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EFFICIENCY OF FOURIER HARMONIC ANALYSIS OF SPERM NUCLEAR SHAPE IN PREDICTING FERTILITY IN WATER BUFFALO BULLS

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ABSTRACT

Fourier harmonic analysis (FHA) assessed the sperm nuclear shape of 31 buffalo bulls with known fertility to identify the bull with top conception rates of 28-36% and lowest conception rates of 13-19%. The FHA model developed for water buffalo, identified harmonic amplitude 1 as critical in selecting optimum cutoff values based on discriminant analysis. Using the model, 44 other bulls were classified as High- (n=12) or Low-fertile (n=10), while the rest (n=22) were mid-fertile bulls. Five bulls from High- and Low-fertility groups were randomly selected for semen quality tests. The tests included fertilization of buffalo oocytes, sperm motility parameters and Hypo-osmotic swelling (HOS) of the plasma membrane integrity. Results showed higher cleavage and blastocysts development with significant difference of hatching rates (*P*=0.021) for bulls that were predicted to be of High- vs. Low-fertility. Progressive motility (67.2±2.9 vs. 51.9±2.9%), straightness (85.4±0.7 vs. 81.3±0.7) and linearity (53.3±0.8 vs. 49.8±0.8) were higher in High- than Low-fertility bulls (*P*<0.05). Further, HOS test showed that High-fertility bulls had higher sperm cells (68.0 ± 6.2) with functional integrity than Low-fertility bulls (59.1 ± 6.2) though difference was not significant (*P*=0.346). The results suggest FHA is a potential tool in predicting bull fertility in water buffalo bulls.

Key words: buffalo, bull fertility, Fourier Harmonic Analysis, semen quality

INTRODUCTION

The success rate of artificial insemination (AI) is very important in order to facilitate the production of genetically improved animals that could supply the need of milk and meat and support the protein requirement of the increasing human population. The average success of AI in buffaloes, however, ranges from 0.5 to 9.8% in a report covering 1998 to 2015 in Samar (Ybañez *et al*., 2017). Artificial insemination has become a critical tool for buffalo genetic improvement and particularly when crossbreeding swamp and riverine buffalo but fertility with AI still remains a problem (Keyolenu *et al*., 2018).

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Improving the success of AI will likely include factors related to both sexes. However, the present paper will only address issues related to the male and in particular with the semen. Fertility of the male can involve both compensable and non-compensable (also called uncompensable) issues associated with the semen (Saacke *et al*., 1994; Evenson, 1999; Saacke, 2008). Routinely, many semen quality analyses are being used and include: semen volume, pH, sperm concentration, motility, viability, and gross morphology. These types of measures identify semen samples with compensable defects that can be overcome by increasing the number of sperm inseminated. A more serious problem for male fertility in AI is a non-compensable issue that cannot be compensated for by increasing the number of sperm inseminated. Chromatin defects are non-compensable issues in semen that have been suggested to be responsible for low fertility of some bull (Saacke, 2008). Different semen evaluation approaches are thus needed to address non-compensable defects in sperm.

Fourier harmonic analysis (FHA) is a tool in predicting bull fertility (Parrish *et al*., 2012). It is a computer aided image analysis approach that measures the subtle shape differences in sperm nuclei by analyzing the nuclei shape expressed as Harmonic Amplitudes or HA. It develops equations to approximate the sperm head curvature and perimeter and expresses results in terms of means and does not differentiate between normal and abnormal, only the mean for a particular male that is related to bull fertility (Parrish *et al*., 2014). This was developed based on the observation that the more brightly/lightly stained sperm for DNA in a population was negatively associated with fertility rate (Parrish *et al*., 1998) and recently granted a US patent (Parrish, 2020). It is known that the sperm head is 90% DNA and the shape of the sperm head is based on the structure and packaging of the DNA (Ballachey *et al*., 1987). Any change in the structure and packaging of the chromatin of the sperm cell should be reflected by a change in sperm shape (Evenson *et al*., 1980; 1994). By FHA, the curvature of a sperm perimeter is explained by Fourier functions, which contain multivariate shape measures known as the harmonic amplitudes (Ostermeier *et al*., 2001a; Parrish *et al*., 2014).

In FHA, the sperm cells are stained with a DNA binding fluorescent dye, epifluorescence microscopy and image analysis then identify non-overlapping sperm nuclei. The perimeter coordinates of sperm nuclei are obtained from 100 sperm per sample and Harmonic Amplitudes (HA) 0-5 computed along with dispersion statistics for each harmonic amplitude (Parrish *et al*., 2014). The approach produces measures of sperm nuclear shape that are independent and orthogonal as reviewed by Duran and Hufana-Duran (2017).

