RESEARCHREPORT

ANIMAL SCIENCE

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Title: *"Evaluation of contributions to seasonal reproductive inefficiency."* **NPB #14-052**

Investigators: Lea A. Rempel[1] and Jeremy R. Miles[1]

Coinvestigator: John Parrish[2]

Institution: [1]USDA, ARS, U.S. Meat Animal Research Center, Clay Center, NE;
[2]University of Wisconsin-Madison

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Industry Summary

Seasonal reproductive inefficiency is globally observed within the swine industry. Even when photoperiod and temperature are controlled, reductions in pregnancies and subsequent farrowing rates are noted. The most obvious contributor to this phenomenon is the young female. In all likelihood, we see greater effects of seasonality on the young dam as a result of her inherent nature to care for herself first and foremost before imparting energies into fostering a pregnancy. However, it has been coming to light that seasonality also has impacts on the male contribution; sperm. Therefore, we comprehensively investigated the basic knowledge of how the primary contributors (male and female) behave physiologically and at a molecular level during heat and cool events.

Semen quality from 12 boars was assessed using percent motile, percent viable, and sperm nuclear shape by Fourier Harmonic Analysis (FHA). Selection of boars for breeding was based upon the amount of nuclear shape change from June (spring collection) and August (summer collection), with 3 boars from the most absolute change and 3 from the least. Breedings took place during summer (August) and winter (January) periods. Gilts were single-time AI following a synchronization protocol using Matrix with semen from either cooled-extended (ExT), cryopreserved from June collection (FrZ spring), or cryopreserved from August collection (FrZ summer).

In order to gain insight into the molecular activity of the sperm itself, we evaluated the transcript activity of candidate genes from motile-rich sperm that had been previously identified as different between summer or winter collected semen and within the epigenetic methylcytosine pathway. Only two transcripts tended to be influenced by treatments. Relative expression of Lectin, Galactoside-Binding, Soluble, 3 (LGALS3) tended to be greater in motile-rich sperm from June collection versus August collection. LGALS3 is necessary for pre-mRNA splicing and is associated with serum testosterone levels in humans. Ribosomal protein L8, a

component of the 60S ribosome subunit and involved in protein synthesis, tended to be less in cryopreserved semen from June in comparison to cryopreserved sperm from August or cooled-extended semen from June.

Fertility, as measured by conception rate at approximately 45 days post-insemination, tended to be influenced by the main effects tested. Semen from the boars with the most HA change tended to have reduced fertility rates in comparison to those with the least HA change. Fertility rates also tended to be less in those gilts bred with semen collected during the summer in contrast to spring or winter collections. And cryopreserved (FrZ) semen tended to yield reduced conception rates in comparison to cooled-extended (ExT) semen.

Influences on litter characteristics from pregnant gilts were less discernible by influence of treatments. Production characteristics including; litter size, fetal weights, placental weights, and placental efficiency were all affected by interaction of the main effects; breeding season, season of semen collection, semen storage, and Harmonic Amplitude (HA) change. Fertility rates tended to be less in gilts inseminated with semen collected during the summer and from boars with the greatest absolute HA change; however, these matings had the largest litter size. Furthermore, cryopreserved semen from August collection period appeared to yield smaller litter sizes. As expected, weights of fetuses were generally less from breedings with the largest litter sizes. Interestingly, fetuses derived from August breedings appeared to be heavier than those from January breedings. Evaluation of placental weights suggested most interactions were similar with the exception of those pregnancies generated using natural estrus detection and 2x insemination, in which placental weights were less. Using placental efficiency as a means of evaluating the uterine environment, it appears the double insemination treatment were somewhat less efficient.

The ultimate product of the male and female contribution is the piglet. To investigate the influence of seasonal parental contributions, the DNA methylation activity, which is an indicator of gene transcriptional activity and can be influenced by epigenetic factors (e.g. temperature, feedstuffs), was measured in fetal liver and placenta. The conversion of 5-methylcytosine to 5-hydroxymethylcytosine is positively associated with increased gene transcript activity. In the current study, we found that the ratio of 5-methylcytosine to 5-hydroxymethylcytosine was favorable for increased gene transcript activation in fetal livers from summer matings that used cooled-extended semen. Whilst the placental methylation ratio was more favorable for increased gene transcript activation from winter matings.

Although we could not control the unseasonably cool summer temperatures, we were able to detect differences in the semen by collection period as well as by breeding season. The use of FHA appears to have provided additional information, beyond current acceptable standards, for semen quality assessment. Nuclear head shape varied by season and storage conditions, and was associated with fertility, as a measure of absolute most or least change in HA from June to August collections, however, no single HA was able to predict fertility. Regardless of HA, expected fertility rates tended to be greater if semen was collected during the cooler seasons (spring or winter) or if cooled-extended (ExT) semen was used. Although litter traits were different, no concise pattern was clearly discernable by treatment; therefore, the use of single insemination was not a detrimental approach to generate pregnancies in the current study. Transcript activity of genes known to be influenced by season of semen collection, tended to be altered in two genes with affiliation to protein synthesis and ribosomal activity, both of which could greatly influence proper sperm viability and/or fertility. Furthermore, general assessment of genomic methylation activity from post-fertilization tissues suggested that epigenetics of season in which semen was collected and breeding season may influence gene activation. These data support that season, even in the absence of extreme heat, may have a deeper molecular influence on infertility within both male and female components. A larger study assessing sperm parameters including the use of Fourier Harmonic methods may provide a greater predictor of fertility that could override seasonal infertility in gilts. However, alterations to the piglet may still be incurred as a result of epigenetic actions upon semen and the uterine environment by season.

Key Findings:

- The use of Fourier Harmonic Amplitude suggests that additional measurements can be made to evaluate semen for a more comprehensive prediction of fertility.
- Following two breeding trials with gilts, cryopreserved semen collected during cooler period (Spring) had greater fertility than cryopreserved semen collected during summer.
- Two motile-rich sperm gene transcripts were altered among semen either collected during Spring and Summer or stored as cooled-extended or cryopreserved.
- Methylation activity was different within fetal livers and placentas between winter and summer breeding seasons, which could result in altered gene activation during fetal development.

Dr. Lea A. Rempel
USDA, ARS, US Meat Animal Research Center
lea.rempel@ars.usda.gov
(402)762-4185

Scientific Abstract

The objective of the current study was to evaluate quality of semen collected from June (spring), August (summer), or January (winter) and either stored and used as cooled-extended (ExT) or cryopreserved (FrZ) for breeding gilts in summer (August) or winter (January). Semen quality evaluation included: % motile, % viable sperm, and Fourier Harmonic Analysis (FHA) of sperm nuclear shape resulting in harmonic amplitudes 0–5 (HA0-5). Six boars were selected for breeding by least- and most- absolute nuclear shape change between June and August collections. Gilts were artificially inseminated 1x following synchronization in August or January with one of three semen types (August ExT or January ExT, August FrZ, and June FrZ). An additional control group of females (CC) was artificially inseminated following natural heat detection and 2x insemination with ExT semen. Fertility rates tended ($P \leq 0.10$) to be greater in gilts inseminated with 1) least HA change semen, 2) semen collected in cooler months (i.e. spring or winter), or 3) if semen was cooled-extended (ExT) versus cryopreserved (FrZ). Largest litters ($P \leq 0.05$) were observed in CC females bred with semen having most HA change whilst smallest litters were in gilts bred with least HA change, and August FrZ semen at the August breeding event ($P \leq 0.05$). Fetal weights and placental weights were significant ($P < 0.05$) by semen collection time x semen storage x HA change x breeding season. Relative ribosomal protein L8 transcript from motile-rich sperm tended ($P = 0.0521$) to be increased in samples collected in June and cryopreserved in comparison to June collected, cooled-extended or August collected, cryopreserved. Relative lectin, galactoside-binding, soluble, 3 transcript tended ($P = 0.0551$) to be greater from June collected sperm in contrast to August collected sperm. Methylation (5-methylcytosine; 5mC) and hydroxymethylation (5-hydroxymethylcytosine; 5hmC) activity in fetal liver and placenta was measured to estimate gene transcription activity as influenced by epigenetic factors. Fetal livers tended ($P \leq 0.0863$) to have increased 5mC:5hmC when derived from FrZ semen in comparison to ExT semen (18.6 ± 2.61 and 12.0 ± 2.70, respectively) or summer breedings versus winter breedings (20.4 ± 3.03 and 10.1 ± 2.63, respectively). In contrast, placentas derived from summer breedings had reduced 5mC:5hmC versus winter breedings (4.7 ± 0.70 and 1.2 ± 0.74, respectively). The findings in the current study support the occurrence of factors contributing to seasonal infertility even in the absence of extreme heat. Of great interest, male and female components may contribute to epigenetic actions influencing piglet gene transcription.

Keywords: Boar, Cryopreserved semen, Fertility, Fourier Harmonic Amplitude, Season

Introduction

The gilt/sow is a polyestrous species; however, a global observation acknowledges that seasonal infertility occurs. Outcomes of seasonal infertility include anestrus, delayed return to estrus following weaning, increased mid- to late-gestation pregnancy losses in the fall, and reduced farrowing rates in cool months (Almond, 1992; Bertoldo et al., 2011; Stork, 1979; Tast et al., 2002). All of these factors contribute to increasing non-productive sow days, which are those days that a breeding female is neither, gestating nor lactating. In the U.S., non-productive sow days have been estimated at 36 days per year on average (Rix and Ketchem, 2009) with a cost of $1.50-$3.50/head/day. Numerous factors have been identified as contributors to female seasonal infertility including increased day-length, elevated ambient temperatures, and relative humidity. Control of any of these factors influencing female activity ***marginally*** improves reproductive efficiency. Likely there is an additive effect contributing to seasonal infertility.

Most of the focus for seasonal infertility has been placed upon the female. However, semen has a seasonal response as well (Trudeau and Sanford, 1986; Yang et al., 2010). Several labs have shown that sperm concentration decreases while ejaculate volume increases during warm months (Ciereszko et al., 2000; Murase et al., 2007). Murase and others also reported spermatozoa motility was lowest, agglutination scores were greatest, amount of spermatozoa with intact acrosomes was least, and the percentage abnormal sperm was greatest during warm months. All of these factors can reduce fertility. At the molecular level, microarray analysis of the messenger RNA from motile sperm-rich fraction of semen collected during summer or winter revealed 33 annotated genes that had modified abundance based upon season (Yang et al., 2010). Candidate transcripts were biologically classified within various processes related to fertility including, but not limited to, metabolic processes, gamete generation, signal transduction, fertilization, and locomotor behavior.

Combination of male and female gametes creates the embryo and extra-embryonic structures (placenta). Environmental influences such as, pollutants, **climate**, or diet, can alter the epigenetic configuration (changes in chromatin that alter gene expression in the absence of mutations in DNA). DNA methylation patterns (basis of chromatin structure) in human extra-embryonic tissues have been linked to irregular fetal growth and early pregnancy loss (Koukoura et al., 2012; Yin et al., 2012). Modification of gametes and uterine environment due to heat stress may alter the placenta and fetus to the extent of hindering or even precluding pregnancy.

In conclusion, it is evident that there is a **vast knowledge gap** for the basic science underlying the contributions of male and female components to seasonal infertility. Our preliminary data confirmed the successful use of fixed-time AI with frozen/thawed semen at USMARC and indicated putative methylation pattern differences in placenta and fetal tissues. We are ideally positioned to undertake the definitive studies presented herein to fully explore the physiology and molecular activities associated with the seasonal infertility phenomenon.

Objectives:

1) Determine quality and effectiveness of semen (collected during spring (June), summer (August), and winter (January) and used as cooled extended (ExT) or frozen/thawed (FrZ) for inseminations) on pregnancy rates and litter characteristics in gilts inseminated during summer (August) or winter (January).

2) Determine the molecular differences in sperm collected in spring, summer, and winter by evaluating expression of seasonal and epigenetic target genes in those sperm.

3) Assess the contribution of seasonality (both sire and dam) and semen storage (ExT and FrZ) upon DNA methylation activity of embryonic and extra-embryonic tissues.

Materials and Methods

All experimental procedures and techniques were reviewed and approved by the U.S. Meat Animal Research Center (USMARC) Animal Care and Use Committee (EO# 5438-31000-091-07). Procedures for handling animals complied with the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010).

