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Quantifying Sperm Nuclear Shape with Fourier Harmonic Analysis and Relationship to Spermatogenesis and Fertility

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Abstract

A method is presented that describes the shape of sperm nuclei, Fourier Harmonic Amplitude (FHA) analysis. Sperm are stained with DNA binding fluorescent dyes, imaged with epiflourescence microscopy, image analysis used to identify sperm nuclei, the perimeter of nuclei obtained for 100 sperm per sample and Harmonic Amplitudes (HA) 0-5 computed along with dispersion statistics for each HA. The approach produces objective measures of sperm nuclear shape that are independent of object rotation and orthogonal. Bull fertility has been classified as low, <1sd below the mean, or high, $\geq$ 1 sd above the mean. In individual studies, we identified groups of bulls with low fertility and all the rest, high fertility and all the rest, or high and low fertility bulls. Consistently, the means of HA0-HA5 or variances of these harmonics have been found related to bull fertility, $p<0.05$. When multiple ejaculates were collected on bulls, the variation among bulls accounted for >84% of the total variation, while the ejaculates accounted for <9%. Within an interval of a few weeks, a single ejaculate is sufficient to determine nuclear shape statistics from FHA analysis on a bull. The FHA approach was also able to differentiate ($p<0.05$) bull semen samples that had reduced *in vitro* fertility either due to environmental heat stress or scrotal insulation. In the boar, the FHA approach could identify low fertility boars and those semen samples impacted by scrotal insulation. To examine the impact of scrotal insulation on spermatogenesis, we performed testicular morphometry on control or insulated bulls and boars. There was reduced numbers of Sertoli cells ($p<0.05$) following scrotal insulation of bulls and loss of primary spermatocytes ($p<0.05$) in the boar. Testicular gene expression experiments following scrotal insulation in the bull found decreased expression of TIMP2. The disruption to the seminiferous epithelium due to scrotal insulation may explain resulting changes to sperm nuclear shape and reduced fertility.

1.0 Introduction

The evaluation of semen quality has been to either understand the cause of, or predict, difference in fertility among males. Approaches have involved biochemical, bio-physical, mechanistic, and morphological evaluation [1,2]. There have been many advances in semen quality measures mostly to establish approaches that are

objective and not subjective. For example, while motility of sperm can be estimated subjectively by visual observation, it is now common for motility to be evaluated with computer assisted sperm analysis (CASA) [3,4]. In contrast the evaluation of sperm morphology is generally subjective [5]. Sperm are typically fixed, perhaps stained and then observed either in a wet mount or after drying on a slide. Key is that sperm are then classified as either normal or abnormal, based on a subjective interpretation of specific sperm characteristics. Multiple observers can classify the same sperm differently, depending on their interpretation of normal and abnormal. The same observer may even classify the same sperm differently on successive occasions. It was our objective to develop new methodology that more completely describes sperm head shape. Sperm morphology is thought to relate to male fertility, as abnormalities are likely associated with failure of sperm to complete the physiological events in the female reproductive tract leading up to fertilization and then sustaining embryo development. While the classification of normal and abnormal sperm have produced statistics on a male that have been related to fertility, we believed this could be improved upon utilizing fluorescence microscopy and computational power of modern computers. We began our work by first considering fertility in the bovine when using artificial insemination. Compensable and uncompensable semen traits have been proposed and for most bulls, semen is inseminated into females at numbers sufficient to overcome compensable traits [6,7]. This would leave uncompensable traits as the primary reason for differences in fertility among bulls. An uncompensable trait would be one in which the sperm reach the site of fertilization in numbers sufficient to achieve penetration and activation of the oocyte. The zygote and/or embryo would then initiate development but ultimately fail. We have hypothesized that failure of development would be due to problems related to the sperm nucleus although other aspects of how the oocyte is activated could also be involved. Results supports nuclear/DNA problems associated with low fertility bulls exist such that entry into S-phase of the zygotes sired by these bull's are delayed and shorter than zygotes sired by higher fertility bulls [8]. Where our research has diverged from others is that we thought it likely that problems with the nucleus/DNA would likely cause only small changes to the sperm nucleus as major abnormalities described by others often occur only in dead sperm [9]. We thus sought to find a way to derive equations which would describe the shape of the sperm nucleus, be able to determine small changes to shape in the sub-micron range and be objective.

2.0 Fourier Harmonic Analysis

Other researchers have sought to describe sperm head shape, essentially the nucleus shape, using measures of length, width and area [10-13]. These descriptors only partially describe shape and are not really related to how the human eye would identify differences between objects. Instead the human eye focuses on the curvature at the edge of the object. In the case of sperm, it is likely the nucleus is the object we would wish to describe. Our approach has been to label sperm with a DNA-binding fluorescence dye such as Propidium Iodide, Hoechst 33342 or Yoyo-1. Sperm are then evaluated with epifluorescence microscopy, image capture and digital analysis to obtain the perimeter coordinates of any sperm nuclei without any interference of sperm structures in the tail. An

example of the nuclear perimeter coordinates of an average bovine sperm is shown in Figure 1. Generally most individuals think of sperm related to a Cartesian coordinate system. If the perimeter coordinates are converted from Cartesian to Polar coordinates and plotted in a rectangular polar coordinate graph, a repeating curve appears each time the radial distance is moved from 0 to 2π radians. In the polar coordinate graph, peak distances represent the radial distance from the centroid to the anterior or posterior regions of the nucleus. The minimum distances represent centroid to lateral edge of the nucleus. The perimeter data in polar coordinates can be quantified using Fourier series. The equations consist of a summation of sin and cosine terms at various frequencies and angles [9,14]. Reduction of the Fourier series to just a summation of cosine terms leaves amplitudes times cosine functions of angles summed over various frequencies. The maximum number of frequencies is n/2, in which n is the number of coordinate points on the perimeter of the object. In our approach, we statistically interpolate the actual data to obtain perimeter locations at equally spaced angles at 1-degree intervals. The evaluations of the amplitudes that describe the magnitude of shape components at each frequency are termed Harmonic Amplitues (HA). We thus refer to our approach as Fourier Harmonic Amplitude (FHA) analysis of sperm nuclear shape. Phase angles adjust the general angle, sliding a typical cosine function to the left or right in the rectangular polar coordinate graph such as seen in Figure 1. While phase angles are calculated, they are only used in the FHA approach to generate average sperm nuclear shapes. Phase angles do contain species information but appear similar within a species (Parrish, personal observation). Several aspects of FHA analysis are important to note: results are independent of object rotation, harmonic amplitudes are orthogonal components of shape, HA 0-5 are sufficient to describe differences in sperm nuclear shape, and small differences in the nuclear shape are detectable due to averaging of data from 100 sperm per sample [9,14].

