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Mid-piece Length of Spermatozoa in Different Cattle Breeds and its Relationship to Fertility

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Abstract: Recently positive correlation has been found between oxygen consumption (ZO_2) in bull spermatozoa and non-return rates and concluded that an increase in ZO_2 , characteristic of the freeze/thaw process, was possibly associated with mitochondrial membrane damage during this procedure: alternatively, sperm may be hyperactivated through the capacitation-like effects of freezing/thawing. We speculated that the morphology of spermatozoa may be associated with their rate of ZO_2 and fertility: for example, sperm mid-piece length where mitochondria are located. Such a relationship has not been investigated before, particularly in context of commercial cattle breeding programmes and bull fertility characteristics. Sperm biometry was performed on ejaculates obtained from 34 bulls representing six breeds: Holstein (yearlings and mature), Friesian, Belgian Blue, Aberdeen Angus, Charolais and Limousin. Five ejaculates were collected from every bull and from each sample a semen smear was fixed and stained with eosin/nigrosin: the mid-piece length of 40 sperm with normal morphology was measured in every sample. Data were analysed by breed, age and within each bull. Significant differences ($p < 0.01$) between ejaculates in 9/34 bulls was found, as well as differences ($p < 0.001$) between individual bulls within the same breed. The average mid-piece length for Aberdeen Angus was 13.35 μm , for Belgian Blues and Limousin around 13.8 μm and for Charolais 13.68 μm : for dairy breeds such as Holstein and Friesian it was about 13.4 μm . The mean value of mid-piece length for breed was compared with their 49 day non-return rate; a negative correlation ($r = -0.53$) was found in black and white dairy breeds.

Key words: Mitochondria, ATP, ejaculates, bulls, non-return rates

INTRODUCTION

Sperm are highly differentiated, specialised cells that transfer a haploid set of paternal chromosomes to the female gamete at fertilisation. To achieve this, they must traverse the length of the female genital tract, bind to oviduct epithelium to undergo capacitation, attach to the zona pellucida and finally penetrate the oocyte itself (Foote, 2003).

Spermatozoa obtain energy for their motility through glycolysis and/or respiration, provided that appropriate substrate is available (Krzyszosiak *et al.*, 1999). Rikmenspoel (1965) estimated that 70% of Adenosine Tri-Phosphate (ATP) produced by bovine spermatozoa is used for motility, the precise proportion being related to the incubation temperature: for example, at 18 and 37°C around 9 and 58% of sperm respectively showed progressive motility (Hammerstedt and Hay, 1980). Spermatozoa generate most of their energy by glycolysis rather than oxidative phosphorylation and a number of

sperm-specific enzymes involved in the glycolytic pathways are located in the fibrous sheath (Miki *et al.*, 2004). Mitochondria that produce ATP through oxidative phosphorylation are located in the sperm mid-piece, found at the extreme anterior end of the flagellum (Turner, 2003; Ford, 2006). This function is crucial because it correlates with the energy status of spermatozoa, their motility and potential for achieving successful fertilisation (Casey *et al.*, 1993; Kasai *et al.*, 2002).

Hyperactivated motility in sperm is an essential precursor to successful fertilization that requires both increased ATP synthesis and immediate availability within mitochondria (Suarez and Ho, 2003; Quill *et al.*, 2003). Their flagellar movements become asymmetrical and whip-like, have high amplitude and low frequency and this facilitates their detachment from the oviduct epithelium to position sperm in the ampulla prior to fertilization (Turner, 2003).

Biometric differences in sperm morphology between mammalian species were described by Cummins and

Woodall (1985), who measured lengths of mid-piece, caput or head and principal piece for most species except primates and bats. Gage (1998) found a correlation between mid-piece and flagellar lengths for the 179 species he investigated. More recently, Aggarwal *et al.* (2007) observed a wide variation in the length of the mid-piece between buffalo bulls of different breeds and suggested that sperm biometric characteristics might be associated with male fertility. It is in this context that sperm competition has been considered in those species where two or more males compete for opportunities to mate with a single female, such as in canines and fertilize ova. Sperm mid-piece volume may be indicative of a higher mitochondrial content and is significantly larger in these species (Anderson *et al.*, 2005): there is also a positive correlation between it and testis size. Mid-piece volume is correlated positively with motility in domestic fowl, possibly dependent upon an endogenous substrate (Froman and Feltmann, 1998) and in primates that exhibit multiple-partner mating activity (Anderson and Dixson, 2002; Dixson *et al.*, 2004). Generally, sperm with longer flagella tend to swim faster to reach the ova sooner and achieve fertilization compared to those with shorter tails when ejaculates from different males intermingle in the same female tract (Gomendio and Roldan, 1991): there is a positive correlation between sperm length and its maximum velocity. By contrast, sperm from the Iberian red deer stag have long mid-pieces but swim relatively slowly (Malo *et al.*, 2006), suggesting that their activated spermatozoa with shorted mid-pieces may exhibit greater motility.