Semen Collection, Storage, and Evaluation

Semen was collected from 12 boars at a commercial facility (International Boar Semen, Eldora, IA) under their conditions and preparation methodologies. Cooled extended (ExT) semen was collected from boars in June 2014, August 2014, and January 2015 for standard assessment and Fourier Harmonic Analysis (FHA). A subset of boars were selected for breeding based upon least (N=3) or most (N=3) absolute change in nuclear head shape between June (spring) and August (summer) ExT semen samples as described within Nuclear Shape Assessment via FHA.

Studies on the impact of heat stress on boar semen have shown differences in semen quality parameters, including nuclear shape change, occur from 19 to 33 days after a given heat event (Gibbs, 2013). No temperature data from the boar stud facility was available, but a weather station located within 15 miles from the stud recorded daily temperatures (US Climate data, v. 2.2 beta). Weekly maximum temperature means are reported in Figure 1 for 8 weeks prior through time of collection as well as the 19-33 d period prior (shaded). During the 19-33 d prior to semen collection in June 2014, mean (SE) daily high temperature was 17.9 °C (1.88) and the period prior to August semen collection was 25.9 °C (0.71). There were five days within the period prior to the August collection with recorded high temperatures of at least 29.0 °C (0.78) of which, two days were consecutively 30.8 °C (0.25). The average high temperature for the period prior to the January 2015 semen collection (ExT, only) was -1.8 °C (1.32).

Sperm Motility, Morphology and Viability Assessment

Percent motile was determined with computer assisted sperm analysis (CASA), performed on a Hamilton Thorne (v.10.9i, Waltham, MA, USA) for June ExT samples, and on a Minitube Androvision® fitted with an Olympus CX41 microscope for June frozen-thawed and August cooled and frozen-thawed. Motility assessment was performed on semen samples diluted 1:1 in semen extender (IMV Technologies, Maple Grove, MN) and warmed to 37 ℃ in a water-bath for 10 minutes. Multiple motility parameters were possible to record with both systems but total percent motile was chosen as a parameter least sensitive to differences of the two systems.

Viability was determined with fluorescent staining (Live Dead Sperm Viability Kit, Molecular Probes, Eugene, OR) with Syber14 and propidium iodide (PI), as described by Garner et al. (1995). Briefly, cooled or frozen-thawed semen was diluted in semen extender (40 million sperm/mL) and placed in a 37ºC water bath. Five µL Syber14 (40 µM in DMSO) was added and the sample was incubated for 10 min, at which point 10 µL PI (2.4 mM) was added and an additional 5 min of incubation was allowed. A 10 µL drop of stained semen was placed on a slide and observed under an epifluorescent microscope. Those cells appearing green (SYBR14) were counted as live and red (PI) as dead. A minimum of 200 sperm were counted per sample and results were expressed as percent live.

Nuclear shape was determined by FHA (Figure 2) described below. Morphology was subjectively evaluated on phase contrast images of the same 100 individual sperm that were identified for use in FHA. The proportion of sperm with abnormal morphology including head, mid-piece and tail abnormalities, as well as retention of cytoplasmic droplets, was determined.

Nuclear Shape Assessment via FHA

Nuclear shape assessment was conducted via image analysis of fluorescently stained nuclei and varied by semen type. Slide preparation consisted of staining semen with Hoechst 33342 (Molecular Probes, Eugene, OR) only for ExT samples, and both Hoechst 33342 and YOYO-1 (Molecular Probes, Eugene, OR) for FrZ samples, as described by Willenburg (2008). The use of YOYO-1 allowed the exclusion of membrane-compromised or dead sperm from FHA for FrZ samples. This was necessitated by the large percent of sperm dead upon thawing in frozen semen that was not present in the cooled semen. Sperm viability, a measure of membrane integrity, has been deemed crucial to successful fertilization (Gadella, 2008; Januskaukas et al., 2003), as well as to FHA evaluation of cryopreserved semen and the corresponding relationship to fertility

(Parrish and Ostermeier, 1998; Parrish et al., 2006; Parrish et al., 2014). Sample concentrations were adjusted to 40 million sperm/mL for each staining procedure.

Briefly, FrZ sperm in 750 µL aliquots were stained with Hoechst 33342 (8 µM) and YOYO-1 (1.1 µM) and incubated (37° C for 30 min) prior to fixation. Excess dye was removed via centrifugation (10 min at 700 X g) of stained sperm through 45% Percol® in Bovine Gamete Medium-3 (BGM-3; Parrish et al.. 1988). The supernatant was discarded and the sperm pellet resuspended with 500 µL BGM-3. The sample was then centrifuged in a micro-centrifuge at 6,000 X g for 8 s. The sperm pellet was then resuspended with 250 µL BGM-3. Fixation was achieved by the addition of 250 µL of 0.4% Paraformaldehyde in 2.9% NaCitrate, 3 mg/mL BSA (PFA solution) for 5 minutes. Samples were then centrifuged again at 6,000 X g for 8 s, the sperm pellet resuspended with 750 µL of PFA solution, centrifuged again at 6,000 X g for 8 s, and finally the sperm pellet resuspended with 750 µL of water. A 10 µL drop of the sperm-water solution was dried onto a slide for each sample. Slides were mounted with 3.5 µL of 0.22 mM triethylenediamine dissolved in one part HEPES buffered saline and nine parts glycerol and covered with a no. 1, 18X18 mm coverslips. Slide preparation for FHA of cooled semen samples was the same as for frozen-thawed with the following exceptions; the PFA solution was added directly to extended semen 1:1, sperm were only stained with Hoechst 33342, and no Percol® wash was performed.

Images were acquired with a QIClick digital camera (Q-Imaging, Surrey, BC V3S 6K3, Canada) on a Nikon Microphot FX microscope (Frank Fryer Co., Carpentersville, IL.) fitted for phase contrast and epifluorescent microscopy. Each field was captured under phase-contrast and fluorescent conditions, frozen-thawed samples with two dyes having two fluorescent images. Each dye was illuminated through the corresponding filter cube: Hoechst 33342, excitation 365 ± 20 nm, dichromatic mirror 400 nm, emission > 400 nm; and YOYO-1, 450-490 nm excitation, 510 nm dichromatic mirror and >520 nm emission. A minimum of 120 viable, non-YOYO-1 staining sperm cells were imaged per sample when the 2 dyes were present and 120 total sperm when only Hoechst 33342 was used.

Fourier harmonic amplitudes (HA) were obtained for sperm nuclei as described by Ostermeier et al (2001). Briefly, computer assisted image analysis was used to identify and determine nuclear perimeter coordinates of Hoechst 33342 stained sperm. A series of proprietary macros were written for the open source software ImageJ (Rasband, 1997-2012). For frozen-thawed samples, the image acquired from YOYO-1 staining was overlaid on the Hoechst 33342 image, allowing the operator to review and delete sperm which had both YOYO-1 and Hoechst 33342 staining. For all samples (ExT and FrZ), Hoechst 33342 fluorescent images were the basis for determination of nuclear perimeter coordinates. Perimeter information was then evaluated with Statistical Analysis System 9.3 (SAS, Cary, NC; 2011) on 100 randomly selected sperm per sample, data converted from Cartesian to Polar coordinates, Fourier Series constructed for each sperm, and Mean Harmonic amplitudes (HA) 0 – 5 determined for each sample. To visualize how sperm were impacted by changes in harmonic amplitudes and experimental treatment, a mean sperm nuclear shape was first required. A total of 100 sperm from each of the boars were oriented in the same direction, anterior head pointing to the right. A combination of automated approaches and individual sperm observation was required to achieve identical orientation. Next perimeter coordinates of each sperm nuclei were converted in SAS from Cartesian to Polar coordinates. The mean Polar coordinates of nuclei, at equally spaced angles of 1 degree, were computed as the average nuclear perimeter. A Fourier series was fit to the average nuclear perimeter data, condensed to a series of just cosines, and HA0-20 as well as phase angles 0–20 determined (Ostermeier et al., 2001).

Gilt and Sample Collection

Landrace-Duroc-Yorkshire gilts at USMARC were selected across six farrowing weeks for use in the August 2014 breeding (Experiment 1; summer) and from two farrowing weeks for use in the January 2015 breeding (Experiment 2; winter). Ninety-four gilts and 84 gilts respective to experiments had shown some form of estrous behavior (e.g. standing, riding, swollen vulva, interest in boar) with fenceline boar presence at least twice over a 45 day period prior to experiments. Gilts were at least 220 days of age at time of insemination and were randomly assigned to treatment groups. Altrenogest (Merck Animal Health, Summit, NJ) was administered for 15 days to synchronize gilts for timed single insemination (d-15 to d0; H.D. Guthrie, personal communication). Gilts received an oral dose (15 mg) via a drenching gun to ensure accurate administration daily at 1000 CST. To induce ovulation, gilts were administered a single *im* injection of 750 IU chorionic gonadotropin; Experiment 1) Chorulon (Merck Animal Health, Summit, NJ) or Experiment 2) chorionic gonadotropin (Sigma-Aldrich, St. Louis, MO) in the evening 5 days following the cessation of altrenogest treatment (d5; 1900 CST). At the time of the second trial, Merck had temporarily shut down the production of Chorulon; therefore, we acquired chorionic gonadotropin from another source (Sigma-Aldrich).

At insemination (0700 CST; d7), fenceline presence of a boar was provided, breeding groups were distributed equally, and only one gilt refused service (Experiment 1; N=93). Insemination doses were as follows; ExT semen, 3 x 10^9 sperm per dose and FrZ semen, 5 x 10^9 sperm per dose adjusted to a volume of 50 mL with USA851 X-Cell Extender (IMV Technologies USA, Maple Grove, MN). As a means of ensuring the synchronization procedure was effective, nine contemporary gilts detected in natural heat were bred within 7 days by double insemination procedures with ExT semen (CC Summer; N=9) for Experiment 1 and nine CC gilts were bred under similar conditions for Experiment 2 (CC Winter; N=9). Each boar used for breeding was represented at least once within the CC groups for each experiment. Daily temperatures were recorded approximately 2 miles from the USMARC swine facility (US Climate v. 2.2 beta). In the 3 d prior to and 3 d following breeding (7 d total), the mean daily high temperature (SE) in August was 31.4 °C (0.44) and in January 2015 was 10.6 °C (1.30). Whilst the mean daily temperature (±SE) from time of insemination until pregnancy ultrasound (approximately 30 d) was 25.0 °C (1.15) in Summer 2014 and 5.1 °C (1.55) in Winter 2015.

Conception was determined by ultrasound at approximately 30 d post-insemination. All open gilts were processed at the USMARC abattoir to verify pregnancy status and evaluate uterine development at approximately 35 d post-insemination. Of note, 30 gilts in Experiment 1 and nine gilts in Experiment 2 had shown signs of estrous prior to the trial, appeared to respond to altrenogest and hCG procedures, were inseminated, presented open at ultrasound; however, upon examination at slaughter were identified as prepubertal. Since these animals could not physiologically conceive at time of insemination they were excluded from the analyses.

All gilts determined to be pregnant by ultrasound were processed at the abattoir at approximately 45 d post-breeding to evaluate pregnancy status, uterine development, and litter size. At time of slaughter, a blood sample in 4% EDTA was collected and plasma was isolated by centrifugation and stored at -80°C. The reproductive tract was excised from the dam shortly after death, *corpora lutea* were counted to determine ovulation rate, the uterus was opened along the antimesometrial surface; litter size (alive and dead) was recorded, each living fetus and companion placenta was weighed. A portion of fetal liver and companion placenta were collected from the smallest, an average, and largest live piglet from each litter. All tissues were snap frozen in liquid N then stored at -80°C until analysis.

Motile Rich Sperm Cell Processing and RNA Isolation

Motile rich sperm was isolated from ½ of a single dose of semen (FrZ; $5x10^9$ cells/dose and ExT; $3x10^9$ cells/dose; International Boar Semen, Eldora, IA) as described by Yang et al., 2010. Briefly, cryopreserved semen was diluted with extender as per distributor recommendations. Extended semen (FrZ and ExT) was incubated at 37°C for 30 minutes to settle non-motile sperm and cellular debris. The upper half of semen was collected and placed into a 50 mL conical, washed with equal parts 1x RNase-free PBS, then centrifuged at 300 x g for 10 minutes, 18°C. The upper half of diluted semen was collected and placed into a new conical, washed with 1x RNase-free PBS, then centrifuged at 1,200 x g for 5 minutes at 18°C at which point the motile sperm was in the formed pellet. Supernatant was removed and checked for motile sperm by microscopy before discarding. The pellet was washed 5X with 1x RNase-free PBS then centrifuged at 1,200 x g, 5 minutes at 18°C, the final pellet was snap frozen in liquid N and stored at -80°C until RNA isolation.

