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Morphometry of head spermatozoa of sexually matured Malayan Gaur Bulls (*Bos gaurus hubbacki*)

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Abstract
The present study was conducted to measure various parameters of head morphometric of the Malayan gaur bulls (*Bos gaurus hubbacki*) spermatozoa through image analysis. Spermatozoa head morphometric was quantified in terms of the following morphological features: length, width, head shape (width: length) and head area. A total of 200 spermatozoa with intact acrosomes were selected, stained with eosin-nigrosin, and assessed with bright field microscopy at 1000x magnification. Mean head length, mean head width, head shape and mean head area were 9.06 ± 0.34 µm, 4.70 ± 0.28 µm, 0.51 – 0.53 and 42.60 µm2, respectively. As a conclusion, morphologically the Malayan gaur spermatozoa in this study show the normal features characteristics with the regular oval head morphometry.

Keywords: Malayan gaur, matured, spermatozoa, head, morphometry

Introduction

The Malayan gaur (*Bos gaurus hubbacki*) or Seladang was listed as vulnerable species by the International Union for Conservation of Nature and Natural Resources (IUCN) (Duckworth et al. 2008). The gaurs were reported mainly distributed in the tropical woodlands of Peninsular Malaysia and Southern Thailand (Lydekker 1907). In Malaysia, the population of the gaur was reported to be less than 500 individuals and it is thought to be declining (Read et al. 1994). Therefore, the collective effort is needed for Malayan gaur conservation; the gaur survival and their genetic.

An improvement in the management system for genetic conservation of the gaurs via semen collection, analysis and storage with regards to animal breeding and husbandry would result in an increase in the number of gaur in the wild (Hafiz et al. 2010). On the other hand, increased research activities on breeding management programs such as synchronisation of estrus, improvement of semen collection, semen evaluation and cryopreservation, genetic studies and increase in the use of assisted reproductive biotechnologies could be useful in increasing gaur population without interrupting their natural habitat in the wild (Fazly Ann et al. 2010, 2011; Hafiz et al. 2011; Iswadi et al. 2011, 2012).

Spermatozoa morphology is an interesting parameter and appears to be related to fertility, at least as a tendency when combine with other semen quality parameters (Gravance et al. 1996; Rodriguez-Martinez 2001; Tanghe et al. 2002; Gadea et al. 2004). Many published reports indicated the abnormal bull spermatozoa morphology has been correlated with reduced fertility (Correa et al. 1997; Januskaukas et al. 2003; Rodriguez-Martinez 2003; Love 2011; Bohlooli et al. 2012). In particular,

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the occurrence of abnormal spermatozoa head morphology is associated with lower fertility in the bull (Sekoni and Gustafsson 1987). However, correlation between spermatozoa morphology and fertility have been shown vary widely, and have most often been statistically non-significant when the semen of AI quality grade has been assessed (Rodriguez-Martinez 2003). Garcia-Herreros and Leal (2013) reported there were significant relationships between spermatozoa head dimension and fertility of the bull assessed by spermatozoa morphometry analysis.

The spermatozoa morphometry of wild animal has been a subject of study considerably less frequently. Spermatozoa morphometry, in combination with other objective traits, can be useful for developing a fertility index. According to Gage (1998), spermatozoa morphometry provide information on the evolution of the mammals by understanding the adaptive form of the spermatozoa structure. Various comparative studies were done to investigate spermatozoa morphometry and other animal parameters such as body size, female tract dimensions and spermatozoa competition (Gomendio and Roldan 1993; Harcourt 1991; Hosken 1997). In the domestic bulls, metric criteria for normal spermatozoa head measurements have not been readily applied to fertility assessment. However, large variability in assessing primary spermatozoa abnormalities, including spermatozoa heads, has been found between laboratories (Bishop et al. 1954). While manual assessment of bull spermatozoa head morphometry has been associated with fertility and chromatin structure (Barth 1992; Sailer et al. 1996), the visual measurement methods employed in these limited studies were extremely laborious or supplied a limited amount of information regarding the overall shape of the spermatozoa head. The wide variation in these spermatozoa head measuring methods makes accurate interpretation of the resulting data difficult; hence, various studies revealed contrasting results (Sailer et al. 1996; Gravance et al. 1999; Foote 2003; Beletti et al. 2005). Manual methods of analysis are subjective and highly variable within and between technicians, which may account for these differences. Recently, computer-aided spermatozoa head morphometry appears to be a precise method of assessing spermatozoa head dimensions (Gravance et al. 1995, 1996, 1999; Sundararaman et al. 2007; Phetudomsinsuk et al. 2008). According to Sanchez et al. (2013), the shape analysis of the sperm can reveal variation in biological traits which are highly distinctive between spermatozoa across taxa. Thus a better characterisation of spermatozoa morphometry will lead to an improvement in understanding the spermatozoa biomechanics and hydrodynamic efficiency. The comparison of spermatozoa morphometry for the most frequently used spermatozoa head morphologic parameters of spermatozoa of wild and domestic ruminants with normal fertility according to different publications are presented in *Table 1*.