3.0 Relationships of Sperm Nuclear Shape and Fertility

Initial studies of FHA analysis demonstrated clusters of sperm could be statistically identified with different types of nuclear shape but this did not advance us much beyond what we could do without FHA analysis [14]. The first study to examine the effects of bull fertility on sperm nuclear shape as determined by FHA analysis utilized frozen-thawed semen from 59 bulls collected at a major US bull stud in late spring [9]. The estimated relative conception rate (ERCR, >100 breedings/bull) was the fertility data used and bulls were classified as OK or of poor fertility. Low fertility was defined as those bulls that were <1 SD below the mean for the population of bulls evaluated. The remaining bulls were classified as acceptable. There were 2 dyes used for staining sperm, Hoechst 33342 and YOYO-1, with YOYO-1 used to identify dead sperm. Analysis was thus possible on live, dead or all sperm. Differences between the low and adequate fertility group bulls was greater for live sperm, so this is what is described. Bulls in the low fertility group had increased variance for HA0, HA2, HA4 and HA5 ($p<0.05$) but no difference in mean HA 0-5 values ($p>0.05$). In this experiment, low fertility bulls produced sperm nuclei with more variation in shape than OK fertility bulls but no difference in overall shape.

To get a more accurate estimate of bull fertility a second trial was conducted in which we evaluated 6 bulls that each had over 5000 first service insemination records [15]. There were 3 high fertility bulls with a mean±SEM nonreturn rate of 78±2% and 3 low fertility bulls with a nonreturn rate of 69±1%. A discriminate analysis utilizing the mean HA 0-5 as components of the model found a significant canonical correlation of 0.55 ($p<0.05$) with HA 2, 4 and 5 the most important for differentiating the 2 fertility groups. If the discriminate analysis simply was asked to place the 6 bulls into 2 groups based on shape, it correctly placed all the bulls into the right fertility group and had a canonical correlation of 0.74 ($p<0.05$) for bulls within a group. The FHA approach yields predictors of bull fertility that are related to shape or its dispersion within an ejaculate.

An important question to ask was about the variation among bulls and collections within bulls. Thus we examined 3 collections on 11 bulls within a 2 week interval, partitioned the variance components, and determined the contribution of different components to the overall variation in sperm nuclear shape [9]. It was found that 84 – 98% of the variation was among bulls while 1 – 9 % variation was found within collections within a bull. There were effects of both variations within and among bull ($p<0.05$) but most of the variation is due to differences among bulls. Assuming no environmental impacts occur to a bull, a single ejaculate is sufficient to evaluate sperm nuclear shape at a particular point in time.

At this point the FHA approach appeared to be promising and we conducted a much larger study with 210 bulls representing most of the Holstein bulls in the US for which semen was being commercially collected in May and June of 2001 [16]. Frozen semen was from large commercial bovine artificial insemination companies in the US. Semen was frozen in egg yolk tris, egg yolk citrate or milk based extenders and straws were thawed as per each company's instructions. The fertility data was based on the ERCR value as of May 2003 which is a rolling 3 year average of data collected and normalized to have a population mean of 0. There was a mean±sem of 2449±254 breedings/bull ranging from a high of 21,931 to a low of 50. For the bulls in the study, mean±sd ERCR value was 0.05±1.82. Low fertility bulls (n=32) were defined as those with ERCR values < 1 SD below the mean or <-1.77. Bull's with ERCR values above the cutoff were defined as having acceptable fertility (n=178). The study was designed to determine if the FHA approach could identify bulls classified as having low fertility. The mean, variance, skewness and kurtosis of HA0-HA5 was determined from 1 collection day for each bull in the study. In addition we also determined a variety of other semen quality measures that will not be describe in this review. Discriminant analysis was used to produce a univariate anaysis of variance analysis (ANOVA), a multivariate analysis of variance (MANOVA) and results are shown in Table 1 for the analysis of mean HA0-HA5. The low fertility bulls had increased values for HA2, HA4 and decreased values for HA0 ($p<0.05$). The variations in HA0-5 between the bull fertility groups were only present for variance of HA1 and HA4 and Kurotsis of HA0. The MANOVA analysis found the overall mean shape of sperm nuclei differed between the bull fertility groups ($p<0.01$) but not the dispersion measures, variance, kurtosis or skewness of the HAs ($p>0.05$). The ability to identify mean

shapes being different among bull fertility groups in this experiment and not previous ones may relate to improvements to algorithms used to identify the edge of the sperm during image analysis. In this same experiment other semen quality analyses were performed. There was no differences between bull fertility groups for motile sperm as determined from CASA or percent sperm with intact acrosomes, $p>0.05$. The percentage of morphologically normal sperm and viable sperm decreased ($p<0.05$), and sperm detected as damaged by the sperm chromatin structure analysis [method of 17,18] were increased ($p<0.05$) in semen from the low fertility bulls.

In contrast to differences for low fertility compared to OK fertility, when fertility groups were identified as high fertility (>1sd above the mean, ERCR>1.87, n=29) or OK fertility (ERCR$\leq$1.87, n=181) some individual variables could separate the groups but were not robust and none of the MANOVA results were significant (Parrish, data not shown). The high fertility bulls thus did not have semen with characteristics very different from the remaining bulls.

We did attempt to produce low fertility bull prediction equations from the discriminant analysis on the 210 bulls whose semen analysis was described in Table 1. However, it became apparent that we really needed a training set that had equal numbers of low and adequate fertility bulls with likely numbers in excess of 60 in each population. Further we were concerned that semen was frozen in at least 3 different extenders and under various freezing protocols. It is unclear if such differences have an impact on sperm shape or even the other parameters beyond those due to spermatogenesis or epididymal maturation. Separation of bulls into stud origin would have left too few bulls to evaluate.

We have also examined the ability of FHA analysis to differentiate between sperm from low and adequate fertility boars [19]. Fertility was determined from at least 100 single sire matings per boar and farrowing rates determined. There were 26 boars in the study. Low fertility boars (n=2) were those with farrowing rates < 60% and adequate fertility boars (n=24) having fertility $\geq$60%. This equates to fertility below or above 1 sd below the mean. The average fertility was 44.7%±3.3 for the low fertility group and 72.2%±0.6 for the adequate fertility group. Extended semen was shipped to the lab at 17°C and evaluated with the same procedure as for bulls to determine the live sperm nuclear shape. In addition, % motile sperm was determined prior to shipping by visual observation. There was no difference in the percentage of motile sperm, percentage viable sperm, HA0, HA1, HA3 or HA5 between the two boar fertility groups ($n>0.05$). Similar to the bull, the mean±sem HA2 and HA4 decreased ($p<0.05$) from the low to adequate fertility groups, 1.110±0.020 vs. 1.010±0.009 and 0.224±0.011 vs. 0.168±0.00, respectively. In the bull and boar, sperm are slightly longer and more tapered in the lower fertility males. Additionally in the bull the average size, HA0, is decreased in the sperm from lower fertility males.