Total RNA was extracted from motile rich sperm using Trizol (Life Technologies, Grand Island, NY) according to manufacturer's recommended procedures. Total RNA was quantified and purity assessed with 1 uL of each sample using an Agilent RNA 6000 Pico assay and Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). Purity of the sperm RNA was determined by the absence of somatic cell 18S and 28S rRNA peaks (Figure 3).

Semi-Quantitative Real-Time PCR Evaluation of Seasonality and Methylation Genes

Five hundred pg total sperm RNA was amplified using the MessageBOOSTER cDNA synthesis kit (Epicentre, Madison, WI) according to manufacturer's recommendations with Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) then reverse transcribed into first-strand cDNA for qPCR. A custom-designed RT2 Profiler PCR Array (Qiagen, Valencia, CA) was used to investigate putative sperm-specific gene transcripts altered by seasonal effects (reported in Yang et al., 2010), methylation activity transcripts, and control targets (Table 1). Amplified first-strand cDNA was used at a dilution of 1:250 in the final quantitative PCR reaction. All reactions were performed on a BioRad CFX384 real-time PCR instrument (Hercules, CA) under the following conditions; 95°C for 10 minutes followed by 40 cycles at 95°C for 15seconds, 60°C for 1 minute, with a final melting curve from 65 to 95°C. Data were prepared as the natural log of fold change for statistical analyses.

Methylation Activity of Embryonic and Extra-Embryonic Tissues

Fetal liver and placental genomic DNA was isolated using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) following manufacturer's recommendation from the small, average, and large fetuses from each litter. Genomic DNAs were then pooled within litter for each tissue and assayed in duplicate. 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) were measured using colorimetric *MethylFlash*^{TM} Methylated DNA or Hydroxymethylated DNA Quantification Kits (Epigentek, Farmingdale, NY), respectively. Interassay CV from control samples for 5mC and 5hmC was 7.14% and 13.9%, respectively and intrassay CVs were 12.1% (fetal liver 5mC), 2.4% (fetal liver 5hmC), 9.8% (placenta 5mC), and 7.4% (placenta 5hmC). Briefly, relative quantification was calculated as the percent of 5mC in 100 ng genomic DNA or the percent 5hmC in 200 ng genomic DNA in relation to a single point standard at 5 ng/µl 5mC or 5hmC, correspondingly.

Statistical Analyses

All statistical analyses were conducted in SAS (2011). Semen assessment data (motility, viability, morphology and HA0-HA5) for each semen type were evaluated by ANOVA using a mixed model. Harmonic values by semen type were also analyzed in aggregate using MANOVA within PROC MIXED. Collection season (spring and summer), preparation (ExT or FrZ), and season by preparation interaction were considered fixed effects while boar and associated boar interactions were considered random for all analysis. One boar failed to meet the boar stud's cryopreservation standards resulting in no frozen sample for August. This boar was subsequently omitted from statistical analysis, leaving 11 boars for semen quality comparison.

Harmonic amplitude effect upon conception rates were analyzed using GLIMMIX with a Laplace approximation for maximum likelihood inferences. The model consisted of the fixed effects; change in HA (least or most), collection season (spring, summer, or winter), preparation of semen (CC, ExT, or FrZ), breeding season (summer or winter), and the interaction of HA by breeding season. Boar was designated as a random effect.

Litter size, fetal weight, placental weight, and placental efficiency were analyzed using the MIXED procedure. Fixed effects included; change in HA (least or most), semen collection season (spring, summer, or winter), preparation of semen (CC, ExT, or FrZ), breeding season (summer or winter), and significant interactions. Sire and sire of dam were defined as random effects, whilst dam was defined as repeated. Fetal weight and placental weight were also adjusted for the covariates litter size and days post-breeding at collection.

Quantitative real-time PCR results from motile-rich sperm fraction RNA were analyzed using the MIXED procedure. All data were normalized to a positive PCR control (PPC) and converted to fold change relative to the PPC. Analyses were performed using log transformed data of the target genes. The full model included the fixed effects of change in HA (least or most), type of semen (ExT or FrZ), semen collection season (spring, summer, or winter), interactions; and boar as a repeated effect. In the event that the interactions were not significant, only main effects were tested.

Litter cytosine methylation and cytosine hydroxymethylation measures from placenta and fetal liver were analyzed using MIXED procedure with the fixed effects of change in HA (least or most), type of semen (ExT or FrZ), semen collection season (spring, summer, or winter), breeding season; and boar as a random effect.

Results

Fourier Harmonic Analysis of Semen

Semen samples from 12 boars were collected in June (spring) for analysis by FHA and standard CASA techniques. Semen was also cryopreserved from all boars at this time and stored in liquid N to represent FrZ Spring semen for breeding in August 2014 (summer breeding) and again in January 2015 (winter breeding). Boars were collected again the first week of August (summer) to assess changes in CASA and FHA. Those animals with the greatest and least absolute change in HA0, 2, and 4 were selected for breeding the following week (6 boars; #1-6; Table 2). Cooled extended semen (ExT Summer) and cryopreserved semen (FrZ Summer) were collected from the selected six boars for breeding in August 2014. Additionally, FrZ Summer semen was stored in liquid N for the second breedings in addition to the FrZ Spring semen to occur in January 2015. One boar (#10) was excluded from breeding selection as the semen sample failed to meet cryopreservation quality standards however one cryopreserved sample was collected for transcription analysis.

Selection of boars for breeding was based on changes from June and August measurements using FHA only (Figure 4; Table 2; Table 3). Boars 1, 2, and 3 had the least; 1) HA0-5 average and 2) HA0, 2, and 4 average ranked change in FHA from June and August collections, whilst boars 4, 5, and 6 had the greatest changes in FHA measurements (Table 2; Table 3). Differences in HA values are presented in Table 3 for boars selected for breeding by least and most change. Percent normal and percent motility were determined using CASA techniques (Table 4), but were not used to determine which boars would be used for breeding. Presence of a droplet on sperm was considered abnormal and is reflected in the % Normal values.

Traditional semen quality parameter of motility exhibited an interaction of season and semen preparation with fewer ($P < 0.001$; Figure 5) motile sperm in August as compared to June for cooled semen. There were no differences ($P > 0.05$; Figure 5); however, with frozen-thawed semen between seasons. There was an effect of preparation on the percentage of viable sperm with reduced viable sperm found in frozen-thawed samples ($P < 0.001$; Figure 5). More normal sperm ($P < 0.05$) were observed among the live FrZ preparations than all sperm in the ExT group. Overall changes in sperm nuclear shape results are shown in Table 2 and 3 and Figure 5 for HA0-HA5. There was an interaction of season and semen preparation for HA0, HA2 and HA4 ($P < 0.05$; Figure 5). Both HA0 and HA2 decreased from June to August ExT semen ($P < 0.05$; Figure 5) but increased for June to August FrZ semen ($P < 0.05$). The HA4 increased as well for the June to August ExT semen ($P < 0.05$) but there was no effect of season on the FrZ semen ($P > 0.05$). There were main effects of season on HA1, HA3 and HA5 ($P < 0.05$; Table 5). Main effects of semen preparation were also present for HA1 ($P < 0.05$; Table 5).

Conception Rates in Gilts Following Insemination with Selected Semen

As a basic measure of fertility, conception rates for gilts were determined at pregnancy detection by ultrasound (d30 post-AI) and again at collection (d45 post-AI; Table 6). The table is presented with those boars exhibiting the least change in HA from June to August in the upper half (boars 1-3), whilst those with the most change are presented in the lower half (boars 4-6). Due to unexpected drop outs from females that appeared to respond to synchronization procedures, but were in effect prepubertal, limitations exist for balance by boar. However, we do have similar totals for HA change (Least or Most) and semen type (ExT Winter, ExT Summer, FrZ Spring, FrZ Summer).

Statistical analyses of pregnancy at d45 post-AI indicated that the main effects of HA change, season of semen collection, and storage of semen all had an impact on fertility ($P \leq 0.10$). Pregnancy rates from breedings in the summer or winter were not different ($P = 0.1377$). However, those pregnancies derived from boars with the least change in HA from June to August tended ($P = 0.1025$; Table 7) to have greater fertility success than those pregnancies derived from boars with the most HA change. Furthermore, breedings conceived through the use of semen collected during spring or winter (i.e., June or January) tended (*P* = 0.0775; Table 8) to have improved pregnancy in comparison to semen collected in summer. Preparation of semen had an influence (*P* = 0.0015; Table 9) on fertility regardless of summer or winter breeding or whether semen had been collected during spring, summer, or winter with ExT and CC semen yielding better fertility rates than FrZ. Semen used as cooled extended for either single insemination (ExT) or double inseminations (CC) had similar (*P* = 0.6046) pregnancy rates at 45 d post-AI. Pregnancy rates derived from either CC or ExT inseminations were greater ($P \leq 0.0118$) than those generated from FrZ semen.

Litter Performance

By design, the combination of both experimental trials yielded 16 treatment groups. We report the differences ($P \leq 0.05$) by interaction of main effects; change in HA (least or most), semen preparation (CC, ExT, or FrZ), season of semen collection (Spring, Summer, or Winter), and breeding season (summer or winter) for litter size (Figure 6), fetal weight (Figure 7), placental weight (Figure 8), and placental efficiency (Figure 9).

The largest litters were observed from those gilts bred under the double insemination protocol following natural heat detection (CC) with semen from boars categorized as having the most change in HA ($P \leq 0.0081$; Figure 6). However, overall fertility rates tended to be decreased among gilts bred with semen from boars identified as having most HA change (Table 5). Single inseminations following synchronization suggested that ExT semen was intermediary regardless of semen collection season (Summer or Winter; Figure 6). The smallest litter sizes were derived from Least HA, FrZ Summer semen used in a single insemination in August. Similar small litter sizes were derived from semen with Most HA change and included; FrZ Spring, winter breeding and FrZ Summer winter and summer breedings (Figure 6).

Fetal weights were from single inseminations using FrZ semen were generally smaller ($P \leq 0.05$) than their summer breeding counterparts with the exception of FrZ Spring Least HA change (Figure 7). Double inseminated (CC) Most HA change gilts had the largest litter sizes (Figure 6) and as expected the fetal weights were reduced; however, they were not the smallest (Figure 7). The smallest fetuses ($P \leq 0.05$) were observed in winter breedings, one being derived from Least HA change, CC Winter semen and the other treatment group was FrZ Spring, Most HA change semen (Figure 7).

Placental weights were reduced ($P \leq 0.05$) in those pregnancies generated using ExT semen with Least HA change and double insemination (CC), with summer breeding less than winter breeding ($P = 0.0073$). Placental weights from CC Winter, Least HA change, winter breeding, were similar to both CC, Most HA change breedings ($P \geq 0.1790$). The largest ($P \leq 0.05$) placentas were generated in pregnancies from ExT Winter, Least HA change semen used in winter breeding (Figure 8).

To gain more insight on the dynamic architecture of the relationship between the placenta and the constituent fetus, placental efficiency, defined as the ratio of fetal weight to placental weight, was evaluated (Figure 9). Among those pregnancies generated using Least HA change semen, ExT Summer and FrZ Spring treatments used during summer breeding had greater ($P \leq 0.05$) efficiency than the reciprocal breeding in the winter season (Figure 9).