The FHA approach sought to develop methods to evaluate non-compensable defects in sperm (Parrish *et al*., 2014). The focused was on the evaluation of sperm nuclear shape using FHA with the thesis that minor defects in the sperm chromatin can result in failed embryo or fetal development after fertilization. These potential defects would produce changes in the sperm nuclear shape that were beyond the ability of human visual observation through a microscope. The FHA of sperm nuclear shape was used to mathematically identify bulls of high fertility by quantifying sperm nuclear shape in an objective, statistically orthogonal, highly sensitive and repeatable fashion. Models using these amplitudes have been applied to the prediction of low and high fertility groups in dairy bulls and boars (Ostermeier *et al*., 2001b; Parrish *et al*., 2012). These measures have been related to *in vivo* fertility (Parrish *et al*., 2012).

With the desire to minimize if not eliminate the bull factor effect on the failure of artificial insemination, a technique that could identify the high fertility bull in water buffalo

species is desired. This is to ensure that all buffalo bulls used as donors of semen are just the high fertility bulls. With the positive findings observed in other species in the use of FHA, this study aims to examine the efficiency of FHA in predicting bull fertility in water buffalo species to assess its wider application as tool in predicting bulls of high fertility and establish a tool that could be applied to predict fertility in water buffalo bulls.

MATERIALS AND METHODS

To test the wide application of FHA for buffalo bull fertility determination, semen samples were collected from buffalo bulls at the National Bull Farm of Philippine Carabao Center in Carranglan, Nueva Ecija, Philippines which are used for the nationwide artificial insemination program. Sperm from these semen samples were subjected to FHA analysis. Semen was also subjected to semen quality analyses that included Hypo-Osmotic Swelling (HOS) test, the use of Computer Assisted Sperm Analysis (CASA) parameters, and an *In Vitro* Fertilization (IVF) assay to confirm the potential use of FHA as a predictor of bull fertility.

An FHA model was developed for water buffalo bulls to differentiate high- and low-fertility bulls. It was found that Water Buffalo bulls with known *in vivo* fertility could be repeatably separated into top 32% (*in vivo* conception rates=28-36%) and bottom 16% (*in vivo* conception rates=13-19%) using discriminant analysis ($P<0.05$). The model cutoff points for harmonic amplitude 1 was <0.042 µm and >0.051 µm for high and low fertility bulls, respectively. The FHA measures of harmonic amplitude 0 and 2-5 (Fig. 1) were not predictors of fertility ($P>0.05$). Using the model, the 44 buffalo bulls used as donors of semen for nationwide artificial insemination program at the National Bull Farm of the Philippine Carabao Center in Caranglan, Nueva Ecija were classified finding 12 high and 10 low fertility bulls with 22 classified as mid-fertility bulls. The 22 bulls that were classified as mid-fertility were not used for further analysis. Five bulls from each high and low fertility group were randomly selected. Sperm from these bulls were used to evaluate semen quality

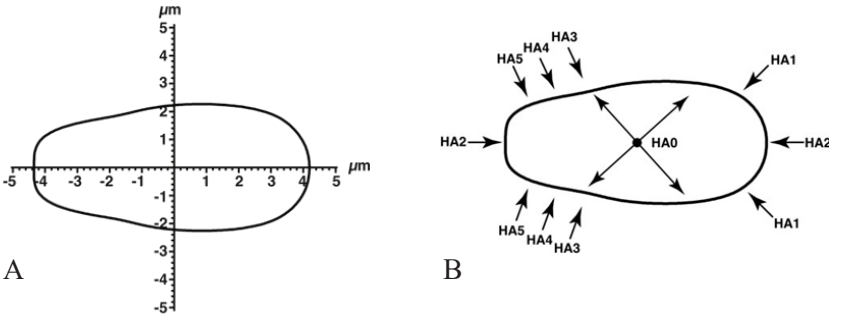

Figure 1. A) Fourier harmonic amplitudes (HA) and B) sperm nuclear measurement. Harmonic amplitude 0 (HA0) is the overall size of the sperm where HA1 through 5 adjust HA0 to reflect nuclear shape via a summation of the various harmonics. The major impacts of specific harmonics are as follows: HA1 reflects the curvature of the anterior portion of the nucleus, HA2 reflects the length and to some extent width, HA4 reflects the width and to some extent the length but also post nuclear pinching, HA3 and HA5 reflect the curvature of the post nuclear region (Parrish *et al*., 2014).

with *in vitro* fertility via fertilization of buffalo oocytes, sperm motility via CASA (IVOS II Hamilton Thorne) and HOS. *In vitro* matured buffalo oocytes were fertilized with capacitated buffalo sperm for 18 hr. There were 5 replicates for each bull in the study with a mean ± SEM of 28.1 ± 1.2 oocytes per replicate. Male pronuclear formation was recorded as evidence of fertilization and cleavage, blastocyst development and hatching rates used to assess the developmental competence of the sired zygotes. The CASA evaluated on 201-1125 sperm per bull with motion analysis following the method described by Hufana-Duran *et al*. (2017). HOS test was carried out on 200 to 300 sperm cells per semen sample (1 straw) and observed in at least five microscopic fields of view. All data are means of the predicted high and low fertility groups, 5 bulls/group, and pooled standard error from the within class variation.