Several methods have been used to assess semen quality, using laboratory parameters to predict the fertilization potential of bulls and correlating these results with actual pregnancy rates achieved in the field. Post thaw motility is assessed most frequently as a measure of sperm viability and is correlated ($r = 0.4-0.5$) with 56 day non-return rates in cows (Januskauskas *et al.*, 2003). Also used are the percentage of viable spermatozoa present in an ejaculate, computer-based estimates of motility characteristics and acrosome integrity that are all correlated significantly with good fertility (Budworth *et al.*, 1988; Kjaestad *et al.*, 1993; Zhang *et al.*, 1998; Januskauskas *et al.*, 2003). Hence, bulls with apparently normal semen that are subfertile are worthy of further investigation using more recently devised molecular techniques (Aggarwal *et al.*, 2007).

Froman and Feltmann (1998) and Froman *et al.* (1999) concluded that mitochondrial function and ATP content of avian sperm were the most important factors affecting

their motility and sperm motility was the primary determinant for optimum fertility. For example, the mean rate of oxygen consumption was $1.14 \mu\text{L O}_2 \text{ min}^{-1}$ in fresh semen ejaculates of high motility compared with $0.62 \mu\text{L O}_2 \text{ min}^{-1}$ in those presenting only moderate motility. Recently, Garrett *et al.* (2008) found a positive correlation between oxygen consumption (ZO_2) in bull spermatozoa and non-return rates and concluded that an increase in ZO_2 , characteristic of the freeze/thaw process, was possibly associated with mitochondrial membrane damage during this procedure: alternatively, sperm may be hyperactivated through the capacitation-like effects of freezing/thawing.

We speculate that the morphology of spermatozoa may be associated with their rate of ZO_2 and fertility: for example, sperm mid-piece length where mitochondria are located. Such a relationship has not been investigated before, particularly in context of commercial cattle breeding programmes and bull fertility characteristics. This present study investigates variation in sperm mid-piece biometry by breed, age and between bulls used in current breeding programmes for dairy and beef herds in both intensive and semi-intensive cattle livestock farms, worldwide.

MATERIALS AND METHODS

Six different breeds were chosen during 2009 for investigation. Holstein and Friesian represented the dairy breeds and Belgian Blue, Aberdeen Angus, Charolais and Limousin the beef breeds used commonly as terminal sires. Five ejaculates were collected and examined from each bull. To prepare semen samples for microscopic examination, $100 \mu\text{L}$ of raw semen was added to $250 \mu\text{L}$ of Eosin Nigrosin stain and, after shaking well, drops of stained semen were placed at one end of a clean microscopic glass slide and a smear made by drawing them out with the clean edge of another glass slide. The slides were labelled with bull identity and date and allowed to dry at room temperature. The mid-piece length of forty morphologically normal sperm per ejaculate was measured, selected from various fields at random on each of five slides, using a $\times 100$ oil immersion objective with a Nikon Labophot 2 Microscope fitted with a digital CCD camera (Penguin-600 CL-CU) with imaging software within the Penguin/Pro application suite (Penguin/Pro Application Suite v.3.5.0, Pixera corporation USA).

Sperm with abnormal morphology of either head or tail and post-ejaculatory injury were excluded so that only those with normal morphology and a distinct mid-piece,