Quantitative Real-Time PCR of Seasonal and Methylation Transcripts from Semen

Of the 40 target genes (Table 1) assessed only nine consistently produced detectable transcripts among all semen types. The nine genes were; Calcium-regulated heat stable protein 1-like (CARHSP), Calcium and integrin binding 1 (aka Calmyrin; CIB1), Ferritin, light polypeptide (FTL), Lectin, galactoside-binding, soluble, 3 (aka Galectin 3; LGALS3), Rab acceptor 1 (RABAC1), Ribosomal protein L10a (RPL10A), Ribosomal protein L8 (RPL8), Ribosomal protein S20 (RPS20), and Sperm mitochondria-associated cysteine-rich protein (SMCP). Transcript abundance was calculated as the relative fold-change (FC) in comparison to the positive PCR control. No differences ($P \geq 0.05$) from the six boars used for inseminations were detected among semen by HA change (Least or Most); therefore, it was dropped from the model. Relative fold-change of LGALS3 transcript tended ($P = 0.0551$) to be greater from June collection in comparison to August collection (-2.3 ± 1.81 and -7.9 ± 1.12 FC, respectively). An interaction ($P = 0.0314$) between semen collection season and semen storage was detected for the transcript RPL8. Motile-rich semen from FrZ Spring (-7.0 ± 1.24 FC) samples tended to have a greater transcription abundance of RPL8 than that of ExT Winter (*P* = 0.0521; -11.1 ± 1.43 FC) or FrZ Summer (*P* = 0.0866; -10.3 ± 1.24 FC).

Methylation and Hydroxymethylation Activity from Fetal and Placental Tissues

Assessment of methylation and hydroxymethylation activity within fetal liver and placental tissues derived from matings was determined. Full models were tested and only one interaction was significant; change in HA by semen storage affected 5mC activity in fetal liver. Fetal liver generated from matings using FrZ, Least HA semen (6.1 % ± 0.61) tended to have reduced 5mC activity in comparison to FrZ, Most HA (*P* = 0.0831; 7.7 % ± 0.69) and ExT, Least HA (*P* = 0.1002; 7.5 % ± 0.61). Main effects of type of semen storage (ExT or FrZ), semen collection temperature (spring, summer, or winter), and breeding season (summer or winter) were tested on remaining methylation activity measures. Placental 5mC activity was effected by breeding season with summer breedings tending to be less (*P* = 0.0781) than winter breedings (5.8 % ± 0.55 and 7.1 % ± 0.58, respectively). Similarly, 5hmC activity in placental tissues was less (*P* = 0.0014) from summer matings in comparison to winter matings (3.5 % ± 0.62 and 6.7 % ± 0.67, respectively). The ratio of 5mC to 5hmC was also evaluated. Semen storage tended (*P* = 0.0863) to influence liver ratios with FrZ semen marginally greater than ExT semen (18.6 ± 2.61 versus 12.0 ± 2.70, respectively). Whilst fetal livers from winter breedings had greater ratios than summer breedings (*P* = 0.0179; 20.5 ± 3.03 and 10.1 ± 2.63, respectively). Placental ratio of 5mC to 5hmC was greater (*P* = 0.0013) in tissues derived from summer breedings versus winter breedings (4.7 ± 0.70 and 1.2 ± 0.74, respectively).

Discussion

In the present study, we evaluated the impact of seasonality, semen storage conditions, and sperm nuclear head shape on conception rate in gilts and subsequent effects on methylation activity in fetal and placental tissues. It is important to point out that the current study was performed during the second coolest summer on record in the last 10 years (US Climate data). The ten year average (2005-2014) for July within 15 miles of the boar stud was 28.1 °C, while the 2014 average was only 25.6 °C. However, during the period of time known to impact spermatozoan development (19-33 d prior to collection; Gibbs, 2013) thermal increases were present for the current study. It is also important to note that modern grow-finish pigs have between 6 and 41% greater heat production than earlier reported genetics (Brown-Brandl et al., 2011) and the maximum thermal neutral zone for growing pigs ranges between 17 °C and 23 °C (Brown-Brandl et al., 2013). Therefore, it appears newer genetics have yielded pigs with greater thermal sensitivities and even though the summer season was cooler, a thermal stress may have occurred in the current study. Furthermore, Barranco and others (Barranco et al., 2013) reported that semen collected during summer months (July-September) was less likely to withstand cryopreservation autonomous of the sperm quality of the ejaculate in comparison to winter (January-March) or spring (April-June) collections from boars housed in temperature-regulated buildings similar to the current study. Since these boars were housed in thermoneutral buildings, ambient temperatures may not be the only contributing factor influencing semen quality from summer periods.

In the current study, gilts were exposed to elevated temperatures preceding and following insemination in August. It has been reported that elevated ambient temperatures at or shortly following insemination of gilts may cause embryonic litter size reduction (Wildt et al., 1975; Wettemann and Bazer, 1985; Wettemann et al., 1988) while others have indicated embryo litter size or embryo survival was not influenced by elevated heat stress (Liao and Veum, 1994; Canady et al., 2013). These latter studies may be a result of modern genetics; however, selection for increased production, such as growth and reproductive output, would likely unfavorably increase sensitivity to environmental stressors (Bloemhof et al., 2012). Although differences were statistically evident within the current study we, unfortunately, did not observe discernable patterns to production traits (number of live fetuses, fetal weights, or placental weights) as a result of treatments. Rather overall fertility, as measured by pregnancy rate, was impacted by the main effects of semen collection season, semen storage, and breeding season.

Cryopreservation has dramatic effects on sperm, resulting in structural and functional changes due to the toxic and osmotic stress induced by cryoprotectants and physical stress caused by cooling, freezing and thawing. The most predominant effects of cryopreservation on sperm are loss of viability, reduction in motility, morphologic changes, and alteration in chromatin structure (Watson, 2000; Thomson et al., 2009). These same changes are also observed as a result of heat stress (Gibbs, 2013) and seasonality (Smital, 2009), all too varying degrees. In the current study, the differences between motility and viability between both preparation and season are consistent with previous studies. The reduction in motility for cryopreserved semen was more severe than previously reported (Barranco et al., 2013; Daigneault et al., 2015). All semen, cooled or cryopreserved, in this study were commercially obtained from the same boar stud and manufacture's recommendations for handling were followed. However, frozen-thawed semen was only incubated for 10 minutes prior to semen quality evaluation, which could limit motile sperm found but should have no impact on viability, morphology or FHA evaluation. Greater change in sperm nuclear head shape in the initial evaluation between June and August and all semen collected from summer months were associated with reduced fertility in gilts in the current study. Furthermore, we found that overall cooled-extended (ExT) semen produced greater pregnancy rates than cryopreserved (FrZ) semen.

Differences between cooled extended and frozen-thawed sperm in both morphology and nuclear shape were observed in the current study. Both of these criteria were evaluated on all sperm for ExT semen but only the live sperm for FrZ semen. It is not clear why these changes occurred. Two possibilities exist. The first is that there is a subpopulation of sperm that are selected to survive cryopreservation that have more normal sperm and sperm with a different nuclear shape. The second is that the cryopreservation process somehow affects the percentage of morphological normal sperm and sperm nuclear shape. While the second option could be true for sperm nuclear shape, there is no reason that cryopreservation improves the percentage of sperm with normal morphology. The first option that a subpopulation of sperm is selected to survive cryopreservation remains possible but we have no data to support or reject this option. It should be noted that we had poor survival from cryopreservation, so a selection of a unique subpopulation of sperm is a distinct possibility worthy of further study.

The HA0 and 2 values for sperm nuclear shape changed within season and by preparation in opposite directions. The HA1 also changed from June to August and ExT to FrZ in opposite directions. It is unknown why sperm nuclei respond to the cryopreservation process differently by season or to season and preparation differently. Heat events and seasonal impacts experienced by the male during spermatogenesis, as well as cryo-damage from the freezing and thawing process, both damage sperm DNA, resulting in measureable impacts on sperm nuclear shape.

Numerous differences in boar semen by season are known to exist but it is unknown how those changes impart susceptibility to cryo-damage on sperm. Reported seasonal differences include protein composition of seminal plasma (Trudeau and Sanford, 1986), higher susceptibility to cryo-damage regardless of pre-freeze quality (Barranco et al., 2013), and membrane composition variations (Ciereszko et al., 2000; Cerolini et al., 2001). Barranco et al. (2013), hypothesized that the differences in freeze-ability by season was linked to seminal plasma variation, the proteins of which have been shown to play a role in protection of sperm from cryoinjury (Hernandez et al., 2007).

The chromatin damage known to result from the cryopreservation process (Baumber et al., 2003; Gandini et al., 2006; Kalthur et al., 2008; Thomson et al., 2009) could be sustained in different manners depending on properties of the seminal plasma proteins and/or membrane compositions that vary by season. Another possibility is that the seasonal differences in sperm membranes impart varying susceptibilities to damage during the freeze-thaw process. This could create a selection process as was suggested earlier; whereby, the membranes of some sperm are preferentially damaged resulting in a selected subpopulation of sperm with intact membranes, on which nuclear shape assessment was performed. If the property that determines membrane susceptibility also associates with nuclear shape, that could explain why nuclei of frozen-thawed semen from June and August had different shapes; however, no membrane property with a link to nuclear shape is known.

Regardless of mechanism, the freezing of semen collected in August resulted in viable sperm with larger nuclei in overall size, longer in length and a greater degree of midline pinching, as measurements HA0, 2 and 4 demonstrate in the current study. The result was harmonic values that more closely resembled those of ExTsemen samples from June. Cooled extended semen from June would be expected to be of high quality and fertilizing capacity as the sperm are not subjected to the damaging effects of warm season heat or freeze-thawing. Previous research in boars has shown HA2 has the strongest correlation to fertility of the six harmonic values, whilst HA0 fell among the lowest correlated (Willenburg, 2008). As August FrZ sperm nuclear shape became more similar to that of the June ExT group, one would expect an increase in fertility. However, the decrease in fertility of August FrZ semen suggests that cryopreservation may impact sperm nuclear shape in ways that do not directly relate to fertility.

The directional difference for nuclear shape change in response to freezing/thawing by season occurred for only two of the six harmonic values. Our data support the findings that subtle differences in all harmonic values together are needed to ascertain fertility (Ostermeier et al., 2001). Determining boars showing more nuclear shape change in cooled semen between seasons correctly identified those of lower fertility. The nuclei of sperm from ejaculates used for insemination from the boars exhibiting the most nuclear shape change between seasons were on average smaller than the sperm nuclei from boars showing the least amount of change, consistent with previous work that found sperm nuclei of boars with unacceptable fertility to have smaller nuclei overall (Willenburg, 2008).

As a means to further characterize potential differences among semen collection season and storage techniques, 32 motile-rich sperm transcripts were tested within our study (Yang et al., 2010) and transcripts within the methylation activity pathway. Sperm RNA has become a credible contributor to transgenerational acquired inheritance either directly influencing the sperm genome or occurring shortly after fertilization influencing the early embryo (Lalancette et al., 2008; Miller, 2014). Lectin, Galactoside-Binding, Soluble, 3 (LGALS3) transcript was greater in motile-rich sperm from June collections than those from August within the current study. These data mimic the microarray outcome from Yang and others in which LGALS3 expression was greater in sperm from winter (January-March) sampling in comparison to summer (July-September). Functionally, a relationship between LGALS3 and mRNA processing has been proposed in which LGALS3 is necessary in pre-mRNA splicing making it a relevant transcript in sperm function (Dagher et al., 1995). Furthermore, Duroc and Yorkshire boars had a dramatic decrease in circulating concentrations of testosterone during summer months in comparison to spring months and a positive relationship between concentrations of serum testosterone and semen volume, sperm concentration, and frozen-thawed sperm viability were also reported (Park and Yi, 2002). Interestingly serum levels of LGALS3 were elevated in females with polycystic ovarian syndrome (PCOS) and had a positive correlation with elevated serum concentrations of testosterone in PCOS subjects as well (Yilmaz et al., 2014). Therefore, the reduced level of LGALS3 transcript from the August collection sperm samples may be influenced by the likelihood that the boars in the current study had reduced circulating concentrations of testosterone.

