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Short Communication: Characteristics of the endangered Javan banteng (*Bos javanicus*) spermatozoa

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Abstract. *Yoelinda VT, Arifiantini RI, Solihin DD, Agil M, Setiadi DR, Hastuti YT, Manansang J, Sajuthi D. 2023. Short Communication: Characteristics of the endangered Javan banteng (*Bos javanicus*) spermatozoa. Biodiversitas 24: 759-766.* Morphology and morphometry of spermatozoa become essential fertility parameters in domestic and wild animals. Such morphological abnormalities can arise from genetic or environmental origins and cause fertility deprivation. Semen collection using transrectal massage has been used in several domestic and wild animals and is known to be simpler, safer, and less painful than artificial vaginas and electro-ejaculation. This study aimed to evaluate morphological and morphometrical features of the banteng spermatozoa head. The objective of this study was also, to evaluate the use of transrectal massage as a semen collection protocol. This study was conducted in Taman Safari Indonesia, Cisarua, Bogor, West Java, involving one banteng (*Bos javanicus*) bull. Semen samples were collected weekly, smeared, and stained with a modified Williams' staining method. This study found ten types of head morphological abnormality, comprising several major defects, with several types known to be the genetic origin. There were also nine types of spermatozoa tail abnormalities and teratoid form abnormalities. The total normal spermatozoa proportion in this study was 54.30±1.74%. Morphometric dimensions of length, width, and area head of banteng spermatozoa were 9.88±0.01 µm, 4.91 µm, and 38.12±0.05 µm^2, respectively, with a tail length of 60.48±0.03 µm. Although our result was limited by the number of samples available for the study, this first report on the banteng bull's sperm morphology and morphometry still need to be considered. The possibility of transrectal massage use for banteng semen collection protocol demonstrated in this study may be helpful to support the conservation approach in terms of breeding program strategy.

Keywords: Banteng, captive, semen, spermatozoa, transrectal massage

INTRODUCTION

Banteng (*Bos javanicus*) is a protected wild animal in Indonesia as listed in the Minister of Environment and Forestry Regulation P.20/MENLHK/SETJEN/KUM.1/6.2018. Banteng is also categorized as an endangered animal according to the International Union for Conservation of Nature and Natural Resources (IUCN) Red Lists (Gardner et al. 2016). In addition, it is also categorized as a prioritized endangered animal by the Directorate General of Nature Resources and Ecosystem Conservation, Ministry of Environment and Forestry of Indonesia (2015). One of the efforts to conserve protected wildlife in Indonesia is through conservation at the existing conservation institutions, such as Safari Park. An essential key for a conservation institution is to breed or carry out the reproductive management of captive animals. However, in some conservation sites, sexually mature male banteng numbers were lesser than females, causing a high incidence of inbreeding (Sawitri and Takandjandji 2012). Therefore, regulating male genetic material exchange is an important step that must be considered.

Semen is a source full of male genetic material that is very valuable and important for understanding the basic reproductive physiology of male animals. Various methods, such as electro-ejaculation, artificial vagina, and transrectal massage, can be used for semen collection. Electro-ejaculation can be applied almost to all animals, both trained and untrained wild animals. This semen collection method has been used on banteng (Yoelinda et al. 2018). Nevertheless, the stress and pain caused by electro-ejaculation are aspects that are against animal welfare (Abril-Sánchez et al. 2019); therefore, some researchers considered that modifications of this method are required (Baiee et al. 2018; Chen et al. 2020). A study showed that semen collection using an electro-ejaculator induced a more significant increase in cortisol concentration and heart rate than transrectal massage (Abril-Sánchez et al. 2017). Another alternative is to use an artificial vagina which working principle is closest to promoting the normal physiology of ejaculation. However, this method has drawbacks, such as it is limited to trained non-disabled animals and requires higher costs for untrained animals (Sylla et al. 2015). Another method that can be used is transrectal massage. The transrectal massage method is easily applicable by directing the operator's fingers specifically to the area of the ampulla of the ductus deferens (Palmer et al. 2004). Research by Abril-Sánchez et al. (2017) shows that this method is less stressful. Furthermore, several studies have shown that the quality of semen, especially microscopically, obtained by an electro-ejaculator is not significantly different from that of transrectal massage (Sarsaifi et al. 2013; Tarmizi et al. 2020).

Characteristics of spermatozoa in various wild animals have been studied and revealed many interesting findings. Morphologically normal spermatozoa consist of a head and tail. Normal spermatozoa heads in mammals are generally oval and flat, consisting of the acrosome and the post-acrosomal section (Chenoweth 2005). The tail consists of the midpiece, the principal piece, and the endpiece (Johnson 2013). Interestingly, spermatozoa morphology serves some unique function, such as in the hydrodynamics of spermatozoa. It means that the design or shape of the head of the spermatozoa, accompanied by force generated by the spermatozoa's tail, will affect the spermatozoa's speed and movement (Nosrati et al. 2015; Soulsbury and Humphries 2022). Any abnormalities in spermatozoa morphology will compromise fertility and even cause sterility in males (Cao et al. 2017; Feyisa et al. 2018; Oehninger and Kruger 2021). The morphology and morphometry of spermatozoa have been studied from various species and showed diversity among species (Villaverde et al. 2013; Cursino and Duarte 2016; Yániz et al. 2016; Rossi et al. 2018; Steinberg et al. 2019; Andraszek et al. 2020). Morphometric dimensions of spermatozoa have also been linked to fertility, inbreeding, and phylogenetic studies (Villaverde et al. 2013; Lawrence et al. 2017).