In a final comparison to *in vivo* fertility, bulls from a progeny test program differed by 1 sd below the mean (low) and 1 sd above the mean (high) were selected. Thawed semen was stained with only Hoechst 33342 and all sperm present were evaluated for FHA of HA0-5 and variation of these harmonics within an ejaculate. Results are shown in Table 2. Both HA0 and HA1 were impacted by bull fertility status, $p<0.05$. While skewness and kurtosis were not impacted by bull fertility group, $p>0.05$ (data not shown), the variance was higher in low fertility bulls for HA0-3, and HA4-5, $p<0.05$. The MANOVA evaluation found both shape as defined by HA0-5 and variation as defined by the variance of HA0-5 were different for the bull fertility groups, $p<0.01$. The definition of fertility groups was different in Tables 1 and 2. Table 2 focused on a large number of both high and low fertility bulls. The assay was also simplified to include evaluation of all sperm. The fertility groups still have different sperm shapes with an increased variation in the sperm shape within low fertility bulls.

4.0 Impact of Heat Stress on Sperm Nuclear Shape and Fertility

Next the impact of summer heat stress and ability of FHA to detect this was examined on semen from 4 bulls housed at a commercial AI center in Wisconsin [16]. The bulls were found to produce semen in August that did not pass quality control standards of the company but then again met those standards in October – November. Semen was frozen at each period, thawed and evaluated or used for *in vitro* fertilization. The percentage of motile and viable sperm was determined at the commercial bull stud using their CASA (5 fields/sample) and flow cytometry (>10,000 sperm/sample) equipment. Sperm morphology was determined on 100 fixed sperm per sample. Since it is not possible to obtain *in vivo* fertility estimates on single ejaculates, *in vitro* fertilization (IVF) was used. The IVF procedure was a described previously [8, 20] with 3 replicates for evaluation of fertilization percentage and 2 replicates to evaluate cleavage and number of nuclei 135 hours post sperm insemination. Results are shown in Table 3. While the percentage of motile sperm and percentage of viable sperm only trended to differ ($p=0.07$), the percentage of sperm with abnormal heads increased ($p<0.05$) and percentage of normal sperm decreased ($p<0.05$) in the summer collected semen. There was no effect of season on secondary or tertiary abnormalities, $p>0.05$ (data not shown). The semen was culled primarily on the poor morphology and the evaluation supports this decision. *In vitro* fertility also demonstrated the reduced ($p<0.05$) ability of even the motile sperm to fertilize oocytes as Percoll separated sperm were used for IVF [20]. The percentage of nuclei in fertilized oocytes at 135 hours after sperm insemination was also decreased ($p<0.05$) in summer derived semen indicating potential DNA damage leading to delayed cleavage events [8, 21]. Finally the summer semen had reduced HA0 ($p<0.05$) indicating sperm nuclei were smaller in size than for sperm from the same bulls in the fall. There was no difference in other HA1-HA5, $p>0.05$ (data not shown). It was previously demonstrated that FHA analysis of live sperm using the Hoechst and Yoyo1 approach, as done in this experiment, obtains the same values as if you first separate sperm with a Percoll gradient [22]. Thus we evaluated the sperm with FHA that were the ones potentially participating in the fertilization of oocytes *in vitro*. This was not true for the morphological exam and most of the abnormal sperm likely are present in the dead sperm population that would have been removed by Percoll

separation. The FHA approach was able to statistically identify sub-standard ejaculates using an objective approach rather than the subjective one of morphology evaluation.

To understand the role of spermatogenesis in sperm nuclear shape and ultimately fertility, we have utilized scrotal insulation models in bulls and boars. Short term scrotal insulation can result in sperm with abnormal morphology, reduced viability and *in vitro* fertility [23–25]. In bulls, we insulated the scrotum of bulls (n=5) for 48 hours, collected semen 3 times/week, (M,W,F) for 2 weeks prior to insulation and 10 weeks following insulation. The semen was then cryopreserved using a standard egg-yolk citrate extender. Data collected prior to scrotal insulation was considered the control and so was pooled and is referred to as day 0. The days >0 then represent semen samples from various days after scrotal insulation was applied. Thawed semen *in vitro* fertility was evaluated by ability of sperm to fertilize oocytes *in vitro*, cleavage of the zygote and embryo development. Sperm nuclear shape was evaluated by FHA analysis. The results of the *in vitro* fertility assessment are shown in Figure 3. Fertilization of oocytes with a standard number of motile sperm resulted in decreased fertilization ($p<0.05$) for days 16, 19, 26, 28, 30 and 33 post insulation. Zygotic cleavage was evaluated at 32 hours as an indicator of early cleavage and 48 hours as an indicator of total cleavage. Early cleavage is related to ability of bovine embryos to form blastocysts [8, 21, 26]. Cleavage ratio decreased ($p<0.05$) for semen from days 19, 21, 28, 30, and 33 post insulation. The number of nuclei was evaluated at 135 hours after sperm addition by fluorescence microscopy of paraformaldehyde fixed embryos, permiabilized with Triton X-100 and nuclei stained with Hoechst 33342. Parthenogenically activated oocytes in this system have no more than 3 nuclei at the 135 hour evaluation time and so all embryos evaluated had more than 3 nuclei (data not shown). The number of nuclei was reduced at 14, 16, 19, 21, 30 and 33 days post insulation. When we examine nuclear shape of sperm with the FHA approach (Figure 4) following scrotal insulation we find impacts ($p<0.05$) on HA0 – HA3 and HA5 with differences occurring over the range of days 19 – 33. It is now possible to determine the cell types impacted by scrotal insulation using a diagram of bovine spermatogenesis as shown in Figure 5 since scrotal insulation was only applied for a short interval of 48 hours. *In vitro* fertility of sperm was decreased by scrotal insulation in a period of time during which round spermatid nuclear condensation, acrosome formation and flagellar elongation are occurring, namely mid spermiogenesis. A second period is impacted by scrotal insulation during the late pacheteyne primary spermatocyte to formation of the round spermatid, namely the end of meiosis. The changes in HA0 – 3 and HA5 indicate impacted by scrotal insulation on mid spermiogenesis and HA0 and HA2 on the end of meiosis. In a scrotal insulation study conducted in boars [27], there were 5 control boars with sham insulation and 5 insulated boars. The FHA analysis of sperm found that scrotal insulation impacted zygotene to pachytene primary spermatocytes and secondary spermatocytes that eventually resulted in ejaculated sperm with changes to HA0 and HA2-HA5. While it is understandable that disturbances to mid spermiogenesis, a period when the nucleus is undergoing reorganization impacts sperm nuclear shape, it is not clear how late meiosis events can also accomplish this.