To our knowledge, limited documents have been published on the analysis of Malayan gaur semen specifically on their spermatozoa. There were a few publication studying of spermatozoa morphology analysis (Hafiz et al. 2010; Iswadi et al. 2012), none were found for the head morphometric analysis of the Malayan gaur spermatozoa. Therefore, this study was conducted to investigate the predictive spermatozoa head morphometric quantitations of Malayan gaur bulls.

Table 1. Means of sperm head morphometric characteristics of wild and domestic ruminants

Species	Number of animals	Length, L (μm)	Width, W (μm)	Area, A (μm^2)	Reference
Plains bison *(Bison bison bison)*	3	9.03 ± 0.32	4.76 ± 0.22	35.64 ± 1.91	Pegge et al. (2011)
Wood bison *(Bison bison athabascae)*	2	9.04 ± 0.44	4.71 ± 0.19	34.72 ± 2.64	Pegge et al. (2011)
Friesian bull cattle *(Bos taurus)*	10	9.12 ± 0.05	4.71 ± 0.02	33.80 ± 0.19	Vicente-Fiel et al. (2013)
Brahman bull cattle *(Bos indicus)*	4	9.43 ± 0.02	5.13 ± 0.01	39.97 ± 0.17	Rubio-Guillen et al. (2007)
Bali bull cattle *(Bos sondaicus)*	10	9.98 ± 0.04	4.92 ± 0.04	n.d.	Arifiantini et al. (2006)
Indian buffaloes *(Bubalus bubalis)*	12	7.59 ± 0.01	4.91 ± 0.01	24.41 ± 0.05	Roy et al. (2008)
Anoa (*Bubalus quarlesi*)	2	7.56 ± 0.06	4.49 ± 0.03	n.d.	Yudi et al. (2010)
Ox *(Bos taurus)*	1	6.77	4.27	n.d.	Cummins and Woodall (1985)

*n.d. = not determined

Materials and methods

Semen collection

Fresh semen samples were collected using by electroejaculation (EEJ) and transrectal massage-electroejaculation (TM-EEJ) techniques from three Malayan gaur bulls at Jenderak Selatan Wildlife Conservation Centre, Department of Wildlife and National Parks, Peninsular Malaysia. Briefly, the EEJ technique was accomplished using an automated semen collection unit with automatic and manual settings (ElectroJac5, Ideal Instruments, Neogen Corporation, USA) and a 66-mm rectal probe with three ventrally oriented electrodes. The voltage used for electroejaculator unit in this study in range of 1 to 6 volt with duration of 00:10 to 15:00 second. The semen was collected as it was emitted from the preputial orifice into a 15 ml graduated test tube covered with heat protector. Immediately after collection, the spermatozoa head morphometry were analysed.

Semen quality

The quality of the freshly collected semen was analysed using the method as reported by Iswadi et al. (2013).

Spermatozoa head morphometry measurements

Semen samples from three sexually mature gaur bulls (total of 35 ejaculates) were evaluated for spermatozoa head morphometry. Spermatozoa morphology was determined using eosin-nigrosin staining protocol with bright field microscopy at 1000x magnification. Measurements for spermatozoa head morphometric dimensions was according to Soler et al. (2005) which were length (L, along the major axis), width

(W, along the shortest axis), head shape (width: length) and area (A) as shown in *Figure 1*. The spermatozoa head morphometry was measured and analysed using Image J software (National Institute of Health, USA). At least 200 spermatozoa were analysed for each preparation from each gaur bull.

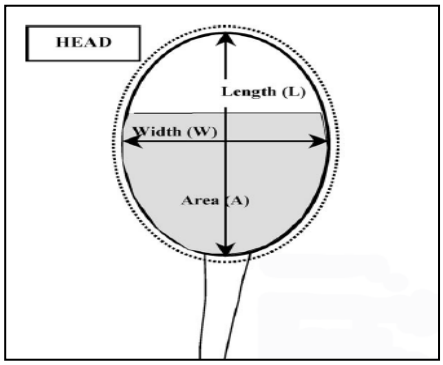

Fig. 1. Sperm head morphometric parameters examined in this study. The length (L), width (W) and area (A) of the head are self-evident. The length, L of the spermatozoa head was measured along the major axis, whereas the width, W of the spermatozoa was measured along the shortest axis

Data analysis

Length and width of the spermatozoa head were statistically analysed using correlation analysis in the Statistical Package for Social Science (SPSS version 13.0, SPSS Inc., USA) software. Data were shown as mean ± SEM and range of values with significant level at $p < 0.05$.