Ribosomal protein L8 (RPL8) is a component of the, larger, 60S subunit of the ribosome and plays a necessary role in ribosome biogenesis (Pöll et al., 2009). Expression of RPL8 tended to be reduced in cryopreserved samples from June in comparison to cooled-extended sperm samples from June as well as cryopreserved sperm from August collections. Yang and others reported increased levels of RPL8 transcript from sperm collected in the winter versus summer (Yang et al., 2010). Numerically, the current study concurred; however, statistically there were no differences between ExT Winter and ExT Summer. In fact, a clear pattern in the relative expression of RPL8 in boar sperm was not evident in the current study with FrZ Summer and ExT Winter having the least relative expression of sperm RPL8 whilst FrZ Spring and ExT Summer had the greatest level of RPL8 mRNA, with ExT Summer not different from any other sample set. Yang and others (2010) only validated the steady-state expression levels of four transcripts reported to be different by microarray analysis between winter and summer sperm collection, which did not include RPL8. Down regulation of RPL8 expression in yeast cells resulted in inefficient production of pre-ribosomal RNA proteins, possibly by 1) altering the 3-dimensional architecture of rRNA domains I and II or 2) influencing the exonuclease digestion of the 5.8S rRNA, 5' end (Pöll et al., 2009). Depletion of RPL8 in Drosophila using RNAi techniques yielded cell proliferation arrest and apoptosis during development (Li et al., 2010). Both studies indicate the importance of RPL8 in downstream events. Of further interest, sperm ribosomal RNA is heavily populated with smaller/shorter RNAs (Miller et al., 1999; Bissonnette et al., 2009) and therefore the relevance of alterations to RPL8 in sperm may influence activity related to sperm viability or fertility.

Not only may spermatozoal RNA influence subsequent offspring, but epigentics may alter paternal genomic DNA (Hughes, 2014). As a part of the current study, one epigentic mechanism, methylation activity, was investigated within tissues derived from semen collected during spring or summer as well as cooled-extended or cryopreserved. Demethylation of 5-methylcytosine, yields 5-hydroxymethylcytosine, which has been positively associated with transcriptional activiation of genes within their promoter regions (Ficz et al., 2011). Increased methylation in the form of 5mC tends to suppress or turn off genes. Therefore, evaluating the ratio of 5mC to 5hmC may provide an indication as to transcript activity. In the current study, the ratio of 5mC to 5hmC in fetal liver tissue was more favorable for potential transcriptional activity from matings using cooled-extended/ fresh semen or matings generated during summer months. Cryopreservation of human sperm did not affect DNA methylation of nine methylation-responsive genes; however, a global genome methylation analysis was not performed (Klaver et al., 2012). Placental ratio of 5mC to 5hmC suggested that pregnancies generated during the winter period had greater transcriptional activity potential. The ratio for each tissue appears juxtaposed by mating season, which in itself generates an intriguing question as to transcript activity of fetal tissue and extraembryonic tissues by breeding season. Ficz and others (2011) proposed that genome hydroxymethylation and methylation were linked to pluripotency and lineage commitment during differentiation of embryonic stem cells; however, our data suggests that environment can plausibly influence this balance. Furthermore, placental formation involving differentiation and subsequent endometrial invasion of trophoblast cells requires a spatio-temporal flux of DNA methyltransferases (DNMTs) and ten-eleven translocation dioxygenase (TET) proteins to coordinate the transcriptional activation of key genes (e.g. *Chorionic Gondaotrophin, Nanog, Snail, E-cadherin, Estrogen Receptor-β*; Logan et al., 2013). Within the current study, real-time quantitative PCR assay of motile-rich sperm did not support the theory of sperm TET and DNMT differences by season of collection or storage methods. It is also interesting to note that day-length has been associated with DNA methylation of promoter regions for target genes of the hypothalamic-gonadal-axis in seasonal breeding mammals (Stevenson and Prendergast, 2013). Although swine are not considered seasonal breeders, season can influence fertility thus a component of the seasonal influence may be based upon methylation activity. Additional investigations into regulatory components of 5mC conversion to 5hmC such as TET proteins and DNMTs may provide further evidence as to whether the differences in ratio are semen storage-specific, tissue-specific, or mating season-specific and if the less invasive placental structure found in swine is replicate to that of mammals with more invasive placental architecture.

Conclusions

Although we could not control the unseasonably cool summer temperatures, we were able to detect differences in the semen by collection period as well as by breeding season. The use of Fourier Harmonic analyses appears to have provided additional information, beyond current acceptable standards, for semen quality assessment. Nuclear head shape varied by season and storage conditions, and was associated with fertility, as a measure of absolute most or least change in harmonic values from June to August collections; however, no single harmonic value was able to predict fertility. A larger scale evaluation of boars may provide a more definitive technique for using Fourier Harmonic analyses to consistently predict fertility rates beyond the use of standard motility and morphology assessment. Regardless of Harmonic value, expected fertility rates tended to be greater if semen was collected during the cooler season or if cooled-extended semen was used. Although litter traits were different, no concise pattern was clearly discernable by treatment; therefore, the use of single insemination was not a detrimental approach to generate pregnancies in the current study. Transcript activity of genes known to be influenced by semen collection period tended to be altered in two genes with affiliation to protein synthesis and ribosomal activity, both of which could greatly influence proper sperm viability and/or fertility. Furthermore, general assessment of genomic methylation activity from post-fertilization tissues suggested that epigenetics of season in which semen was collected and breeding season may influence gene activation. These data support that season, even in the absence of extreme heat, may have a deeper molecular influence on seasonal infertility within both male and female components. A larger study assessing sperm parameters including the use of Fourier Harmonic methods may provide a greater predictor of fertility that could override seasonal infertility in gilts. However, alterations to the piglet may still be incurred as a result of epigenetic actions upon semen and the uterine environment by season.

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Table 1. Gene targets for assessment of sperm RNA from boars collected during the cool season (June 2014) or following a heat event (August 2014) and cryopreserved or cooled/ extended.

Group[1]	Refseq #	Official Gene Name
S	NM_001204772	Bromodomain containing 4 (BRD4)
S	XM_003124608	Calcium-regulated heat stable protein 1-like
S	NM_001129946	Chemokine (C-C motif) ligand 5 (CCL5/RANTES)
S	XM_001926454	Calcium and integrin binding 1 (calmyrin) (CIB1)
S	XM_003127178	DNA-binding death effector domain-containing protein 2-like
S	XM_003132408	DnaJ homolog subfamily B member 8-like
S	XM_003127288	Ferritin, light polypeptide (FTL)
S	XM_001926833	G protein-coupled receptor 161 (GPR161)
S	XM_001927795	Heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa) (GPR78)
S	XM_001925450	H/ACA ribonucleoprotein complex subunit 3-like
S	XM_003132152	Kinesin-like protein KIF15-like
S	XM_003122329	Lipocalin 5 (LCN5)
S	NM_001097501	Lectin, galactoside-binding, soluble, 3 (LGALS3)
S	XM_001929583	Testis-specific serine/threonine-protein kinase 2-like
S	XM_003359573	Titin-like
S	XM_003356757	Activator of 90 kDa heat shock protein ATPase homolog 1-like
S	XM_003132131	Lysozyme-like protein 4-like
S	XM_003127115	Mitogen-activated protein kinase kinase kinase kinase 1 (MAP4K1)
S	XM_003131921	Misshapen-like kinase 1-like
S	NM_001031795	Rab acceptor 1 (prenylated) (RABAC1)
S	NM_001097477	Ribosomal protein L10a (RPL10A)
S	XM_001924929	Ribosomal protein L8 (RPL8)
S	NM_001129954	Ribosomal protein S20 (RPS20)
S	XM_003121339	Sestrin-1-like
S	NM_001008685	Sperm mitochondria-associated cysteine-rich protein (SMCP)
S	NM_214011	Sperm adhesion molecule 1 (SPAM1)
S	NM_001198920	Spermatogenesis associated 3 (SPATA3)
S	NM_001025223	Speedy homolog A (Xenopus laevis) (RINGO/SPDY1)
S	NM_214238	Small proline-rich protein (SPRR1B)
S	XM_003124592	Stannin-like
S	XM_003121984	Testis-specific kinase 1 (TESK1)
S	XM_003128044	Testis-specific kinase 2 (TESK2)
M	NM_001243640	DNA methyltransferase 1 associated protein 1 (DMAP1)
M	NM_001032355	DNA (cytosine-5-)-methyltransferase 1 (DNMT1)
M	NM_001097437	DNA (cytosine-5-)-methyltransferase 3 alpha (DNMT3a)
M	XM_001928593	DNA (cytosine-5-)-methyltransferase 3 beta(DNMT3b)
M	XM_001928303	Ten-eleven translocation oncogene 1 (TET1)
M	XM_003129278	Ten-eleven translocation oncogene family member 2 (TET2)
M	XM_003125027	Ten-eleven translocation oncogene family member 3 (TET3)
M	NM_001162885	TRNA aspartic acid methyltransferase 1 (TRDMT1/DNMT2)
C	NM_213978	Beta-2-microglobulin (B2M)
C	NM_001032376	Hypoxanthine phosphoribosyltransferase 1 (HPRT)
C	XM_003360434	Ribosomal protein, large, P1 (RPLP1)
C	SA_00133	Pig Genomic DNA Contamination
C	SA_00104	Reverse Transcription Control (x2)
C	SA_00103	Positive PCR Control (x2)

[1] C-Control; M-Methylation; S-Seasonality in semen

Table 2. Ranked changes from June and August collections, within each Harmonic amplitude measurement (HA0-5), total average rank, and average rank for HA0, 2 and 4.

Boar[1]	HA0	HA1	HA2	HA3	HA4	HA5	Average Rank	Average HA0, 2, 4

1	1	1	2	4	8	1	2.83	3.67
2	3	10	5	1	1	5	4.17	3.00
3	2	9	8	2	3	3	4.50	4.33
4	7	3	10	6	11	7	7.33	9.33
5	10	11	9	3	9	9	8.50	9.33
6	8	5	4	10	12	11	8.33	8.00
7	5	8	1	9	5	8	6.00	3.67
8	6	2	12	11	2	2	5.83	6.67
9	4	4	7	5	6	6	5.33	5.67
10[2]	12	12	11	12	7	12	11.00	10.00
11	11	7	3	8	10	10	8.17	8.00
12	9	6	6	7	4	4	6.00	6.33

HA – Harmonic amplitude

[1] Boars 1-6 used for breeding; boars 1-3 identified as least HA change and boars 4-6 identified as most HA change.

[2] Excluded from breeding selection, did not meet standard collection procedures in August.

Table 3. Harmonic amplitudes (HA0-HA5; in microns) for semen samples of boars selected for breeding by least and most difference in HA values by season.

Category	Season	HA0	HA1	HA2	HA3	HA4	HA5
Least change	June	2.841	0.0795	1.085	0.0309	0.224	0.024
	August	2.818	0.0791	1.079	0.0310	0.232	0.026
	Difference	-0.023	-0.004	-0.006	0.0001	0.008	0.002
Most change	June	2.808	0.092	1.075	0.035	0.216	0.032
	August	2.743	0.098	1.066	0.038	0.240	0.042
	Difference	-0.065	0.006	-0.009	-0.003	0.025	0.010

Mean harmonic values (HA0-HA5) by season for those boars selected for breeding, boars with the least amount of nuclear shape change between season (upper half; n=3), and those with the most change (lower half; n=3 each). Selection process used sum of ranks of absolute difference in harmonic values for individual boars.

Table 4. CASA assessment of boar semen collected in June and August from 12 boars.

Boar[1]	%Normal (June)	%Normal (August)	%Change in Normal from June to August	%Motile (June)	%Motile (August)	%Change in Motility from June to August
1	32.4	94.0	61.6	79.3	59.4	-19.9
2	38.4	72.0	33.6	94.1	93.9	-0.2
3	32.6	91.0	58.4	60.8	58.5	-2.3
4	79.7	74.0	-5.7	46.9	70.9	24.0
5	59.3	85.0	25.7	56.5	75.6	19.1
6	31.6	84.0	52.4	93.4	77.8	-15.6
7	40.0	92.0	52.1	59.4	54.1	-5.2
8	51.1	48.0	-3.1	82.1	50.3	-31.7
9	42.8	77.0	34.2	27.6	40.8	13.2
10[2]	38.7	64.0	25.4	50.0	40.0	-10.0
11	16.2	95.0	78.8	90.0	94.7	4.7
12	57.9	85.0	27.1	49.3	61.7	12.4

CASA – Computer Assisted Sperm Analysis

[1] Boars 1-6 used for breeding; boars 1-3 identified as least HA change and boars 4-6 identified as most HA change.

[2] Excluded from breeding, did not meet standard collection procedures in August.

Table 5. Effect of season (June or August) and preparation (cooled extended or frozen-thawed) on semen quality parameters[a].