To support our finding on impacts of scrotal insulation on spermatogenesis, we performed histological evaluation of testes using testicular morphometry [28]. Included in the study were control (N=3) or scrotal insulated (N=3) bulls. We found that the number of Sertoli cells decreased ($p<0.05$) and Sertoli cell index (germ cells/Sertoli Cell) increased ($p<0.05$). In a similar study we evaluated sham control (N=2) and scrotal insulated (N=3) boars. This time the height of the seminiferous epithelium decreased ($p<0.05$) along with the number of preleptotene to leptotene and pachytene primary spermatocytes ($p<0.05$). There was also increased ($p<0.05$) debris in the lumen of the semeniferous tubules in scrotal insulated boars. The studies on the impacts of scrotal insulation to the testes histology support that scrotal insulation disrupts the function of the seminiferous epithelium, Sertoli cells and development of germ cells that could result in the changes to sperm nuclear shape and reduced fertility.

In the bull scrotal insulation for histology, RNA was extracted from testes of control (N=3) or insulated (N=3) bulls and expression arrays containing 19,000 genes [29]. The set of genes evaluated was 200 and focused on apoptosis, DNA repair, hypoxia, and heat stress. There were 6 gene transcripts identified as different between control and insulated bulls testes. However on verification with real time PCR, only one, Tissue Inhibitor of Matrix Metalloproteinase-2 (TIMP-2) trended to differ ($p=0.06$) and it was down regulated in scrotal insulated bull testes. It is known that TIMP-2 is involved in preventing extracellular matrix remodeling and is located on the surface of Sertoli Cells [30], up regulated by FSH[30, 31], down regulated in infertile mice and humans [32], down regulated and has altered expression with heat stress [30, 33]. Further, TIMP-2 is involved in preventing apoptosis in male germ cells [31] and clearly plays a regulatory role in spermatogenesis [34]. The effects of scrotal insulation and elevated temperature may therefore be due to changes in TIMP-2 expression and alteration of the relationship between the Sertoli cells and associated germ cells. In addition to testicular TIMP-2, Ax and coworkers have identified the TIMP-2 protein in seminal plasma and bound to sperm as one of the proteins recognized by an antibody that is diagnostic of bull fertility [35] and others have found a relation between TIMP-2 RNA in sperm and bull fertility. One may speculate that artificial supplementation of FSH could potential negate the effects of heat stress on production of sperm with altered sperm nuclear shape and reduced fertility.

5.0 Conclusions

The analysis of sperm nuclear shape using FHA analysis has developed slowly due to complexity of mathematics, image analysis and statistical techniques for evaluation. Despite limitations, we have shown that sperm nuclear shape as evaluated with the FHA is related to both bull and boar fertility. The FHA approach is also able to identify differences in sperm from bulls based on lifetime ERCR values and due to seasonal impacts on individual ejaculates. When investigating effects of scrotal insulation to simulate heat stress on bulls, it was found that FHA detected sperm nuclear shape changes that were associated with changes in in vitro fertility. The current results of testicular gene expression changes during scrotal insulation in bulls suggests that TIMP-2 expression is disrupted and may lead to altered germ cell-Sertoli cell interactions that result in sperm nuclear shape changes and reduced fertility.

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References

[1] Graham EF, Schmehl MKL, Nelson DS. 1980. Problems with laboratory assays. In: *Proc 8th Tech Conf Artif Insem Reprod,* National Association of Animal Breeders, Columbia MO. p 59.

[2] Rodriguez-Martinez, H. 2003. Laboratory semen assessment and prediction of fertility: still utopia? *Reprod. Domest. Anim*. 38:312–318.

[3] Holt WV, O'Brien, Abaigar T. 2007. Applications and interpretation of computer-assisted sperm analyses and sperm sorting methods in assisted breeding and comparative research. *Reprod. Fert. Dev*. 19:709-718.

[4] Martinez-Pastor FEJ, Tizado JJ, Garde LA, de Paz, P. 2011. Statistical Series: Opportunities and challenges of sperm motility subpopulation analysis. *Theriogenology* 75:783-795.

[5] Barth AD and Oko RJ. 1989. Abnormal Morphology of Bovine Spermatozoa. Iowa State University Press, Ames.

[6] Saacke, R.G., Nebel, R.L., Karabinus, D.S., Bame, J.H. and J. Mullins. 1988. Sperm transport and accessory sperm evaluation. In: *Proc. 12th Tech. Conf. Artif. Insemin. Reprod*., National Association of Animal Breeders, Columbia MO. pp.7.

[7] Saacke, R.G., Nadir, S., Dalton, J., Bame, J., DeJarnette, J.M., Defelos, S. and R.L. Nebel. 1994. Accessory sperm evaluation and bull fertility-an update. In: *Proc. 15th Tech. Conf. Artif. Insemin. Reprod.,* National Association of Animal Breeders, Columbia MO. pp. 57-67.

[8] Eid LN, Lorton SP, and Parrish JJ. 1994. Paternal influence on S-phase in the first cell cycle of the bovine embryo. *Biol. Reprod*. 51:1232-1237.

[9] Parrish JJ, Ostermeier GC, Pace MM. 1998. Fourier harmonic analysis of sperm morphology. In: *Proc. 17th Tech. Conf. Artif. Insemin. Reprod.,* National Association of Animal Breeders, Columbia MO. pp 25-31.

[10] Auger J, Dadoune JP. 1993. The nuclear status of human sperm cells by TEM image cytometry: changes in nuclear shape and chromatin texture during spermiogenesis and epididymal transit. *Biol Reprod.* 49: 166–175.

[11] Gravance C, Liu I, Davis R, Hughes J, Casey P. 1996. Quantification of normal head morphometry of stallion spermatozoa. *J Reprod Fertil.* 108:41–46.

[12] Park K, Yi W, Paick J. 1997. Segmentation of sperms using the strategic Hough transform. *Ann Biomed Eng.* 25:294–302.

[13] Aziz N, Fear S, Taylor C, Kingsland C, Lewis-Jones D. 1998. Human sperm head morphometric distribution and its influence on human fertility. *Fertil Steril.* 70:883–891.

[14] Ostermeier GC, Sargeant GA, Yandell BS, Parrish JJ. 2001(a). Measurement of bovine sperm nuclear shape using Fourier Harmonic Amplitudes. *J. Andrology.* 22: 584-594.

[15] Ostermeier GC, Sargeant GA, Yandell BS, Evenson DP, Parrish JJ. 2001(b). Relationship of bull fertility to sperm nuclear shape. *J. Andrology.* 22: 595-603.