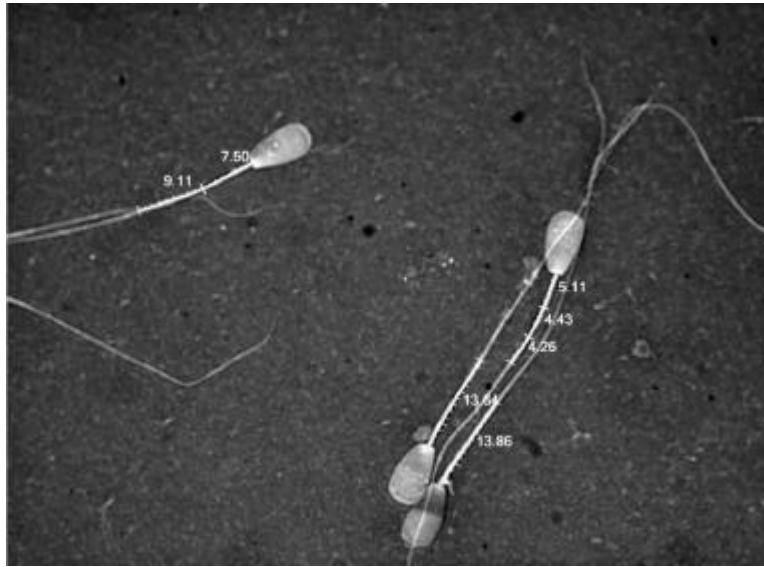


Fig. 1: Spermatozoa with both straight and bent mid-pieces that were measured either as a single straight line or the sum of several measurements

either straight or curved, were measured (Fig. 1). Before taking the measurement, the captured digital image of the individual spermatozoon was magnified to a level where the mid-piece could be distinguished clearly from the principal piece. Measurements (in microns) were made of those sperm with straight flagellum by dragging the cursor from the distal point of the mid-piece to its anterior end at the base of head region: where the flagella were bent, mid-pieces were measured using a series of short straight lines that described their curve, the sum of these being considered as a single value.

Data and statistical analysis: The estimated mid-piece lengths were recorded and organized into Excel files with the independent variables being breed and ejaculate number. After cleaning, the data were investigated using one-way Analysis of Variance (ANOVA) in Minitab; relationships between independent variables were investigated using linear correlation coefficient. Significance was accepted at a p-value <0.05 and results presented as Mean±SD, except where stated.

RESULTS

Differences between ejaculates from the same bull: The mid-piece length was compared between young Holstein bulls less than two years old with older, mature animals (Fig. 2a-c). Nine of the 34 bulls examined (31%) showed significant variation between their own ejaculates: four Holsteins (F = 2.48, p<0.05; F = 3.75, p<0.01), one Friesian

(F = 2.92, p<0.05), two Belgian Blues (F = 2.98, p<0.05), one Aberdeen Angus (F = 2.54, p<0.05) and a Limousin (F = 2.71, p<0.05). The ejaculates obtained from Friesian bulls showed least variation between collections whereas one Holstein bull (AHO2) showed just over 2% variation in mid-piece length (F = 5.89, p<0.01).

Differences between breeds: There were significant differences (p<0.001) between bulls within the same breed: for example, between juvenile Holstein bull 2 and the other four within that group and two Belgian Blue bulls - 2 and 5-that had significantly shorter mid-pieces than the other three (Table 1). Despite this intra-breed variation, there was no significant difference in mid-piece length between the breeds. Sperm from Belgian Blue and Limousin bulls presented with mid-pieces of similar length that were longer than the other breeds investigated: those with the shortest length were from juvenile Holstein bulls.

Mid-piece length and 49-day non-return rates: Data were analysed from 5850 individual cow breeding records that were served using semen collected from the five mature Holstein and five Friesian bulls evaluated in this study. The overall 49-day non-return rate for the Holstein bulls was 73% and for Friesians 80%. However, within this dairy bull sample the non-return rate was lower by 5% from the breed average when mid-piece length was significantly longer (r = -0.53): the small numbers of bulls involved prevented meaningful statistical relationships being established.

