination with sexed sperm

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Flow cytometry sorting of X- and Y-chromosome bearing sperm has been emerging as a promising technology to alter the sex ratio in progenies of mammals in the resent years. The objective of this study was to evaluate the efficiency of surgically artificial insemination (AI) by using the sexed sperm to produce sex-preselected piglet. Three recipients were inseminated with X-sorted sperm, all of which were pregnant and delivered a total of 12 piglets from which 11 were females (91.67%, 11/12), while 40% of piglets were female in the control group which were inseminated with unsorted sperm. One sow inseminated with Y-sorted sperm farrowed 6 male piglets (100%, 6/6) while the control group only derived 57.14% of male piglets. The total of sex identification reached 94.4% (17/18) and the piglets born from sexed sperm were healthy. No abnormality in term of weaning weight or other phenotype was observed comparing to those derived from unsorted sperm. There was no significant difference in pregnancy and farrowing rates between sows inseminated with sexed and unsexed sperm, but the litter size of sows inseminated with sexed sperm were lower than those inseminated with unsexed sperm. Results of this study indicate the feasibility of piglet production from sperm sorted by flow cytometry.

P54

DNA fragmentation in sex-sorted, frozen–thawed boar sperm

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Sperm sex-sorting process involves several stressful steps, which can exert a negative effect over sperm DNA integrity and then affect embryo viability. Reduced fertility and increased early pregnancy loss have been related to the application of sex-sorted frozen–thawed boar sperm. The objective of the present study was to evaluate DNA fragmentation of boar spermatozoa undergoing sex-sorting and subsequent cryopreservation. Sperm rich fractions (SRF) from three healthy and fertile hybrid boars (three SRFs per boar) were split into three aliquots to be subjected to three different handlings: (1) cryopreservation at 1000×10^6 sperm/ml in standard 0.5 straw protocol; (2) cryopreservation at 20×10^6 sperm/ml in standard 0.25 straw protocol; and (3) sex-sorted and cryopreservation at 20×10^6 sperm/ml in a standard 0.25 ml straw protocol. The fragmentation of sperm DNA was assessed using a commercial variant of the sperm chromatin dispersion test (Sperm-Sus-Halomax®; Halotech DNA SL, Madrid, Spain) at 5°C and at 150 min after thawing, and showed as percentage of sperm with fragmented DNA. All sperm samples exhibited a low percentage of sperm with DNA fragmented, always under 3%, irrespective of handling used.

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However, significant differences ($p < 0.05$) among handling were observed. Sperm samples frozen at 1000×10^6 sperm/ml showed higher percentages of sperm with DNA fragmented (1.8 ± 0.2 and 2.8 ± 0.1 at 5°C and 150 min post-thawing, respectively) than those either frozen at 20×10^6 sperm/ml (0.6 ± 0.1 and 0.9 ± 0.1 at 5°C and 150 min post-thawing, respectively) or sex-sorted and frozen at 20×10^6 sperm/ml (0.1 ± 0.1 and 0.3 ± 0.1 at 5°C and 150 min post-thawing, respectively). Although the low overall DNA damage obtained in the present study seems to have poor biological significance, we conclude that frozen at low sperm concentration resulted in a lower rate of DNA fragmentation, and sex-sorting handling did not affect negatively the DNA integrity of sex-sorted and cryopreserved boar sperm. Supported by MICINN (AGL2008-04127/GAN), Seneca Foundation of Murcia (GERM04543/07) and Sexing Technologies (Texas, USA).

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