Main Effect	**Semen Season and Preparation**	
Season	**June**	**August**
HA1 (µm)	0.841 ± 0.013	0.940 ± 0.014 ****
HA3 (µm)	0.033 ± 0.001	0.037 ± 0.002 **
HA5 (µm)	0.030 ± 0.002	0.034 ± 0.002 **
Preparation	**Cooled extended**	**Frozen-thawed**
Morphology (%)[b]	67.9 ± 3.8	76.4 ± 2.4 *
Viability (%)	73.0 ± 2.1	22.7 ± 1.9 ****
HA1 (µm)	0.092 ± 0.002	0.086 ± 0.003 ****

[a] Values are the means ± standard error. Values between columns differ: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Variables for which an interaction of season by preparation was observed are while those for which there were no differences, $P > 0.05$, are not shown.

[b] Morphology is expressed as percent normal.

Table 6. Categorical data reflecting the experimental design and outcome.

Day 45 Collection		**Experiment 1 – Summer[1]**					**Experiment 2 – Winter[1]**					**Total[2]**
BOAR	HA change	ExT Summer[2]	FrZ Summer[2]	FrZ Spring[2]	CC Summer[2]	Exp 1[2]	ExT Winter[2]	FrZ Summer[2]	FrZ Spring[2]	CC Winter[2]	Exp 2[2]	
1	Least	2/3	5/5	4/5	2/2	13/15	3/4	2/3	4/4	1/1	10/12	23/27
2	Least	3/3	1/3	3/3	1/1	8/10	4/5	1/4	1/4	2/2	8/15	16/25
3	Least	3/3	1/3	0/3	0/1	4/10	3/5	0/5	1/5	1/1	5/16	9/26
Total[2]		8/9	7/11	7/11	3/4	25/35	10/14	3/12	6/13	4/4	23/43	48/78
Pregnancy Rate (%)		88.9	63.6	63.6	75.0	71.4	71.4	25.0	46.2	100.0	53.5	61.5
4	Most	3/4	1/5	2/3	2/2	8/14	2/5	3/4	3/4	1/1	9/14	17/28
5	Most	2/4	0/2	1/1	0/2	3/9	5/5	0/4	0/5	2/2	7/16	10/25
6	Most	3/5	1/3	3/5	0/1	7/14	2/3	2/4	0/3	2/2	6/11	13/25
Total[2]		8/13	2/10	6/9	2/5	18/37	9/13	5/12	3/11	5/5	22/41	40/78
Pregnancy Rate (%)		61.5	20.0	66.7	40.0	48.6	69.2	41.7	27.2	100.0	53.7	51.3
Total by Semen Type & Collection Season[2]		16/22	9/21	13/20	5/9	43/72	19/27	8/24	9/25	9/9	45/84	88/156
Pregnancy Rate (%)		72.7	42.9	65.0	55.6	59.7	70.4	33.3	36.0	100.0	53.6	56.4

[1] Breeding season of experimental trial

[2] Reported as number pregnant over number bred.

Table 7. Pregnancy rate at 45 d post-AI by change in Harmonic Amplitude from June to August.

HA Change	Least	Most
Total	48/78	40/78
Pregnancy Rate (%)†	61.5	51.3

†differ by 0.10.

Table 8. Pregnancy rate at 45 d post-AI by season in which semen was collected.

Semen Collection Season	Spring/Winter	Summer
Total	50/81	38/76
Pregnancy Rate (%)†	61.7	50.0

†differ by 0.10.

Table 9. Pregnancy rate at 45 d post-AI by semen storage (cooled extended or cryopreserved).

Semen Preparation	ExT[1]	FrZ	CC[1]
Total	35/49	39/90	14/18
Pregnancy Rate (%)	71.4[a]	43.3[b]	77.8[a]

[1] Cooled extended semen used in a synchronized single insemination (ExT) or naturally detected estrus with double insemination (CC).

[ab] Pregnancy rates with different letters differ by 0.05.

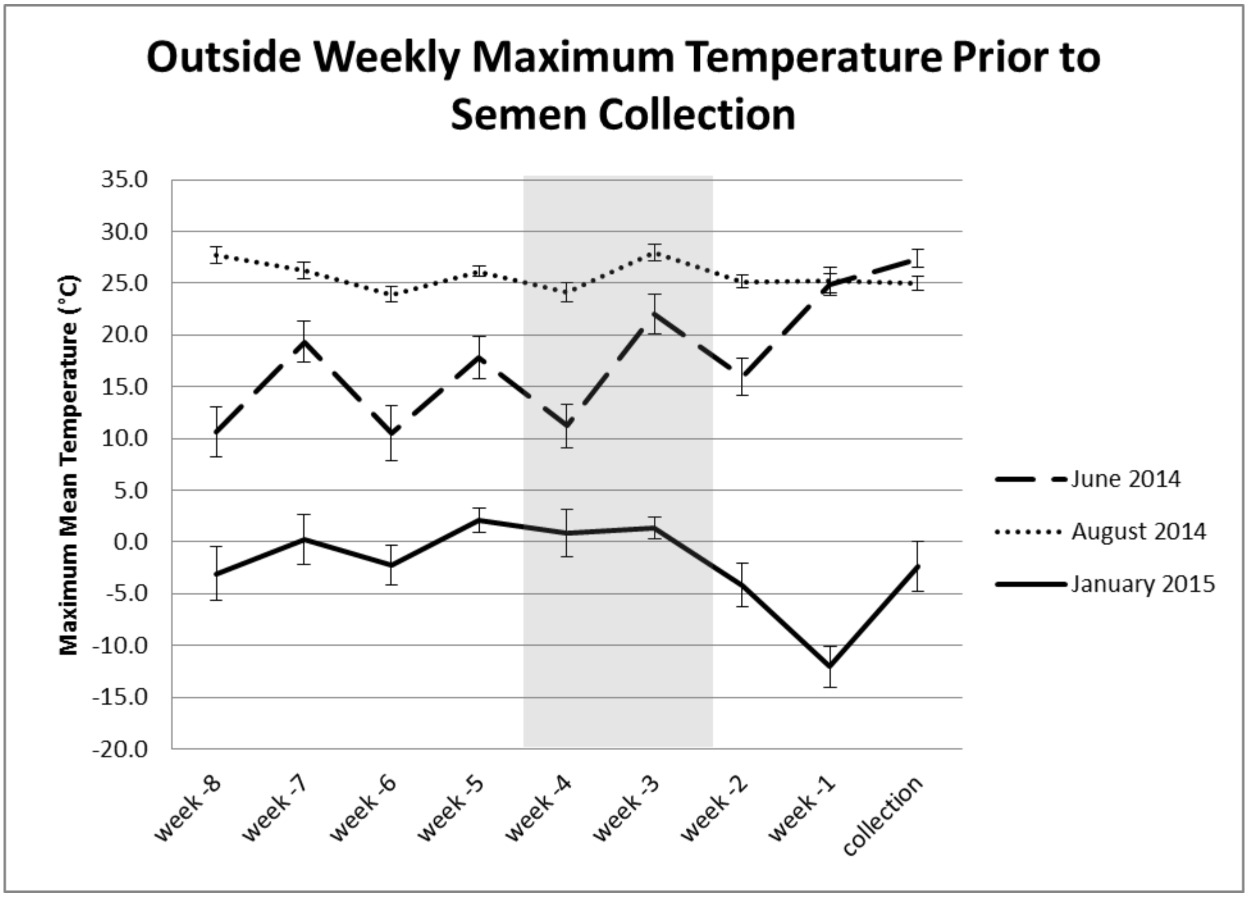

Figure 1. Maximum outdoor temperatures prior to semen collection in June (— -), August (----), or January (—). Grey area represents the period of time prior to semen collection that has the greatest thermal impact upon sperm quality. Temperatures are reported as weekly means ± SEM.

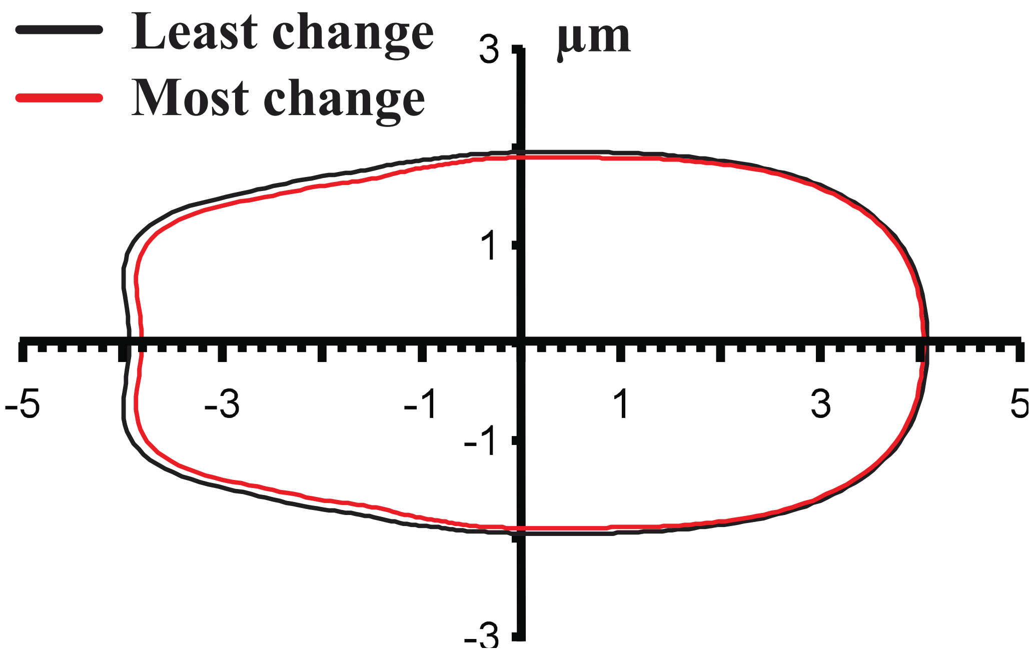

Figure 2. Impact of season on sperm nuclear shape among boars showing least and most amount of change HA between cooled semen collections by season. Six total boars were selected that met commercial boar stud quality standards, based on exhibiting the most (n=3 boars) or least (n=3 boars) amount of nuclear shape change between June and August cooled extended semen collections. The average nuclear shape of all 12 boars from June was used as a base from which the HA0-5 were replaced with HA0-5 means from August cooled extended semen of those boars showing the least and most change separately. A new Cartesian graph was derived to illustrate the manner of change for these two groups from June. Boars whose semen showed a greater degree of shape change between seasons (red) had smaller nuclei on average than did those showing less change (black).

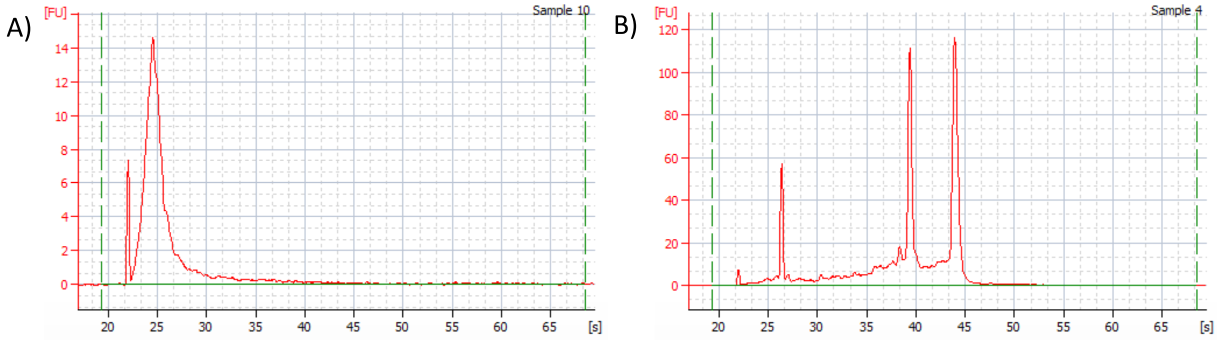

Figure 3. Representative electropherograms of sperm RNA (panel A) and somatic cell RNA (panel B) ran on an Agilent Bioanalyzer using picoChip arrays. The first peak that appears at approximately 22 seconds is the embedded marker; peak at approximately 25 seconds is derived by small RNAs, tRNAs, and 5S and 5.8S rRNA; and peaks at 40 and 45 seconds are 18S and 28S rRNA, respectively, of which sperm does not contain. Absence of peak disturbances from approximately 42 through 65 seconds indicates no genomic DNA contamination. [FU] – fluorescence, [s] – seconds.