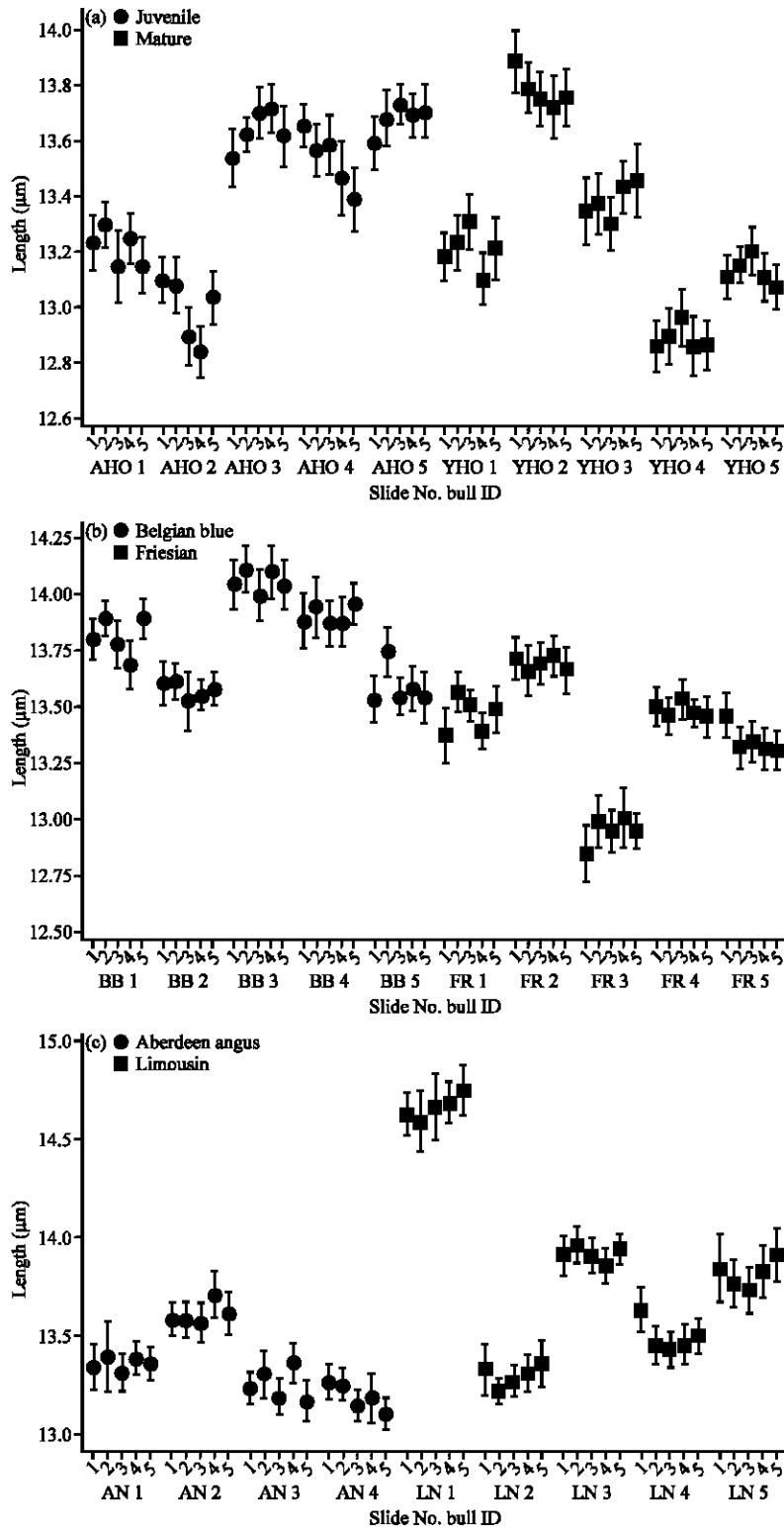


Fig. 2: Variation in sperm mid-piece length between ejaculates within five different bull breeds: (a) Holsteins juvenile and Holsteins mature (b) Belgian Blue and Friesian and (c) Aberdeen Angus and Limousin

Table 1: Mean mid-piece length (SD) of 200 spermatazoa examined from five ejaculates, each obtained from either juvenile and mature Holstein or five other breeds of bull

Breed of bull	Bull					F-value	p-value
	1	2	3	4	5		
Holstein (juvenile)	13.21 (0.08)	13.78 (0.06)	13.38 (0.06)	12.89 (0.05)	13.13 (0.5)	84.4	<0.001
Holstein (mature)	13.22 (0.06)	12.99 (0.12)	13.64 (0.07)	13.53 (0.11)	13.68 (0.05)		
Friesian	13.46 (0.08)	13.69 (0.03)	12.95 (0.06)	13.48 (0.03)	13.35 (0.06)	115.7	<0.001
Aberdeen angus	13.36 (0.03)	13.61 (0.06)	13.25 (0.08)	13.19 (0.07)		42.6	<0.001
Belgian blue	13.81 (0.9)	13.58 (0.04)	14.06 (0.05)	13.91 (0.04)	13.59 (0.09)	51.8	<0.001
Charolais	13.53 (0.04)	13.59 (0.05)	14.16 (0.09)	13.36 (0.03)	13.86 (0.03)	183.7	<0.001
Limousin	14.67 (0.06)	13.30 (0.05)	13.93 (0.04)	13.49 (0.08)	13.82 (0.07)	231.2	<0.001