Results and discussion

Briefly, results of the semen quality parameters recorded were semen volume (0.2 – 8.5 ml), semen pH (6.58 – 7.95), progressive motile sperm (16 – 70 %), sperm concentration (105 – 3400 x 10^6 spermatozoa/ml), sperm viability (80 – 86 %) and sperm normal morphology (85 – 87.5 %) respectively. In line with the results in this study, Mahmood et al. (2014) show the comparable result of semen quality parameters recorded in breeding bulls.

The reproductive performance of the gaur bulls were determined by performing the AI using the semen collected in this study. As a result of the AI performed in the Kedah-Kelantan crossbred cattle, 13 of 21 cattle were detected pregnant, 11 cattle gave birth to a healthy calves while two calves were dead during birth as reported by Fazly Ann et al. (2011), Hafiz et al. (2011) and Iswadi et al. (2011). The result indicated the semen collected in this study were from sexually mature and fertile gaur bulls.

The results of spermatozoa head morphometry are presented in *Table 2*. The range of values of the Malayan gaur spermatozoa was length, L = 8.24 – 9.96 µm, width, W = 3.97 – 5.54 µm, width/length = 0.51 – 0.53, area, A = 42.60 μm^2. The mean of length, $\underline{L}$ = 9.06 ± 0.34 µm and mean of width, $\underline{W}$ = 4.70 ± 0.28 µm. The spermatozoa morphometric analysis results in this study were close to the other bull of the family of Bovidae and genus of Bos such as *Bos sondaicus, Bos indicus* and *Bos taurus*. In line with our results, Arifiantini et al. (2006) found that the mean of length, $\underline{L}$ = 9.98 ± 0.04 µm and mean of width, $\underline{W}$ = 4.92 ± 0.04 µm in spermatozoa of *Bos sondaicus*. Other study by Rubio-Guillen et al. (2007) show the results of mean of length, $\underline{L}$ = 9.43 ± 0.02 µm, mean of width, $\underline{W}$ = 5.13 ± 0.01 µm and area, A = 39.97 ± 0.17 μm^2 in *Bos indicus*. Vecente-Fiel et al. (2013) show the results of mean of length, $\underline{L}$ = 9.12 ± 0.05 µm, mean of width, $\underline{W}$ = 4.71 ± 0.02 µm and area, A = 33.80 ± 0.19 μm^2 in *Bos taurus*.

Table 2. Result of spermatozoa head morphometry of Malayan gaur bulls

No. of bulls	Mean length of the spermatozoa head, L (µm±SEM)	Mean width of the spermatozoa head, W (µm±SEM)	Range			Area (A) (μm^2)
			Length, L (µm)	Width, W (µm)	Head shape (width: length)	
1	$(8.92 \pm 0.31)^a$	$(4.44 \pm 0.25)^a$	8.38 – 9.91	3.97 – 4.99	0.47 – 0.50	39.60
2	$(9.06 \pm 0.33)^b$	$(4.75 \pm 0.17)^b$	8.24 – 9.69	4.28 – 5.15	0.52 – 0.53	43.04
3	$(9.20 \pm 0.33)^c$	$(4.91 \pm 0.21)^c$	8.45 – 9.96	4.55 – 5.54	0.54 – 0.56	45.17
Mean						
Σ(x)	(9.06 ± 0.34)	(4.70 ± 0.28)	8.24 – 9.96	3.97 – 5.54	0.51 – 0.53	42.60

In row: a,b,c indicate significant differences of spermatozoa length and width at $p < 0.05$.

Spermatozoa are exceptionally variable in morphology and even within closely related species spermatozoa may vary widely in shape and size (Arifiantini et al. 2006; Rubio-Guillen et al. 2007; Pegge et al. 2011; Vicente-Fiel et al. 2013). Substantial differences among head sizes and dimension within a species have been reported. This was reported due in part to biological variation (Beatty 1970). The great diversity of forms in spermatozoa structure and the specialized nature of spermatozoa have motivated the study of the evolutionary associations between and within mammalian spermatozoa morphometry. According to Sanchez et al. (2013), the evolution in size and shape of spermatozoa may be driven by two main selective forces: spermatozoa competition and female reproductive biology. Spermatozoa competition reported had been associated with an increase in total sperm dimensions and elongated head (Gomendio and Roldan 2008; Tourmente et al. 2011). On the other hand, spermatozoa morphology has been identified as a characteristic that can be used to predict a male's ability to fertilise an egg in competitive and non-competitive contexts (Ho et al. 2007; Tsakmakidis et al. 2010; Vasan 2011). Morphological assessment of spermatozoa also has importance in the investigation of biotechnology to cryopreserve spermatozoa (Gravance et al.1998), fertility status (Ostermeier et al. 2001) and sire selection (Foote 2003; Purwantara et al. 2010) of the bull.