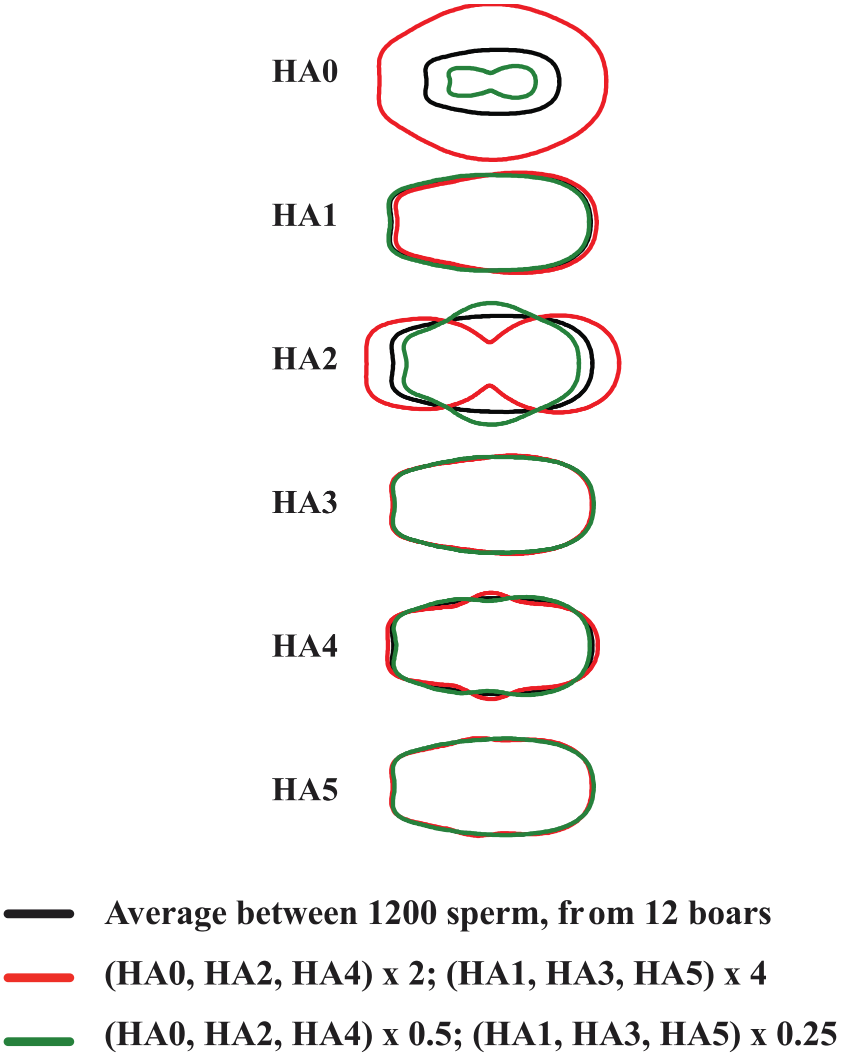

Figure 4. Difference in boar sperm nuclei by harmonic amplitude values. One hundred sperm from each of 12 boars cooled extended semen from June were averaged for each of 20 harmonic values and a Cartesian coordinate line was constructed (black). The six harmonic values (HA0-HA5) were then modified one at a time to illustrate changes in nuclear shape change in response to harmonic value manipulation. Harmonic values were multiplied by 2 and 0.5 (HA 0, 2, 4) or by 4 and 0.25 (HA 1, 3, 5). Nuclear shape response to increased harmonic amplitudes, are denoted in red, and decreases in green.

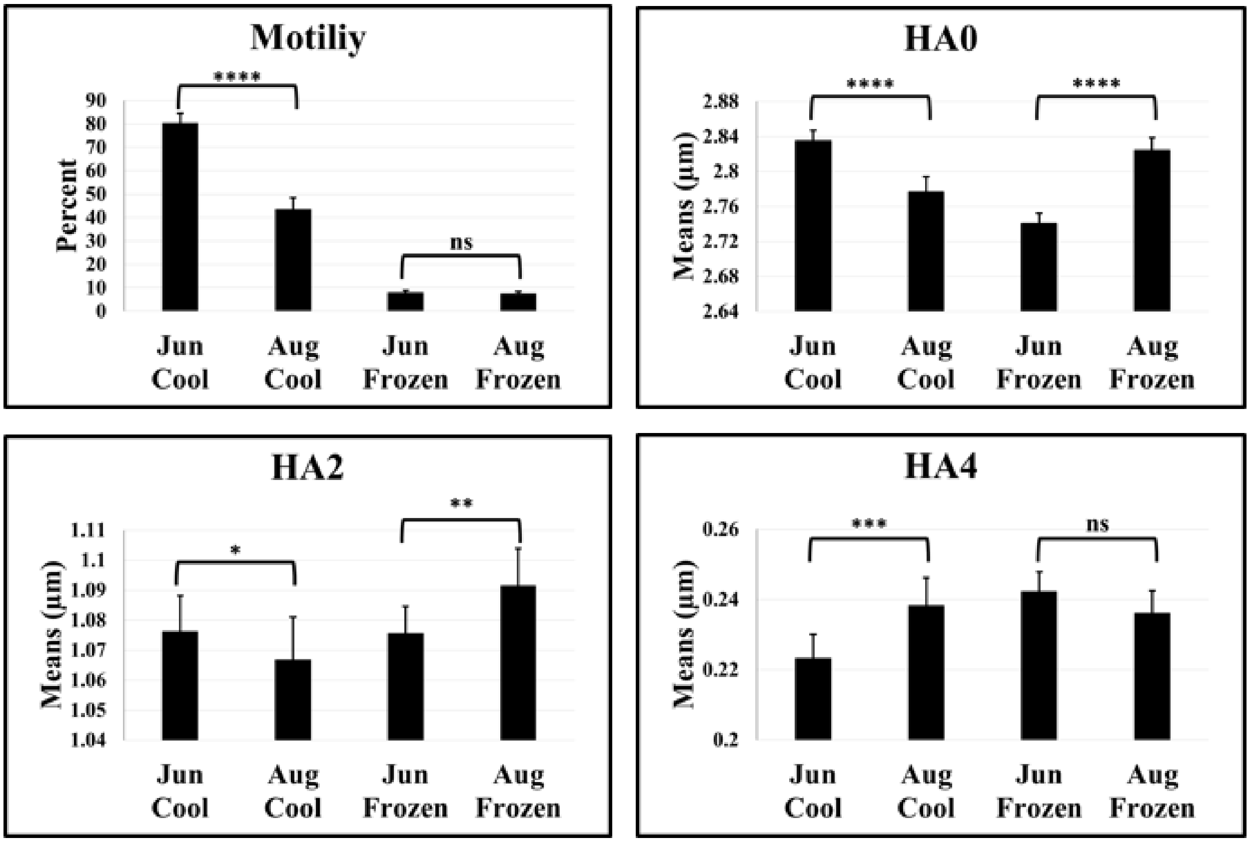

Figure 5. Effect of the interaction of preparation by season on semen quality parameters among four semen types. Semen cooled extended from Spring (Jun Cool), cooled extended from Summer (Aug Cool), frozen-thawed from Spring (June Frozen) and frozen-thawed from Summer (Aug Frozen). Values are the means ± standard error. Values between bracketed bars differ: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$; or ns, not significant.

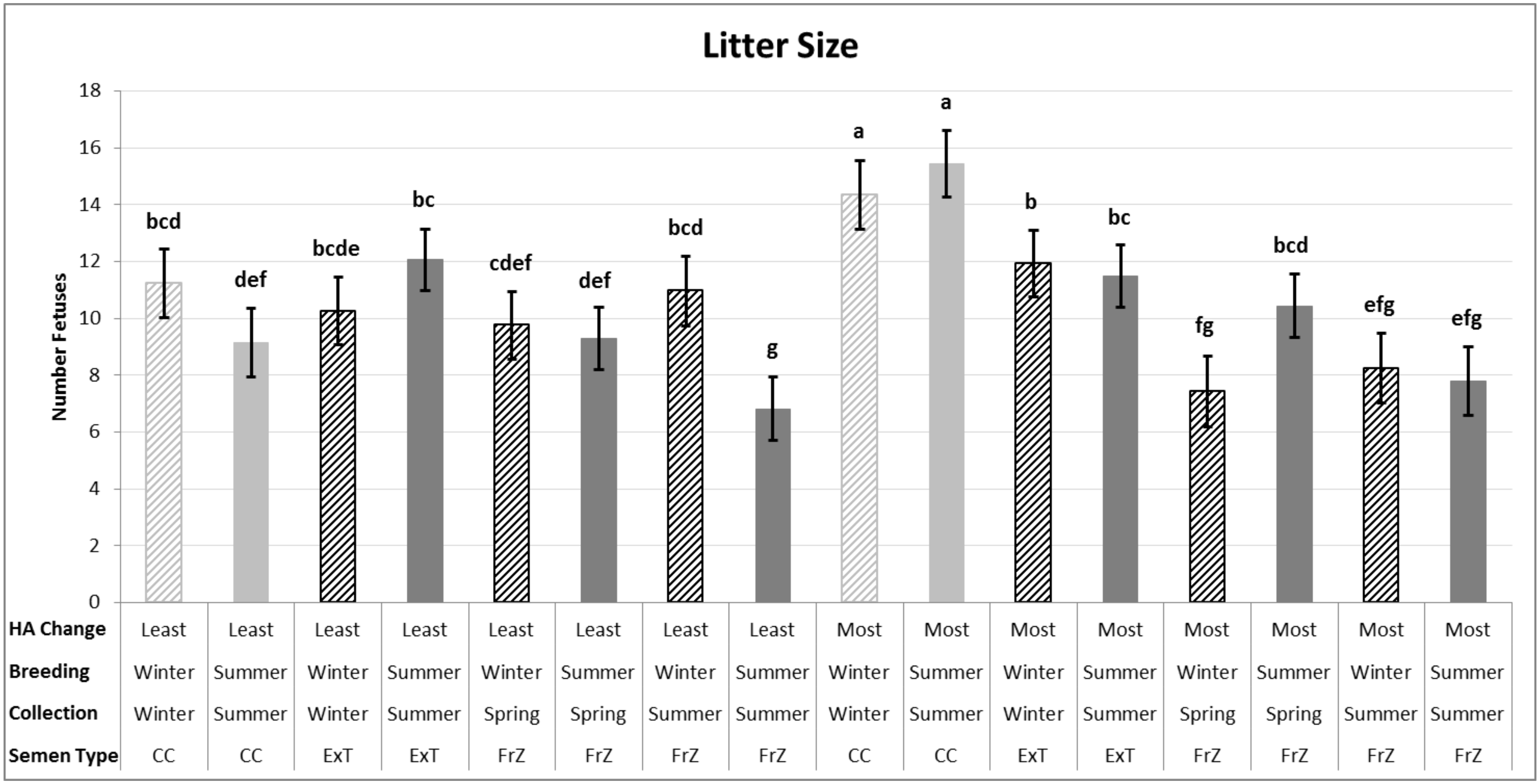

Figure 6. Least square means of litter size. Litters derived from matings using semen with least or greatest HA (HA Change); summer or winter breeding (Breeding); semen collected during spring, summer, or winter (Collection); and storage conditions (Semen Type). Values are LSM ± SE. Values are significantly different at $P < 0.05$and are denoted by different letters. Hatched bars represents winter breeding and solid bars represent summer breeding. Light grey represents 2x insemination control gilts (CC) and black and dark grey represent synchronized, 1x insemination gilts with cooled extended (ExT) or cryopreserved (FrZ) semen.