Semen samples were prepared for FHA. Semen sample with 160 X 10^6 sperm/mL was mixed 1:3 semen to diluent (2.9% NaCitrate + 3% Bovine Serum Albumin, Fraction V) to reach the ideal sperm concentration of 40 X 10^6 sperm/mL. Sperm were then stained with Hoechst 33342 (H1399, Sigma Chemicals) and Yoyo-1 (Invitrogen by Thermo Fisher Scientific) for 30 min at 37°C. Samples were then processed to obtain a semen sample for imaging. Sperm cell images were taken using a Nikon Diaphot (Nikon instrument Inc., Tokyo, Japan) microscope equipped for phase contrast microscopy (Fig. 2A), epifluorescence (Fig. 2B-F), and computer aided image analysis. The image passing through a 100x Nikon Flour, 1.30 numerical aperture, phase contrast 4, oil-immersion objective and a 1.25x magnifier into an intensified charged coupled device camera was captured. Images of not less than 200 sperm cells per sample that are not overlapping was taken. Semen samples and images were sent to University of Wisconsin-Madison for FHA.

For the FHA analysis, Cartesian coordinate perimeter points were gathered on all sperm except ones which are overlapping. These Cartesian coordinates converted to polar coordinates and harmonic amplitudes derived as described by Ostermeier *et al*. (2001b). For each animal, mean HA and heritability coefficients from 100 of all of the sperm, including the dead sperm, were compared to the mean HA and heritability coefficients of 100 live sperm. With the use of YOYO-1 stain and Boolean algebra, the selection and removal of dead sperm were made possible. The phase contrast image was used to attain primary and secondary abnormalities. The sperm head shape from FHA is related to nuclear size for HA0, the anterior head curvature for HA1, length for HA2, and posterior curvature for HA3 to 5.

In vitro fertilization and embryo development assay were carried out to check the male and female pro-nuclear formation and the developmental competence of the resultant zygotes after using the High- and Low-fertility semen samples for IVF. The IVF was done using oocytes collected from slaughter-house derived ovaries. Collected oocytes were classified and selected based on the classification published by Hufana-Duran *et al*. (2008) and *in vitro* matured, fertilized and cultured to check the male and female pro-nuclear formation and development to pre-implantation stages following the methods described earlier (Hufana-Duran, 2009).

Characteristics of sperm motility were assessed via CASA (Ivos-Ultimate; Hamilton Thorne Biosciences, Beverly, MA, USA) as described by Hufana-Duran *et al*. (2017) with the set-up set for bull sperm analysis. The parameters recorded were motility (%), progressive motility (%), average path velocity (VAP, μm/s), progressive velocity or straight–line (rectilinear) velocity (VSL, μm/s), track speed or curvilinear velocity (VCL, μm/s),