Decreasing fertility due to poor semen morphology has been observed in bulls (Sekoni and Gustafsson 1987). Spermatozoa head abnormalities reported have been associated with early embryonic loss, lowered fertility and embryo quality (DeJarnette et al. 1992). An attempt to assess the predictive value of bull fertility, the morphometric dimensions of spermatozoa head from shape parameters (L, W and A) needed mathematical combinations with others semen quality assessment (e.g. concentration, motility, viability, acrosome integrity and etc). Considering that many assays test only a single attribute, it is unlikely that the fertility will be predicted accurately because many successive steps must occur for fertilisation to succeed. The use of multivariate analysis would help to discriminate potential fertility because this combines the functional information regarding different capacities of the spermatozoa. A combination of selected semen tests, therefore, yields a higher accuracy than a single test in the prediction of fertilizing capacity.

Traditionally, spermatozoa head have been analysed manually using one dimensional parameter such as length, width and area (Davis and Gravance 1994). However, the wide variation in the spermatozoa head measuring methods makes accurate interpretation of the resulting data difficult. Some possible causes could be related to the fact that certain spermatozoa characteristics cannot be analysed by the

classical spermiogram. With the classical spermiogram, it was possible to detect very low quality samples and eliminate them as being associated with poor fertility (Mandal et al. 2012). However, this is not an accurate way to distinguish samples with excellent fertility from those with low fertility. This could be related to the very limited variation in these parameters in mature fertile bulls, or to the fact that these tests do not properly evaluate the functionality of the spermatozoa. Manual methods of analysis are subjective and highly variable within and between technicians, which may account for these differences. Computer-aided spermatozoa head morphometry appears to be a precise method of assessing spermatozoa head dimensions. Determination of spermatozoa morphology by light microscopy by human eye suffers from subjectiveness, with different technicians often achieving different results on the same series of smears. Increasingly, computer aided spermatozoa head morphometry appears to be a precise method of assessing spermatozoa head dimensions (Jagoe et al. 1986; Katz et al. 1986). Computerised methods focus on evaluating the measurements that are able to quantify and classify spermatozoa morphology correctly and offer repeatable and objective method of assessing bull spermatozoa head morphometry within and between technicians.

So far, information regarding spermatozoa head morphometry and fertility of bull semen after artificial insemination is scarce. Sailer et al. (1996) reported that variation of morphometry measurements is likely a sensitive biomarker related to fertility potential and abnormal chromatin structure. Some evidence for a relationship between spermatozoa morphology and fertility in bulls has been presented (Soderquist et al. 1991,1997; Rodriguez-Martinez 2003; Chenoweth 2005; Al-Makhzoomi et al. 2008). Therefore, a complete morphological examination is recommended when bulls are introduced into the AI station and during subsequent regular routine examinations (Al-Makhzoomi et al. 2008). Conducting a semen morphology examination once before entering bulls into AI use, proved to be beneficial in predicting the suitability of a young bull for breeding. Further studies in semen morphology examination in Malayan gaur bulls may help to screen-out overtly poor-quality ejaculates collected from this species.

Conclusion

As a conclusion, the spermatozoa morphometric analysis results in this study show normal features characteristics with the regular oval head morphometry. Interestingly, the spermatozoa morphometric analysis data generated in this study shown that the morphometry of the Malayan gaur spermatozoa was close to the other bull of the family of Bovidae and genus of Bos. In the future, Malayan gaur spermatozoa morphometric analysis, in combination with other objective traits, can be useful for developing a fertility index of the bulls. Furthermore, the generation of new knowledge in molecular and genomic technology will help us to evaluate the potential fertility of bull ejaculates more accurately.

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Abstrak

Kajian ini dilakukan bertujuan untuk mengukur pelbagai parameter morfometri kepala sperma dari seladang (*Bos gaurus hubbacki*) melalui analisis imej. Morfometri kepala sperma diukur berdasarkan terma-terma bentuk morfologi berikut: panjang, lebar, bentuk (lebar:panjang) dan keluasan kepala sperma. 200 sperma dengan akrosom yang masih berkeadaan baik dipilih, diwarnakan dengan pewarna eosin-nigrosin dan dianalisis menggunakan *bright field* mikroskopi pada pembesaran 1000x. Purata panjang, lebar, bentuk dan keluasan kepala sperma adalah seperti berikut: 9.06 ± 0.34 µm, 4.70 ± 0.28 µm, 0.51 – 0.53 dan 42.60 μm^2. Kesimpulannya, morfologi sperma seladang daripada hasil kajian ini menunjukkan ciri-ciri bentuk normal dengan morfometri kepala yang bujur tetap.