[16] Parrish, J.J., L. Enwall, A. Kaya, C. Pawshe, M.A. Siddiqui, M. Shamusuddin. 2006. Sperm shape research: an update. In: *Proc. 21st Tech. Conf. Artif. Insemin. Reprod.,* National Association of Animal Breeders, Columbia MO. pp. 19-26.

[17] Evenson, D. P. 1999. Loss of livestock breeding efficiency due to uncompensable sperm nuclear defects. *Reprod. Fertil. Dev*. 11: 1-15.

[18] Evenson, DP, Larson, KL and LK Jost. 2002. Sperm chromatin structure assay: Its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. *J. Andrology.* 23:25-43.

[19] Parrish J, Schindler J, Willenburg K, Enwall L, Kaya A. 2012. Quantitative sperm shape analysis: What can this tell us about male fertility. In: *Proc. 24th Tech. Conf. Artif. Insemin. Reprod.,* National Association of Animal Breeders, Columbia MO. pp. 74-80.

[20] Parrish JJ, Krogenaes A, Susko-Parrish JL. 1995. Effect of bovine sperm separation by swim-up or Percoll on success of in vitro fertilization and embryo development. *Theriogenology*. 44: 859-869.

[21] Ward F, Rizos D, Corridan D, Quinn K, Boland M, Lonergan P. 2001. Paternal influence on the time of first embryonic cleavage post insemination and the implications for subsequent bovine embryo development in vitro and in vivo. *Molec Reprod Devel.* 60: 47-55.

[22] Geiger LN. 2003. Bovine sperm nuclear morphology related to breeds, sperm separation methods and scrotal insulation. M.S. Thesis. University of Wisconsin.

[23] Ross AD, Entiwistle KW. 1979. The effect of scrotal insulation on spermatozoa morphology and the rates of spermatogenesis and epididymal passage of spermatozoa in the bull. *Theriogenology.* 11:111-129.

[24] Vogler CJ, Saacke RG, Bame JH, DeJarnette JM, and McGillard ML. 1991. Effects of scrotal insulation on viability characteristics of cryopreserved bovine semen. *J Dairy Sci.* 74:3827-3835.

[25] Vogler CJ, Bame JH, DeJarnette JM, McGilliard ML, Saacke RG. 1993. Effects of elevated testicular temperature on morphology characteristics of ejaculated spermatozoa in the bovine. *Theriogenology* 40:1207-1219.

[26] Hillery-Winhold F. 1991. The influences of individual bulls on bovine in vitro fertilization and embryo development. M.S. Thesis. University of Wisconsin.

[27] Gibbs, K. 2013. Determining The effect of Scrotal Insulation on Sperm Production in the Boar, M.S. Thesis, University of Wisconsin.

[28] Yagoda, K. 2013. The Effects of Short Term Heat Stress on Spermatogenesis and Seminiferous Tubule Morphometry in the Bull and Boar, M.S. Thesis, University of Wisconsin.

[29] Schindler, J. 2013. Effect of scrotal insulation on testis gene expression. M.S. Thesis. University of Wisconsin.

[30] Charron M, and Wright W, 2005. *Protease and Protease Inhibitors*. In: Sertoli Cell Biology, Vol. 1. Eds: M.K. Skinner and M. Griswold, Elsevier Academic Press, London, pp. 121 – 148.

[31] Yao P-L, Lin Y-C, Richburg JH. 2011. Transcriptional suppression of Sertoli Cell Timp2 in rodents following mono-(2-ethylhexyl) Phthalate exposure is regulated by CEBPA and MYC. *Biol. Reprod.* 85:1203-1215.

[32] Rockett JC, Patrizio P, Schmid JE, Hecht NB, Dix DJ. 2004. Gene expression patterns associated with infertility in humans and rodent models. *Mutation Research* 549:225-240.

[33] Newton LD, Kastelic JP, Wong B, Van Der Hoorn F, Thundathil J. 2009. Elevated testicular temperature modulates expression patterns of sperm proteins in Holstein bulls. *Molec Reprod Devel* 76:109-118.

[34] Slongo ML, Zampieri M, Onisto M. 2002. Expression of matrix metalloproteases (MMP-2, MT!-MMP) and their tissue inhibitor (TIMP-2) by rat Sertoli cells in culture: implications for spermatogenesis. *Biol. Chem.* 383:235-239.

[35] McCauley TC, Zhang HM, Bellin ME, Ax RL. 2001. Identification of heparin binding protein in bovine seminal fluid as tissue inhibitor metalloproteinase 2. *Molec Reprod Devel* 58:336-341.

[36] Amann R. 1962. Reproductive Capacity of Dairy Bulls III. The effect of ejaculation frequency, unilateral vasectomy and age on spermatogenesis. *Amer J Anat* 110:49 – 67.

[37] Johnson L. 1994. Spermatogenesis in the bull. In: *Proc.15th Tech. Conf. Artif. Insemin. Reprod.,* National Association of Animal Breeders, Columbia MO. pp 9-27.

Table 1. Harmonic Amplitude (HA) 0 – 5 within bull fertility group

	Mean ± sem[a]	
Criteria	Low[b]	Acceptable
HA0	3.111 ± 0.018	3.155 ± 0.007*
HA1	0.119 ± 0.004	0.117 ± 0.001
HA2	1.111 ± 1.013	1.079 ± 0.005*
HA3	0.120 ± 0.003	0.126 ± 0.001
HA4	0.236 ± 0.009	0.215 ± 0.002**
HA5	0.090 ± 0.004	0.086 ± 0.001

[a] Values are in microns and the sem reflects variation among bulls within a group.

[b] MANOVA found the cull group to be different from the acceptable group, $p<0.05$.

*,** Means within a row differed, $p<0.05$ or $p<0.01$, respectively.

Table 2. Harmonic amplitude (HA) 0 – 5[a] and associated variances[b] are shown for bulls of differing fertility.[a]

	Mean ± SEM (n=4)	
Criteria	Low (N=54)	High (N=53)
Fertlity Group[b]	-4.7 ± 0.3	4.1 ± 0.1
HAO	2.927 ± 0.010	2.975 ± 0.009**
HA1	0.143 ± 0.003	0.134 ± 0.003*
HA2	1.082 ± 0.010	1.090 ± 0.007
HA3	0.129 ± 0.002	0.124 ± 0.002
HA4	0.233 ± 0.006	0.230 ± 0.004
HA5	0.097 ± 0.003	0.093 ± 0.003
Var HA0	3.219 ± 0.138	2.691 ± 0.189*
Var HA1	187.9 ± 11.00	134.2 ± 10.54***
Var HA2	26.99 ± 1.744	16.97 ± 1.760***
Var HA3	0.546 ± 0.028	0.444 ± 0.044
Var HA4	0.131 ± 0.007	0.105 ± 0.009*
Var HA5	0.036 ± 0.002	0.028 ± 0.001***

[a] Sperm were only stained with Hoechst 33342 and no other stains. The HA data represents all sperm in the ejaculate, live or dead.