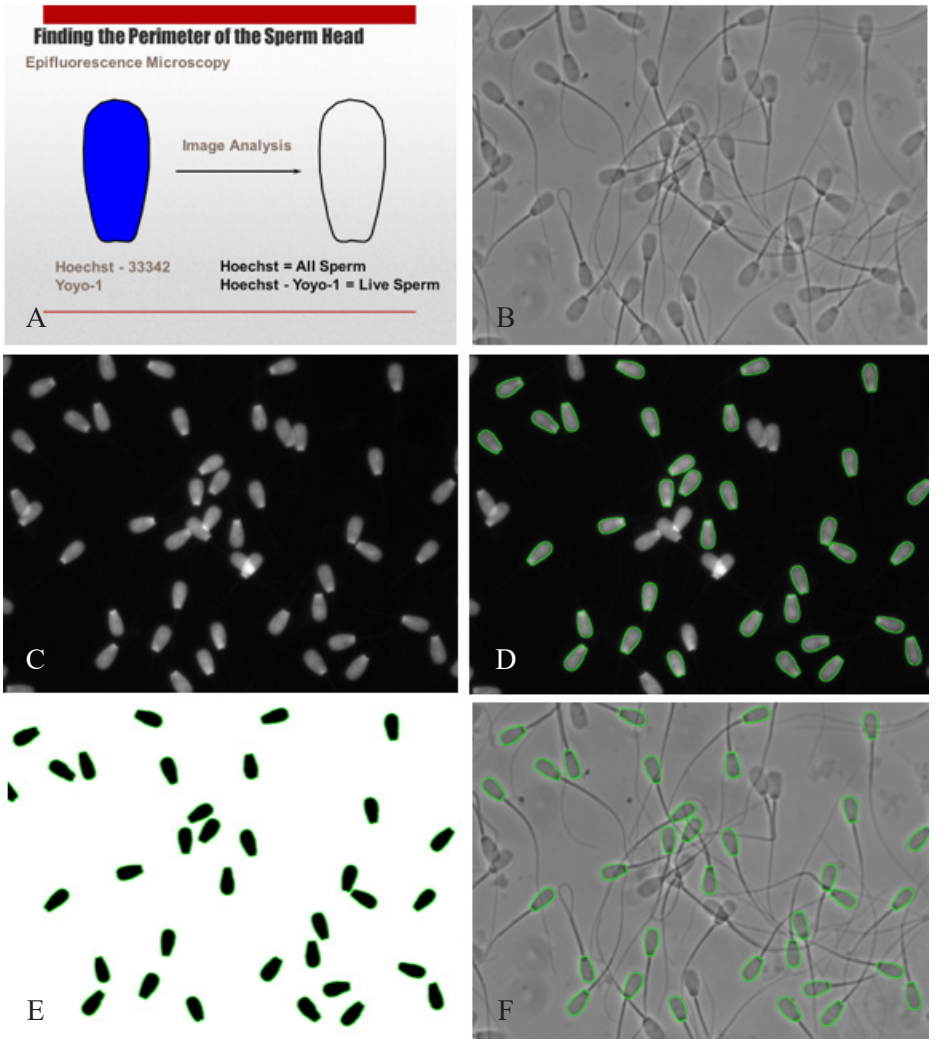

Figure 2. Sperm images for Fourier Harmonic Analysis. A) Description of image preparation and analysis. B) Phase contrast image of sperm. C) Hoechst image. Objects identified as sperm nuclei were thresholded and identified by custom software in ImageJ. Overlapping sperm were not included in analysis. D) Sperm perimeters from the ImageJ analysis overlayed on Hoechst image. E) Mask of sperm nuclei used to obtain perimeter coordinates of each sperm nuclei. F) Perimeter outlines placed on phase contrast image to demonstrate how the software was able to identify sperm nuclei. Not shown were images of Yoyo-1 labeled sperm which were dead. In a similar way they were identified, masks were overlayed on the object image (E), dead Yoyo-1 staining sperm deleted to leave only the live sperm to evaluate.

amplitude of lateral head displacement (ALH, μm), beat cross frequency (BCF, Hz), straightness (STR), and linearity (LIN) examined.

The HOS test was done to check the integrity of the sperm plasma membrane of the sperm cells of classified High- and Low-fertility bulls following the methods described by Hufana-Duran *et al*. (2015) in water buffalo. Briefly, buffalo spermatozoa were exposed to 150 mOsm fructose-sodium citrate solution at 37º C for 45 min and then examined at 40X magnification. The percentage of HOS-reacted spermatozoa out of 200 spermatozoa was recorded.

Data were expressed in average and percentages. Levene's test was used to test the homogeneity of variance. The IVF, embryo development, HOS and CASA data were analyzed with Statistical Analysis Systems software (SAS version 7–1; SAS Institute, Cary, NC) using a mixed model analysis of variance (ANOVA; Jackson and Brashers, 1994) with bulls within fertility group (within class factor) considered random and fertility group as fixed. Data were considered significant if $P<0.05$. Since there were only 2 groups per data type, a significant ANOVA result indicated means were different.

RESULTS AND DISCUSSION

Table 1 presents the results on semen quality and predicted fertility of bulls. There was no difference in the HOS test of 68.0 ± 6.2 vs. 59.1 ± 6.2 ($P=0.35$). A trend was observed for differences in fertilization (83.5 ± 4.4 vs 70.1 ± 4.4; $P=0.07$), cleavage (73.5 ± 4.6 vs. 60.1 ± 4.6; $P=0.07$), blastocyst development from all oocytes inseminated (27.4 ± 2.8 vs. 18.5 ± 2.8; $P=0.06$), and blastocyst development from cleaved oocytes (37.3 ± 2.6 vs. 29.5 ± 2.6; $P=0.06$) for the bulls with predicted High- vs. Low-fertility, respectively. Hatching as a percentage of blastocyst (38.1 ± 3.2 vs. 25.1 ± 3.2) was significantly higher ($P=0.02$) in High- vs Low-fertility bulls. The CASA measures differed for progressive motility (67.2 ± 2.9 vs. 51.9 ± 2.9), straightness (STR) (85.4 ± 0.7 vs. 81.3 ± 0.7) and LIN (53.3 ± 0.8 vs. 49.8 ± 0.8) for sperm from High vs Low predicted fertility bulls ($P<0.05$). There was no effect of VAP, VSL, VCL, ALH or BCF in relationship to bull fertility group ($P>0.05$).

The results showed that while there was no significant difference on the HOS test for functional integrity of the sperm plasma membrane, the fertility group effects on *in vitro* fertilization and embryo development rates between the High- and Low-fertility bulls were either very close, $P<0.07$ and $P>0.06$ or significant $P=0.02$, with the developmental competence evidenced by hatching rate of the sired zygotes higher ($P=0.02$) in High-fertility than in Low-fertility FHA predicted bulls. These results suggest that High-fertility bulls classified by the FHA have a better plasma membrane with functional integrity that resulted in higher penetration of the oocytes as evident by the higher fertilization rate (83.5 ± 4.4 vs. 70.1 ± 4.4). Plasma membrane integrity is important for fertility (Watson, 1995; Ramirez *et al*., 2009) because plasma membrane plays a key role in maintaining and intracellular stability within sperm (Hammerstedt *et al*., 1990) and damaged sperm would leak ATP resulting in loss of motility/fertility. The sperm membrane is important in keeping both the biochemical and structural integrity of spermatozoon (Cabrita *et al*., 1999) and membrane integrity is crucial for optimal sperm function (Khan and Ijaz, 2008). It is fundamental in ensuring fertilizing capacity because only a sperm with an intact plasma membrane can fertilize an oocyte (Yanagimachi, 1994). Even though cryopreservation affects the plasma membrane (Hammerstedt *et al*., 1976; Ugur *et al*., 2019), the FHA classified High-fertility bulls had