DISCUSSION

Variations in the length of mammalian spermatazoa are due mostly to variation in tail length (Cummins and Woodall, 1985) and the size of the mid-piece contributes to such species differences. Anderson and Dixson (2002) found that mid-piece volume was greater in primates whose normal mating patterns involved multiple rather than single partners. Earlier, Gomendio and Roldan (1991) had found similar differences between monoandrous and polyandrous species: in rodent and other species where males face sperm competition after mating with a single female, spermatazoa are longer than in species where only a single dominant male will mate with a female e.g. some wild cats and deer. The longer the sperm flagellum, the greater the forces generated by its motions (Katz and Drobins, 1990) and its requirement for immediately available energy. This present study is the first to record variation in mid-piece length of a single species, the bovine, associated with collecting their ejaculates over a period of time. Whilst no significant differences were found between different breeds of bull, Aggarwal *et al.* (2007) found such variation within eight various breeds of buffalo. Results from this study showed significant variation in mid-piece length between ejaculates collected from nine of the 34 bulls investigated. The mid-piece lengths we obtained were similar to those found by Venkataswami and Vedanayagam (1962) who found an average mid-piece length of $12.41 \pm 0.85 \mu\text{m}$ for buffalo and $14.19 \pm 1.57 \mu\text{m}$ for domestic cattle. It is unlikely that the variation found in this present study occurred because of measuring technique since spermatazoa with straight tails could be measured as one entity and sperm with severely bent tails, due to cold shock, were positively excluded. In any case, the SD in this present study was ten-fold less than that recorded by the Indian researchers, suggesting this current computer-aided technique is robust. However, no attempt was made biochemically to distinguish between spermatazoa that were either non-apoptotic or undergoing programmed cell death: mid-piece morphology and mitochondrial volume is affected by apoptosis (Hendricks and Hansen, 2009) and this may have contributed to some variation found in this study.

Age influences mid- piece length in bulls. Cummins and Woodall (1985) found that sperm of bulls over 10 years old possess mid-pieces around 4% longer than younger fully mature males. In this present study, Holstein bulls under 18 months old had mid-pieces 1% shorter than older males, suggesting a slow, progressive lengthening of the mid-piece from puberty through to old age. Male reproductive potential generally falls with increasing age, associated with two factors. First, there is greater opportunity for mutations to adversely affect the mitochondrial genome and impair oxidative phosphorylation. The mid-piece is crucial for capacitation that involves tyrosine phosphorylated proteins, two of which are phospholipid hydroperoxide glutamate peroxidase and ATP synthase beta subunit: both have a primary role in sperm motility (Shivaji *et al.*, 2009) and disruption of their synthesis will reduce male fertility. Second, mitochondria are a source of Reactive Oxygen Species (ROS) which is a normal product of cellular respiration: normally these are removed from cells by antioxidant defence mechanisms. In sperm, ROS can accumulate within the mid-piece and damage mitochondria: the likelihood of this occurring increases as males grow older (Desai *et al.*, 2010). Further preliminary data from this present study associated Non-Return Rates (NRRs) in cows bred with semen collected from specific bulls whose mid-piece lengths were measured: longer mid-pieces correlated with up to a 5% reduction in NRR in the dairy bulls investigated. The relationship between mid-piece length, its mitochondrial volume and the efficiency of oxidative phosphorylation should be investigated further.

There are several other factors that affect mid-piece length in bulls. For example, the mid-piece length of mature spermatazoa in pure-bred black-and-white cattle is 2% longer than in crossbred Friesian x Sahiwal animals (Sarder, 2005). Heterosis is well known as a positive influence for reproductive efficiency in cattle, particularly in reducing embryonic mortality (Heins *et al.*, 2008) but the contribution of the male gamete to such improved fertility is unknown. Also, sperm from unimproved Indo-Pak zebu breeds tend to have mid-pieces 2-9% longer than European cattle (Venkataswami and

Vedanayagam, 1962): again, the significance of this in terms of breeding efficiency is not known.

This study has described differences in spermatozoa mid-piece length in different ejaculates collected from the same bull and variation between breeds. These preliminary results suggest that further work is necessary to relate mid-piece length with mitochondrial density and volume and energy metabolism related to sperm motility, capacitation and fertility potential of known beef and dairy bulls.

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