[b] Data and bulls were from progeny testing at Alta Genetics Inc. and 108 testing herds spread across the US. Fertility values represent conception rates with pregnancy confirmed along with DNA parentage of offspring verified. There was a mean ± sem breeding of 2368±324 and 1124±137 in the high and low fertility group respectively. The population fertility of bulls from Alta Genetics Inc. is adjusted to 0.

*,**,*** The means within a row differ, $p<0.05$, $p<0.01$ or $p<0.001$, respectively.

Table 3. Impact of summer vs. fall collected semen on semen quality parameters, *in vitro* fertilization and FHA analysis.[a]

	Mean ± SEM (n=4)[b]	
Criteria	Summer	Fall
Motile (%)[c]	30 ± 3	45 ± 6[h]
Viability (%)[c]	40 ± 2	56 ± 3[h]
Morphology[d]		
Abnormal heads (%)	28 ± 5	14 ± 1*
Normal (%)	55 ± 6	76 ± 1*
Fertilization (%)[e]	65 ± 4	76 ± 2*
Embryo Nuclei #[f]	10.8 ± 0.1	16.8 ± 0.1*
FHA[g]		
HA0	3.07 ± 0.04	3.20 ± 0.05*

[a] Semen provided by Alta Genetics, Inc.

[b] The sem reflects variation among bulls and within a season.

[c] Analysis done at Alta Genetics using CASA for motility analysis and flow cytometry for live:dead analysis (Sybr-Green and Propidium Iodide) at thawing of semen.

[d] Done on paraformaldehyde fixed sperm. There was no effect of season on secondary or tertiary defects, p>0.1.

[e] There were 50 oocytes/replicate and 3 replicates per bull per season. Data is for monospermic (normal) fertilization.

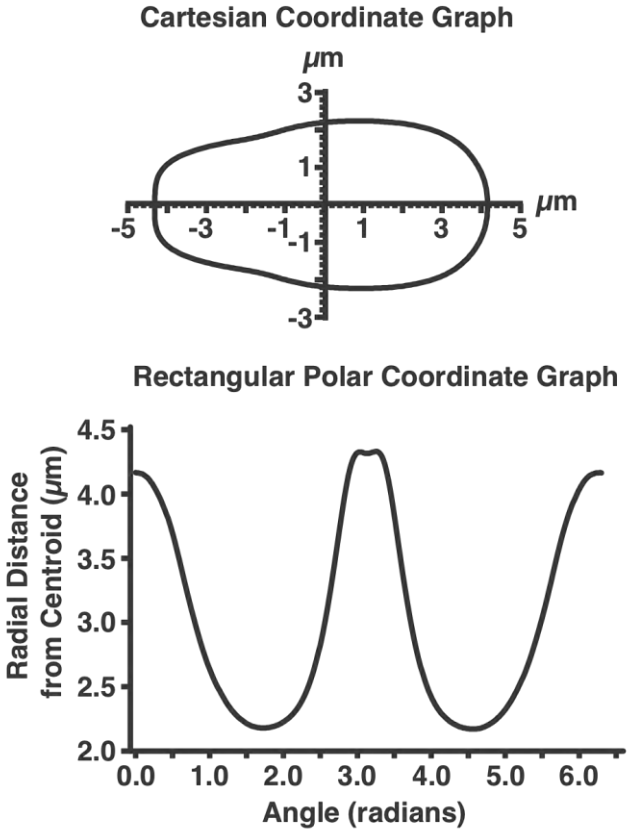
[f] There were 50 oocytes fertilized/replicate and 2 replicates per bull per season.

[g] Values are in microns and there was no effect of season on HA1-HA5, p>0.1.

[h] Indicates season differed, p=0.07

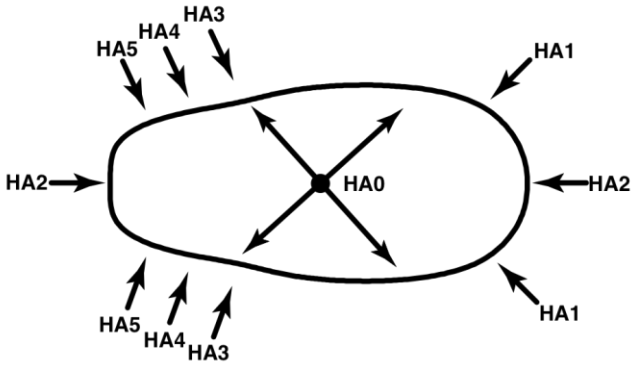
[*] Indicates season differed, p<0.05.

Figure 1.



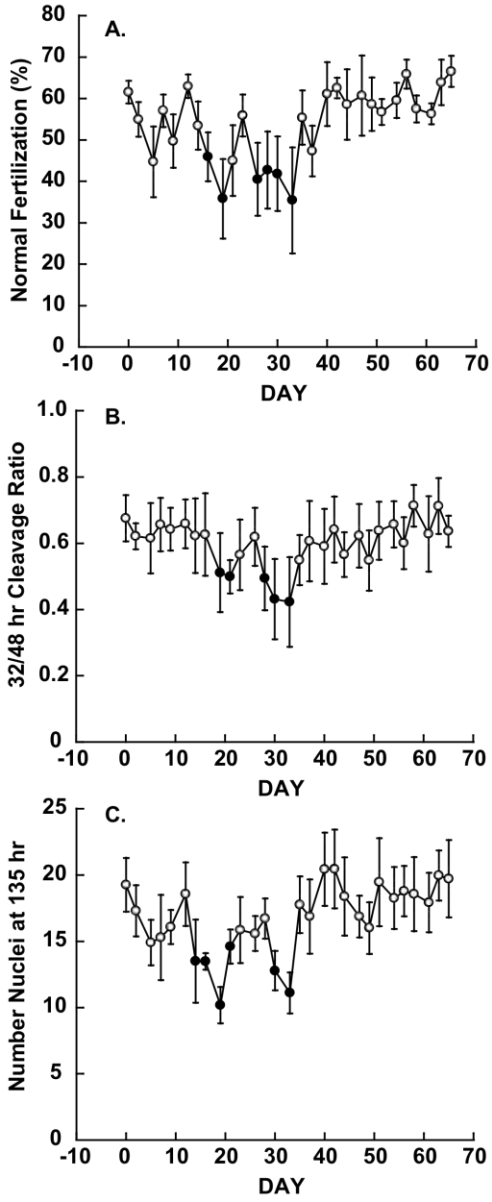
The average shape of sperm from 5 bulls is shown. Sperm nuclei were by image analysis of epifluorescent images. Sperm were stained with Hoechst 33342. The shape is derived from averaging the perimeter coordinates of 100 sperm/bull. The Cartesian coordinate graph is in microns (μm). The same average sperm is shown in the rectangular polar coordinate graph with the y-axis the radial distance in μm from the centroid to each perimeter point at specific angles in radians. The value for 0 radians is the point crossing the positive x-axis of the Cartesian coordinate graph. The angle then increases by rotating counter clockwise around the sperm perimeter.