Table 1. Semen quality and predicted fertility of High- and Low-fertility bulls selected by Fourier Harmonic Analysis.

Criteria	High-fertility Bull Mean±SEM	Low-fertility Bull Mean±SEM	*P*-value
HOS	68.0 ± 6.2	59.1 ± 6.2	0.346
CASA			
Motility, Progressive (%)	67.2 ± 2.9	51.9 ± 2.9	0.006
Linearity	53.3 ± 0.8	49.8 ± 0.8	0.019
Straightness	85.4 ± 0.7	81.3 ± 0.7	0.005
VAP (μm/s)	149.4 ± 32.1	96.6 ± 32.1	0.278
VSL (μm/s)	91.5 ± 4.9	80.3 ± 4.9	0.140
VCL (μm/s)	218.4 ± 33.2	170.4 ± 33.2	0.336
ALH (μm)	7.0 ± 0.2	7.1 ± 0.2	0.730
BCF (Hz)	33.5 ± 0.8	33.7 ± 0.8	0.868
IVF			
Fertilization (%)	83.5 ± 4.4	70.1 ± 4.4	0.070
Cleavage (%)	73.5 ± 4.6	60.1 ± 4.6	0.074
Blastocyst (% of all)	27.4 ± 2.8	18.5 ± 2.8	0.055
Blastocyst (% of cleaved)	37.3 ± 2.6	29.5 ± 2.6	0.064
Hatching (% of blast.)	38.1 ± 3.2	25.1 ± 3.2	0.021

HOS = Hypo-osmotic swelling; CASA = Computer Assisted Sperm Analysis; VAP= path velocity; VSL= progressive velocity; VCL= track speed or curvilinear velocity; ALH= amplitude of lateral head movement; BCF= beat frequency; IVF= *in vitro* fertilization.

better quality plasma membranes than Low-fertility bulls post-thawing.

The *in vitro* fertilization results demonstrate that FHA can classify the bulls according to their fertility. The zygotes sired from High-fertility bulls had higher developmental competence than zygotes sired by Low-fertility bulls which following the trend of increased fertilization percentage, cleavage percentage, blastocyst development and then led to a higher hatching rate (38.1 ± 3.2% vs. 25.1 ± 3.2%; $P<0.05$).

The decreased developmental competence observed among oocytes sired by Low-fertility bulls supports earlier reports (Ostermeier *et al*., 2001a) indicating that Low-fertility bulls classified through FHA have sperm cells that have fragmented DNA and lower ability to sire zygotes with developmental competence (Parrish *et al*., 2012; 2014).

These results conform to the observations in other livestock species such as bull and boar (Parrish *et al*., 1998; Ostermeier *et al*., 2001b; Parrish *et al*., 2012). The bulls of high fertility produced embryos that are more likely to reach the morula/blastocyst stage (Hillery *et al*., 1990; Hillery-Weinhold, 1991; Parrish and Eid, 1994) and develop normally when provided with the appropriate environment. The relationship of higher field fertility and ability to sire more embryos *in vitro* have not always been confirmed (Utt, 2016). A key goal here is not to produce as many embryos as possible but to limit numbers of sperm used for *in vitro* fertilization assays so as to minimize polyspermy. The mechanism involved in

significantly higher developmental competence among oocytes sired by High-fertility bulls is the viability of the sperm DNA necessary for normal development. FHA used the sperm nuclear shape of each bull in classifying them as High- or Low-fertility. The sperm nuclear shape shows the DNA of the sperm cells and DNA accounts 90% of the sperm head (Ballachey *et al*., 1987). The DNA shape exhibits the normal or fragmented DNA. The fragmented DNA inhibits the sperm to fertilize an egg and or if fertilization occurred, cannot support the development of an embryo full-term (Eid *et al*., 1994; Ostermeier *et al*., 2001b) with reasons yet to be resolved. It is the failure of motile sperm to sustain fertilization and pregnancy (Eid *et al*., 1994).