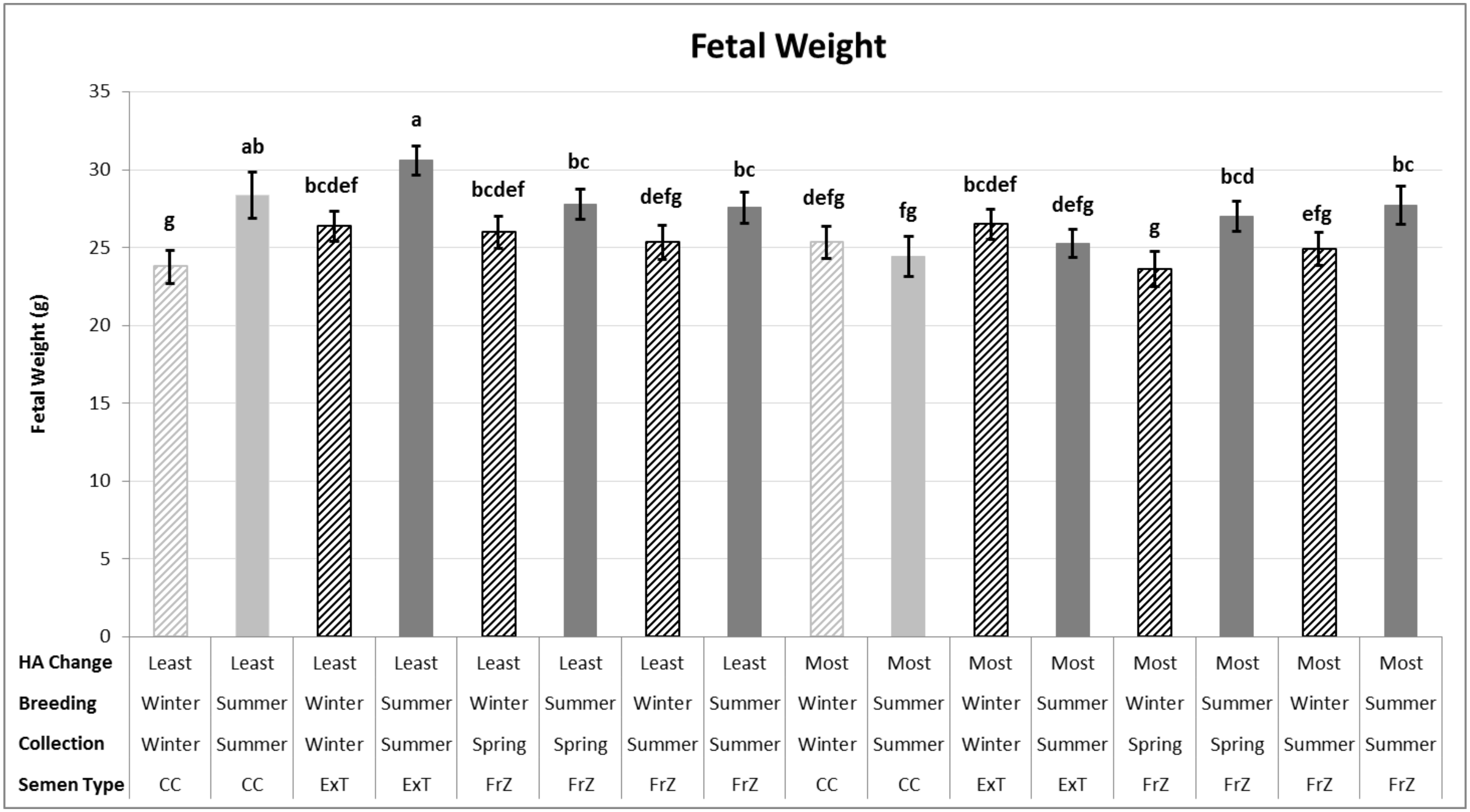

Figure 7. Least square means of fetal weight. Litters derived from matings using semen with least or greatest HA (HA Change); summer or winter breeding (Breeding); semen collected during spring, summer, or winter (Collection); and storage conditions (Semen Type). Values are LSM ± SE. Values are significantly different at $P < 0.05$and are denoted by different letters. Hatched bars represents winter breeding and solid bars represent summer breeding. Light grey represents 2x insemination control gilts (CC) and black and dark grey represent synchronized, 1x insemination gilts with cooled extended (ExT) or cryopreserved (FrZ) semen.

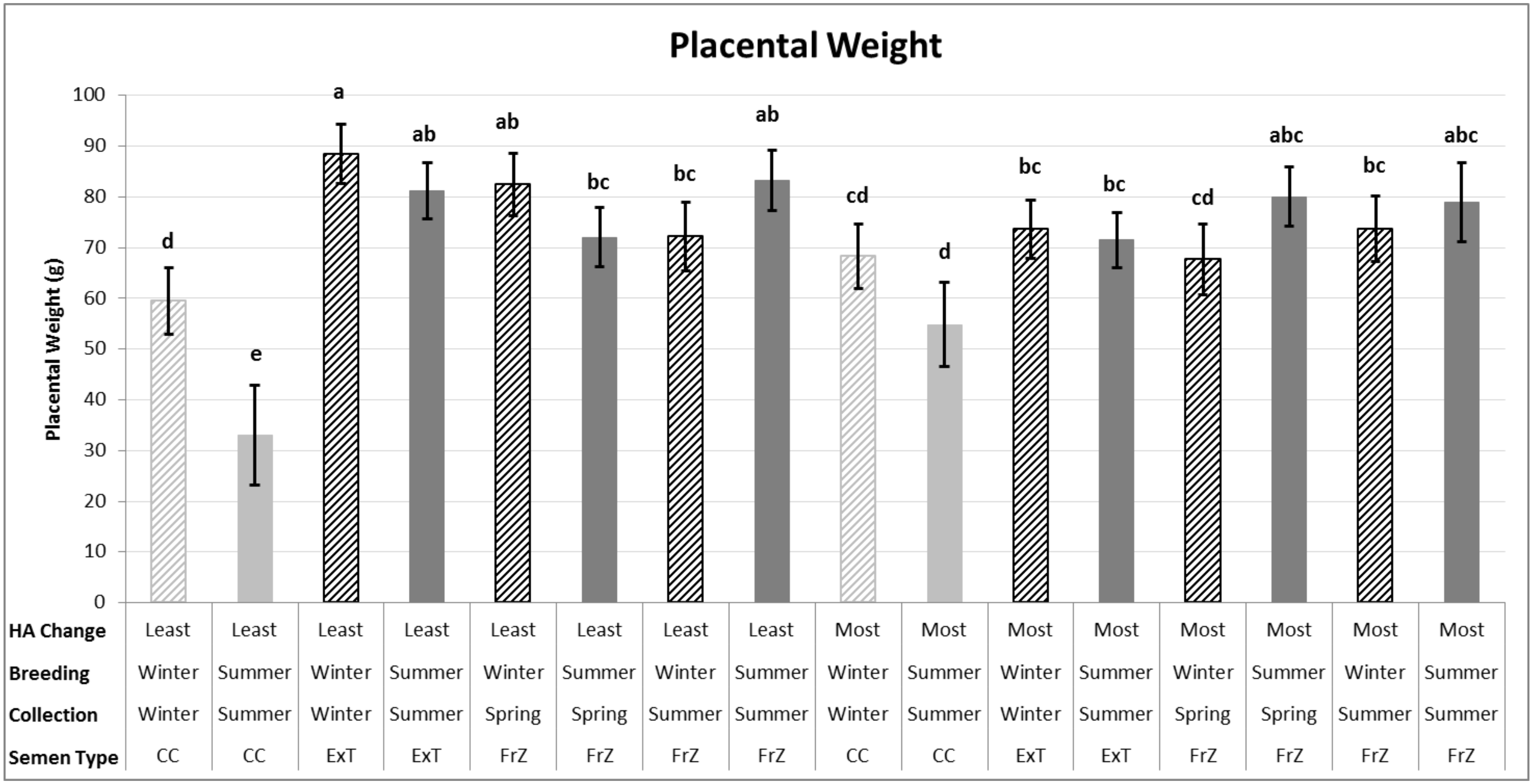

Figure 8. Least square means of placental weight. Litters derived from matings using semen with least or greatest HA (HA Change); summer or winter breeding (Breeding); semen collected during spring, summer, or winter (Collection); and storage conditions (Semen Type). Values are LSM ± SE. Values are significantly different at $P < 0.05$and are denoted by different letters. Hatched bars represents winter breeding and solid bars represent summer breeding. Light grey represents 2x insemination control gilts (CC) and black and dark grey represent synchronized, 1x insemination gilts with cooled extended (ExT) or cryopreserved (FrZ) semen.

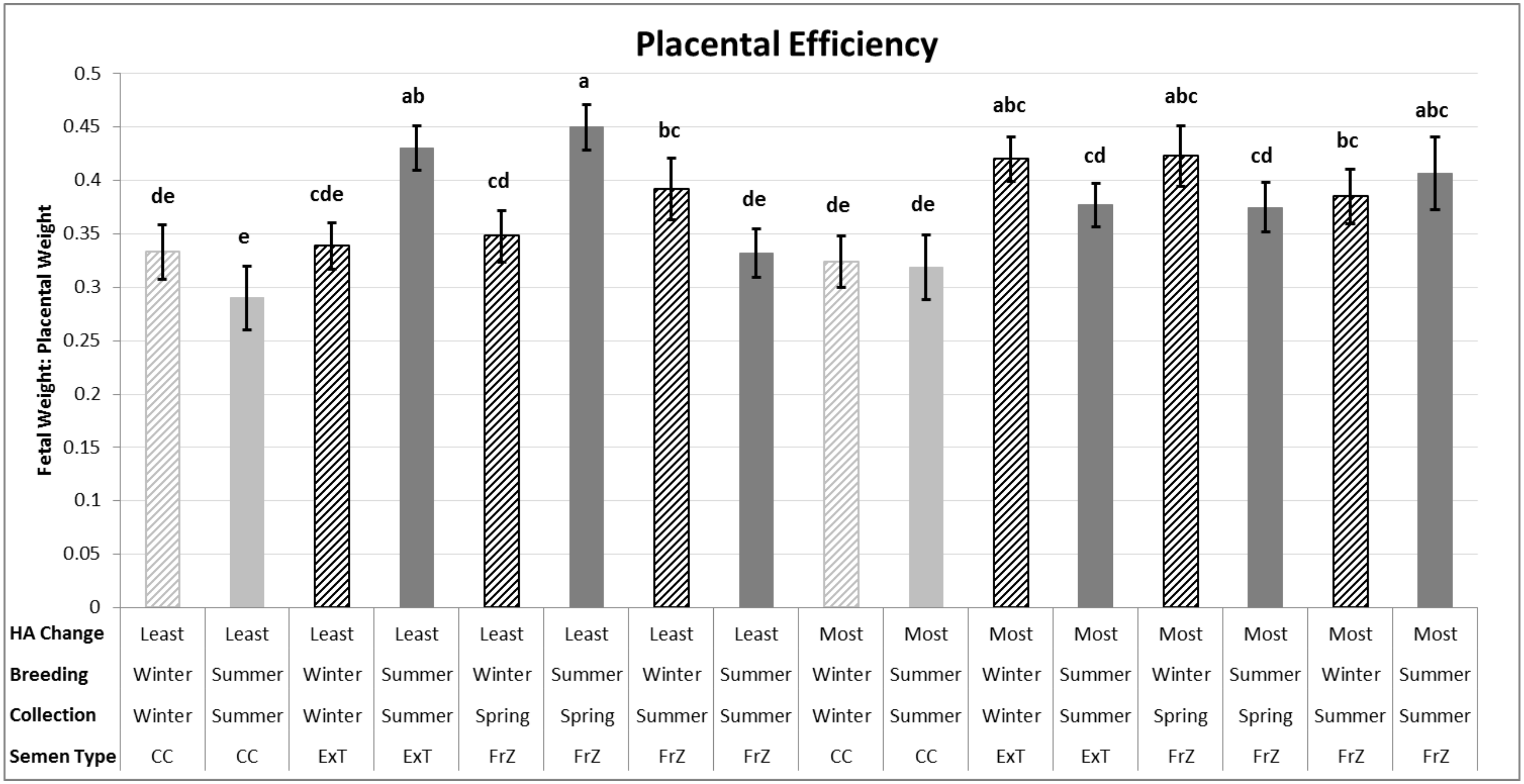

Figure 9. Least square means of placental efficiency. Litters derived from matings using semen with least or greatest HA (HA Change); summer or winter breeding (Breeding); semen collected during spring, summer, or winter (Collection); and storage conditions (Semen Type). Values are LSM ± SE. Values are significantly different at $P < 0.05$and are denoted by different letters. Hatched bars represents winter breeding and solid bars represent summer breeding. Light grey represents 2x insemination control gilts (CC) and black and dark grey represent synchronized, 1x insemination gilts with cooled extended (ExT) or cryopreserved (FrZ) semen.