Figure 2.



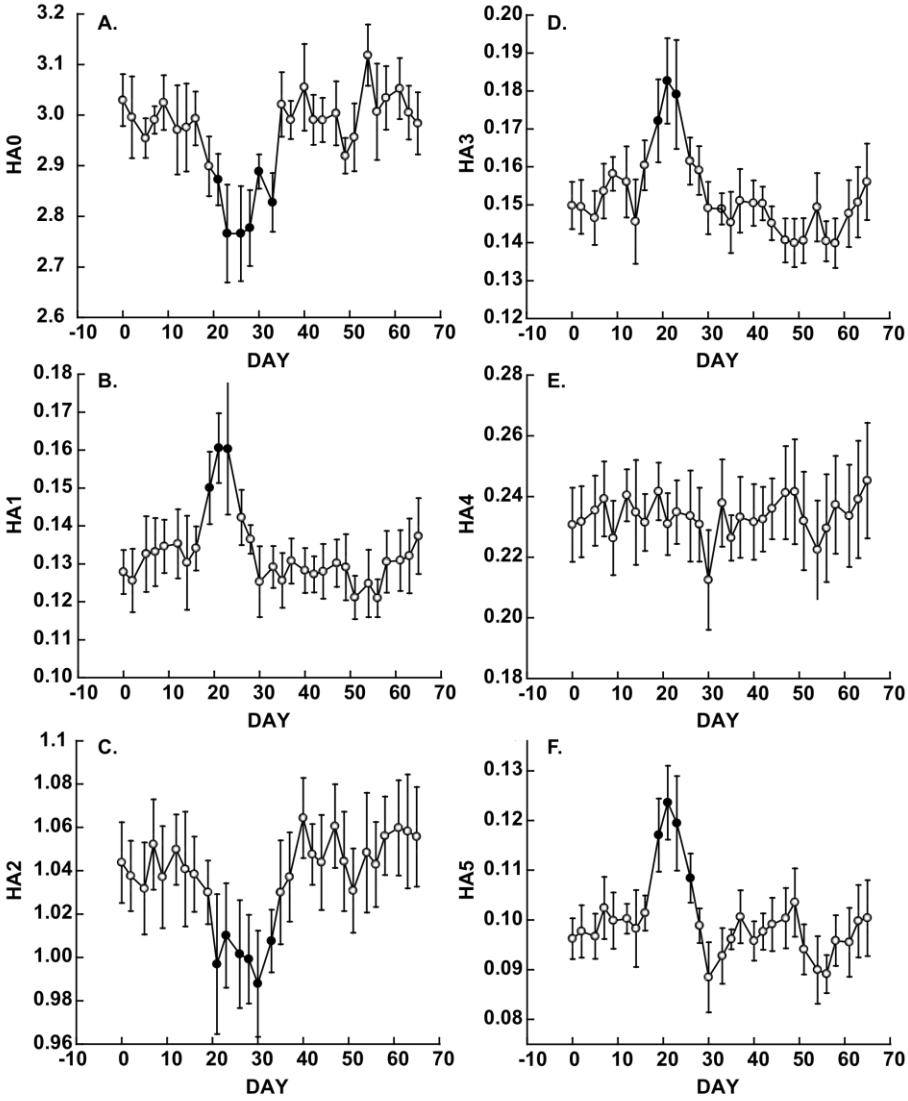
The major impact of the Harmonic Amplitudes (HA) on sperm nuclear shape in the bovine is displayed. The HA0 represents an increase in overall size of the sperm. The HA1 impacts the pinching of the anterior head but also movement of the centroid either more anterior or posterior. The HA2 increases with increasing length of the sperm nuclei but at the same time it also decreases the width. The HA3-5 impacts the pinching in the posterior head and to a lesser extent the anterior head. The impacts of specific HA on the sperm nuclear shape is mathematically precise but general descriptions are more complex than indicated. Further, specific impact of HA may differ among species.

Figure 3.



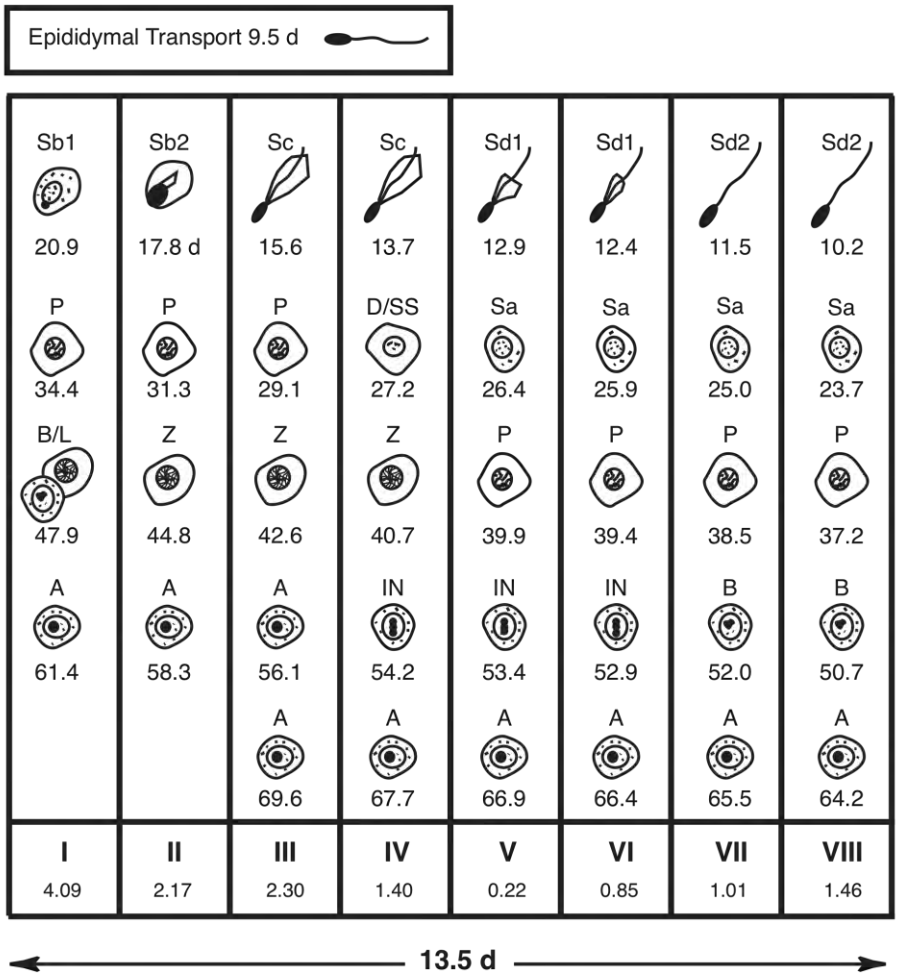
Scrotal insulation impacts on *in vitro* fertility of bulls. The graphs represent the combined means of 5 bulls for (A) normal or monospermic fertilization rate, (B) the 32/48 hour cleavage ratio and (C) number of nuclei in embryos with >3 nuclei at 135 hours of culture. Embryos at 135 hours of culture with 3 or fewer nuclei were considered to be of parthenogenic origin and not included in the data. Error bars represent the standard error for bull effects. In the fertilization assays, sperm penetration of oocytes was evaluated at 18 hours after sperm addition in fixed and orcein stained presumptive zygotes. There were 3 replicates within a bull and day conducted with a minimum of 50 oocytes per replicate with a total of 30,627 presumptive zyotes evaluated. The second experiment evaluated early cleavage and nuclei number. These evaluations were done on the same replicates but only 2 replicates of 50 oocytes within a bull and day were conducted for a total of 20,212 oocytes. Cleavage status was evaluated in live embryos while number of nuclei was done on fixed embryos stained with Hoechst 33342. Days that were different from the day 0, $p<0.05$, are represented by filled circles.