It can be deduced from these results that selection of bulls by FHA using sperm nuclear shape is a potential tool for predicting bull fertility in the buffalo species. Differences in development were detected as early as the first cell cycle and success of cleavage; 73.5% in High-fertility against 60.1% in Low-fertility bulls. It was previously demonstrated that low fertility bulls sire zygotes that enter DNA synthesis (S-Phase) latter suggesting repair of sperm DNA (Parrish and Eid, 1994; Eid *et al*., 1994; Eid and Parrish, 1995). The cleavage results in the buffalo bulls could be the result of an inability of oocytes to repair sperm DNA in sperm from the Low-fertility bulls. Indeed, it has also been shown that low fertility bulls have altered sperm chromatin as identified by the Sperm Chromatin Structure Assay (SCSA; Ballachey *et al*., 1987; Evenson, 2016) that is highly correlated with sperm shape from FHA analysis (Parrish *et al*., 2012; 2014). Further, the SCSA has demonstrated relationships to boar, ram and human fertility (Evenson *et al*., 1980; Evenson *et al*., 1994; Sailer *et al*., 1995; Evenson, 2016). Thus, damaged DNA and abnormal chromatin are two likely explanations for the lower fertility in buffalo bulls classified as Low-fertility by their nuclear shape.

Motility characteristics of the High- and Low-fertility bulls classified by FHA showed that sperm cells of High-fertility bulls have significantly higher ($P<0.05$) progressive motility, linearity, and straightness of movement than the Low-fertility bulls. High progressive motility, linearity of movement, and straightness of direction are important characteristics of sperm motility to effect successful fertilization as shown in higher *in vitro* fertilization rate (83.5 ± 4.4% vs. 70.1 ± 4.4%, $P=0.07$) among *in vitro* matured oocytes sired by High-fertility bulls. Linearity is an important parameter of success in human IVF (Liu *et al*., 1991) and in cattle (Iqbal and Hunter, 1995). Though CASA cannot accurately predict 'fertility' of a semen sample (Amann and Wabershi, 2014), sperm subpopulations exist, and their motility characteristics can assess the status of a sperm sample. This can be expanded to the sample's fertility potential, capitalizing on the heterogeneity (Martinez-Pastor *et al*., 2011). Sperm samples are heterogeneous so a variety of spermatozoa with different motion characteristics can coexist in the same ejaculate (Katz *et al*., 1979; Muino *et al*., 2008). The significant difference on the progressive motility, LIN and STR of the sperm movement among High-fertility bulls guarantee higher capability of the sperm cells to swim in the reproductive tract and perform fertilization. These results suggest that FHA has positive correlation with progressive motility, linearity and straightness of movement assessed by CASA. These results also demonstrate that sperm cells of High-fertility bulls have better motility characteristics than the Low-fertility bulls.

While there was no difference on functional integrity of the sperm plasma membrane between the predicted High- and Low-fertility bulls by FHA, the High-fertility bulls had higher percentage of sperm cells that has good functional integrity (68.0 ± 6.2 vs. 59.1

± 6.2, *P*=0.35). This result suggests that the hypothesized DNA fragmentation among Low fertility bulls exhibit a degree of effect on the functional integrity of the sperm plasma membrane that also supports the observation of lower fertilization rate among Low-fertility bulls when used for *in vitro* fertilization.

In these evaluations, sperm were either examined *in vitro* for physiological characteristics of plasma membrane integrity (HOS), motility (CASA) or ability to fertilize oocytes *in vitro* and ability of zygotes to continue on in development up through hatching. These characteristics of sperm are related to fertility as described by Parrish *et al*. (2014). Briefly, sperm must have an intact plasma membrane to be even capable of fertilizing an oocyte *in vivo* or *in vitro* and hence the relevance of HOS. *In vivo* and *in vitro*, sperm must also be motile to penetrate an oocyte with a zona pellucida hence the need for CASA evaluation. There could well be a question of the importance of straightness and linearity, but it is clear that sperm must be motile and a sample with higher motility has a better probability of fertilizing oocytes. Sperm undergo the acrosome reaction when coming into contact with the zona pellucida such that they can digest a pathway to enter into the perivitelline space and then fuse with the plasma membrane of the oocyte and initiate the first steps of fertilization, activation of the oocyte. A sperm that is not motile or has damaged plasma membranes is not capable of surviving sufficiently long enough after thawing to be able to reach the oocyte and undergo the acrosome reaction. Again, sperm must have an intact and functional plasma membrane when they reach the zona pellucida. Following sperm activation of the oocyte, the sperm must initiate the correct division of the early embryo, formation of a blastocyst and then undergo hatching. Without successfully completing these steps, fertilization fails. These steps require the sperm to initiate a series of calcium waves, formation of pronuclei, and the switch from maternal gene activation to embryonic gene activation. Any abnormality in the sperm head, where the material for initiating the calcium wave and genetic contribution to the embryo will thus lead to failure of the embryo to develop. In these experiments, we have oocytes from many ovaries and thus eliminate any specific maternal contribution of a single female. Any differences seen in oocyte activation and embryo development are thus the result of differences in sperm between the two fertility groups of males. It is acknowledged that there are maternal effects *in vivo* in which all events occur in a single female but that is not what this paper examined.

With the above results, FHA demonstrates potential and efficiency in predicting the fertility of water buffalo bulls through analysis of the sperm nuclear shape. The FHA is a computerized imaging system that analyses the precise and mathematical curvature of the perimeter of the sperm head that is below the accuracy of human vision to perceive. Any change in chromatin structure should be reflected by a change in sperm head shape. While this technique has described the size and curvature of mammalian sperm nucleus such as bull, boar, stallion for over a decade (Parrish *et al*., 2012), the results of this study guarantees the wider application of this technique in predicting bull fertility. Careful analysis indicates that only evaluation of sperm DNA and/or head shape has shown relationships to bovine bull fertility (Parrish *et al*., 2012; 2014) and this study demonstrates FHA use in buffalo bulls.

In conclusion, buffalo bulls predicted via FHA to be of higher fertility have better *in vitro* fertility, functional integrity of plasma membrane, and sperm motion characteristics. Significantly, higher quality embryos were obtained when High-fertility predicted bulls sire embryos *in vitro*. FHA is efficient in predicting bull fertility.

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REFERENCES

Amann RP and Wabershi D. 2014. Computer assisted sperm analysis (CASA): Capabilities and potential developments. *Theriogenology* 81(1):5-17.e3.

Ballachey BE, Hohenboken WD and Evenson DP. 1987. Heterogeneity of sperm nuclear chromatin structure and its relationship to bull fertility. *Biol Reprod* 36(4):915-925.

Cabrita E, Alvarez R, Anel E and Herraez MP. 1999. The hypo-osmotic swelling test per formed with coulter counter: A method to assay functional integrity of sperm membrane in rainbow trout. *Anim Reprod Sci* 55:279-287.

Duran PG and Hufana-Duran DH. 2017. Fourier Harmonic Analysis as tool in predicting bull fertility for improved buffalo breeding. *Proceedings of the International Buffalo Symposium* (Devkota B, Bhattarai N, Gautam G, Sah MK, Luitel H, ed) Agriculture and Forestry University, Rampur, Chitwan, Nepal, November 15-18, 2017. Pp. 118-126.

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.

Eid LN and Parrish JJ. 1995. Duration of G2-phase and onset of M-phase during the first cell cycle of the bovine embryo is dependent on bull *in vivo* fertility. *Theriogenology* 43:205-205.

Evenson DP, Darzynkiewicz Z and Melamed MR. 1980. Relation of mammalian sperm chromatin heterogeneity to fertility. *Science* 240:1131-1133.

Evenson DP, Thompson L and Jost LK. 1994. Flow cytometric evaluation of boar semen by the sperm chromatin structure assay as related to cryopreservation and fertility. *Theriogenology* 41:637-651.

Evenson DP. 1999. Loss of livestock breeding efficiency due to uncompensable sperm nuclear defects. *Reprod Fertil Dev* 11:1-15.

Evenson DP. 2016. The sperm chromatin structure assay (SCSA) and other sperm DNA fragmentation tests for evaluation of sperm nuclear DNA integrity as related to fertility. *Animal Reprod Sci* 169:56-075.

Hammerstedt RH, Amann RP, Rucinsky T, Morse PD Jr, Lepock J, Snipes W and Keith AD. 1976. Use of spin labels and electron spin resonance spectroscopy to characterize membranes of bovine sperm: effect of butylated hydroxytoluene and cold shock. *Biol Reprod* 14:381–397.

Hammerstedt RH, Graham JK and Nolan JP. 1990: Cryopreservation of mammalian sperm: what we ask them to survive. *J Androl* 11:73–88.

Hillery FL, Parrish JJ and First NL. 1990. Bull specific effect on fertilization and embryo development *in vitro*. *Theriogenology* 33:249.

Hillery-Weinhold F. 1991. Sire influence on bovine *in vitro* fertilization and development. *MS Thesis*. University of Wisconsin, Madison, WI.

Hufana-Duran D, Atabay EP, Duran PG, Atabay EC, Takahashi Y, Kanai Y and Cruz L. 2008. Selection of water buffalo oocytes with developmental competence *in vitro*. *Philipp J Vet Anim* Sci 34:149-158.

Hufana-Duran D. 2009. Studies for the improvement of *in vitro* culture systems of oocytes and embryos in water buffaloes. *PhD Dissertation*. Graduate School of Life and Environmental Sciences. The University of Tsukuba, Japan.

Hufana-Duran D, Mallari R, Suba D, Duran P, Abella E and Mamuad F. 2015. Hypo-osmotic swelling test for membrane integrity evaluation of frozen-thawed water buffalo (*Bubalus bubalis*, Linn.) spermatozoa. *Phil J Sci* 144(2):209-219.

Hufana-Duran D, Duran PG, Monson R and Parrish J. 2017. Motility and membrane integrity of ejaculated bovine spermatozoa extended and cryopreserved in L-carnitine Tris-egg yolk extender. *J ISSAAS* 23(1):56-67.

Iqbal N and Hunter AG. 1995. Effect of various capacitation systems on bovine sperm motion characteristics, acrosome integrity, and induction of hyperactivation. *J Dairy Sci* 78:91-102.