Figure 4.



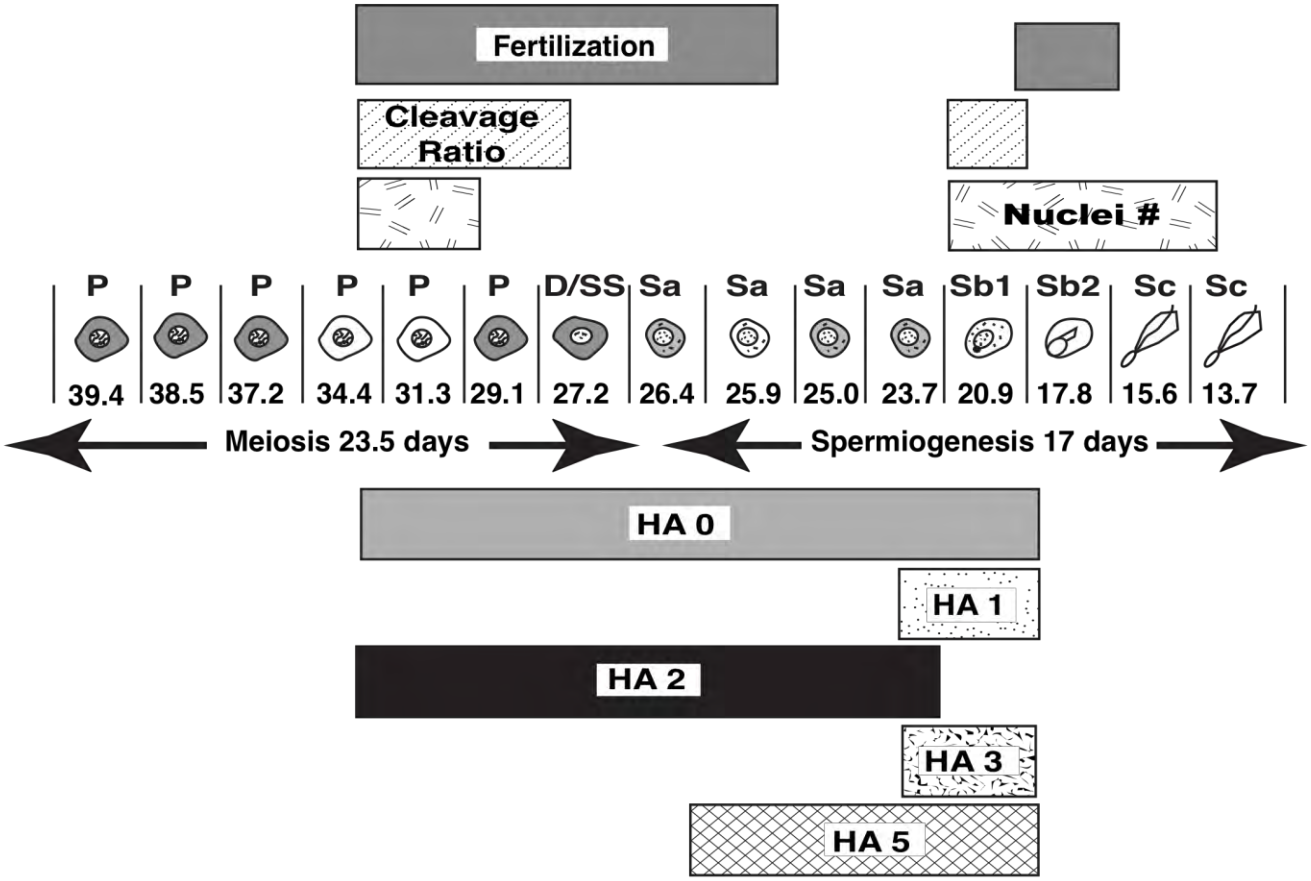
Scrotal insulation impacts on sperm nuclear shape as evaluated by Fourier Harmonic Amplitude (HA) analysis. The graphs represent the combined means of 5 bulls for FHA analysis and the days post scrotal insulation. Error bars represent the standard error for bull effects. Day 0 represents pooled data for semen collected prior to scrotal insulation. Results for HA0 are shown in A., HA1 in B., HA2 in C., HA3 in D., HA4 in E., and HA5 in F. Days that were different from the day 0, $p<0.05$, are represented by filled circles.

Figure 5.



An illustration of the 8 stages of spermatogenesis in the bull is shown (adapted from Amann (36) and Johnson (37)). The length of a cycle is shown at the bottom, with the length in days of each stage indicated below the roman numerals. A diagrammatic illustration of each cell type is shown and represent A, B and Intermediate spermatogonia (A, B, In), primary spermatocytes at leptotene, zygotene, pachytene and diplotene (L, Z, P, D), secondary spermatocytes (SS), spermatids at stages Sa, Sb1, Sb2, Sc, Sd1 and Sd2 (see Johnson (37)). Below each cellular illustration are the days until that particular cell type would be ejaculated. These are based on the length of each stage, cell types within a stage and average epididymal transit time of 9.5 days as indicated at the top left.

Figure 6.



An illustration of the impact of scrotal insulation and its relationship to bovine spermatogenesis is shown. The horizontal bars indicate when fertilization, 32/48 hr early cleavage, and nuclei number from Figure 3, and the Harmonic Amplitudes from Figure 4 were different, $p<0.05$, from day 0.