Jackson S and Brashers DE. 1994. *Random Factors in ANOVA*. Sage University Paper Series on Quantitative Applications in the Social Sciences, 07– 098. Thousand Oaks, CA: Sage Publications 1–65.

Katz DF Erickson RP and Nathanson M. 1979. Beat frequency is bimodally distributed in spermatozoa from t/t12 mice. *J Exp Zool* 210:529-535.

Khan MIR and Ijaz A. 2008. Effects of osmotic pressure on motility, plasma membrane integrity and viability in fresh and frozen-thawed buffalo spermatozoa. *Animal* 2:548–553.

Keyolenu Y, Chukham G, Tolenkhomba TC, Shyamsana NS, Kalyan S and Prava M. 2018. Genetic improvement of swamp buffalo through cross breeding and backcrossing with riverine buffalo. *Int J Livestock Res* 8:30-45.

Liu DY, Clark GN and Baker HWG. 1991. Relationship between sperm motility assessed with the Hamilton-Thorn motility analyzer and fertilization rates *in vitro*. *J. Androl* 12:231.

Martinez-Pastor F, Jorge Tizado E, Julian Garde J, Anel L and de Paz P. 2011. Statistical series: Opportunities and challenges of sperm motility subpopulation analysis. *Theriogenology* 75(5):783-795.

Muino R, Tamargo C, Hildalgo CO and Pena AI. 2008. Identifcation of sperm subpopulations with defined motility characteristics in ejaculates from Holstein bulls: effects of cryopreservation and between-bull variation. *Anim Reprod Sci* 109:27-39.

Ostermeier C, Sargent GA, Yandell BS and Parrish JJ. 2001a. Measurement of bovine sperm nuclear shape using Fourier harmonic amplitudes. *J Androl* 22:584-594.

Ostermeier GG, Sargent GA, Yandell BS, Evenson DP and Parrish JJ. 2001b. Relationship of bull fertility to sperm nuclear shape. *J Androl* 22(4):595-603.

Parrish JJ and Eid L. 1994. *In vitro* fertilization and its relationship to bull fertility. *Proceedings of the 15th Technical Conference on AI and Reproduction*, Milwaukee, WI, pp. 68-73.

Parrish JJ, Ostermeier GC and Pace MM. 1998. Fourier harmonic analysis of sperm morphology. *Proceedings of the 17th NAAB Technological Conference on Artificial Insemination and Reproduction*, Columbia MO, pp. 25-31.

Parrish J, Schindler J, Willenburg K, Enwall L and Kaya A. 2012. Quantitative sperm shape analysis: What can this tell us about male fertility. *Proceedings of the 24th Technological Conference Artificial Insemination and Reproduction*, National Association of Animal Breeders, Columbia MO. pp. 74-80.

Parrish JJ, Ostermeier C, Schindler J, Willenburg K, Enwall L and Kaya A. 2014. Quantifying sperm nuclear shape with Fourier harmonic analysis and relationship to spermatogenesis and fertility. *Association for Applied Animal Andrology 2014 Meeting*, p30 – 49.

Parrish JJ. 2020. Prediction of fertility in males, US Patent App. 16/024,335; Patent granted May 2020.

Ramirez J, Carreras A and Mendoza C. 2009. Sperm plasma membrane integrity in fertile and infertile men. *Andrologia* 24:141-144.

Saacke RG. 2008. Sperm morphology: its relevance to compensable and uncompensable traits in semen. *Theriogenology* 70:473-478.

Saacke RG, Nadir S and Nebel RL. 1994. Relationship of semen quality to sperm transport, fertilization, and embryo quality in ruminants. *Theriogenology* 41:45-50.

Sailer BL, Jost LK and Evenson DP. 1995. Mammalian sperm DNA susceptibility to in situ denaturation associated with the presence of DNA strand breaks as measured by terminal deoxynucleotidyl transferase assay. *J Andro*l 16:80-87.

Ugur MR, Saber AA, Evans HC, Gilmore AA, Hitit Mustafa, Arifiantini RL, Purwantara B, Kaya A and Erdogan M. 2019. Advances in the cryopreservation of bull sperm. *Frontiers in Vet Sci* 6:268-283.

Utt M. 2016. Prediction of bull fertility. *Anim Reprod Sci* 169:37-44.

Yanagimachi R. 1994. Mammalian fertilization. In: Knobil E, Neill JD (eds). 2nd ed. *The Physiology of Reproduction*. New York: Raven Press, p. 189–196

Ybañez AP, Ybañez RHD, Fojas AJG, Malate PLT, Abela JV, Nuñez ES, Royo JT Jr and Lopez IFM. 2017. Retrospective analysis of selected artificial insemination (AI) related practices, maternal factors and success rate of AI in water buffaloes in three rural areas in the Philippines (1998-2015). *Livestock Res Rural Dev* 29, Article #75.

Watson PF. 1995. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Reprod Fertil Dev* 7:871–891.