Furthermore, it also varied among species and evolved by the fertilization mode (Gage 2021; Kahrl et al. 2021). However, reports regarding the characteristics of banteng spermatozoa, including morphology and morphometry, have not been previously reported. Therefore, this study wanted to characterize banteng spermatozoa as a preliminary report.

MATERIALS AND METHODS

Ethical clearance

The procedures conducted in the study have been approved by the Animal Care and Ethics Committee, LPPM - IPB University, Bogor, Indonesia (No.: 165-2019 IPB).

Procedures

Animal sample

During the research period, only one captive-born banteng bull was available and accessible for semen collection in Taman Safari Indonesia, Cisarua, Bogor. The bull was approximately 2.4 years old, weighing around 530 kg, and was sexually inactive. The bull was fed four bunches of grass weighing around 30 kg, roughly 4 kg of sweet potato, 4 kg of pellets, and 4 kg of bean sprouts daily-supplementation of Introvit® and Calphovit® given 40 g each for five days at the beginning of each month.

Animal habituation

The habituation of the banteng bull was carried out by providing feed in a modified restraint chute as the reinforcement for the bull. The zookeeper carefully led the bull to enter the chute. The banteng bull was then trained for transrectal palpation in the chute by authorized personnel. The time taken for the habituation procedure was adjusted according to the bull response for semen collection trials.

Semen collection

The habituated banteng bull was led and encouraged to enter and stay still within the chute with feeds during semen collection. First, the hairs around the banteng bull's prepuce were cleaned and clipped to avoid contamination during semen collection. Next, the prepuce and penile orifice were washed with a warm saline solution and wiped gently with a paper towel. Two people carried out the semen collection; one was a transrectal massage operator. Another operator collected semen using a modified tool outside the chute (Figure 1). The transrectal massage operator wore a lubricated glove before performing the procedure. The transrectal massage procedure referred to Palmer et al. (2004) with modifications. A modified semen collection tool was directed by another operator closer to the bull's prepuce to collect the dripping semen. Semen collection was carried out at intervals of once a week.

Figure 1. Modified semen collection tool. The collection tube assembled to the tunnel end

Spermatozoa morphology and morphometry evaluation

Smeared samples were evaluated with a light microscope (Olympus 33, Japan) at 400x magnification for morphological evaluation. First, a minimum of 500 cells from each smear was evaluated, and spermatozoa abnormalities were counted according to Freneau et al. (2010). Briefly, each normal spermatozoa counted as one record, whereas the frequency of each type of abnormality in each spermatozoon was calculated separately.

A minimum of 200 normal spermatozoa per smear was measured for morphometric evaluation. The evaluation was carried out with an iScope® light microscope (Euromex Microscope, The Netherlands) at 400x magnification. The brightness and contrast of the images were adjusted, and then captured using a Dino-Eye Eyepiece microscope camera (AnMo Electronics Corporation, Taiwan). The images were then analyzed using ImageJ software. The observed variables referred to Arifiantini and Ferdian (2006): spermatozoa head area, length, and width. In addition, the total length of the tail was measured, comprised of the length from midpiece until endpiece.

Data analysis

Morphological and morphometric evaluations of spermatozoa were conducted in three replicates for each collection period. The data are presented descriptively in mean values and standard error of the mean (SEM).

RESULTS AND DISCUSSION

Banteng habituation and semen collection

Banteng habituation was successfully carried out within five months until the first successful semen collection in this study. In this study, 1 to 1.5 mL of semen was successfully obtained by the transrectal massage method per collection attempt (Figure 3). The color of semen obtained varies from transparent, milky white, and cream with a watery consistency. Spermatozoa motility was collected using massage transrectal. The mass movement with the value (+) was only observed from one collection period.

Banteng spermatozoa morphological evaluation

From spermatozoa morphological evaluation, several types of abnormality were found in this study (Table 1). The total of the abnormal spermatozoa heads proportion were 22.80±1.17%. In this study, several major abnormalities, such as: abnormal contour, double head, knobbed acrosome, narrow at the base, piriformis, round head, tail stump, and dag-like defect were found. Some of those major abnormalities, namely knobbed acrosome, round head, tail stump, and dag-like defect, were genetic-related (Chenoweth 2005). In addition, this study found that banteng semen collected with transrectal massage had a total abnormal spermatozoa tails with a proportion of 29.89±2.06%. The total normal spermatozoa proportion in this study was only around 54.30±1.74%.

Banteng spermatozoa morphometric evaluation

The results of the morphometric evaluation of banteng spermatozoa are presented in Table 2.

Discussion

Habituation is the process of adapting animals to certain stimuli to learn to identify repetitive events that are harmless. This process is necessary, especially for wild animals that are unfamiliar with human encounters for the animals and human safety. Animal habituation for semen collection was reported in rhinos, camels, macaques, and even manatees (Agil et al. 2008; El-Hassanein 2017; Cowart et al. 2020; Houser et al. 2021). The habituation time needed for semen collection in rhinos was for two months (Agil et al. 2008), whereas, in the yak (*Poephagus grunniens*). the time was not reported (Das and Sarkar 2004). The habituation process reported by Houser et al. (2021) showed mixed results among individuals from the same species regarding the time needed to progress from each stage. These findings imply the presence of the influence of species or individuals in their response to the habituation process. The collection of banteng semen by the transrectal massage method has not been previously reported. This method has been used in swamp buffalo (*Bubalus bubalis*), rhinos (*Dicerorhinus sumatrensis*), elephants (*Elephas maximus*), and pangolins (*Manis javanica*) (Arifiantini and Ferdian 2006; Agil et al. 2008; Kiso et al. 2013; Tarmizi et al. 2020).

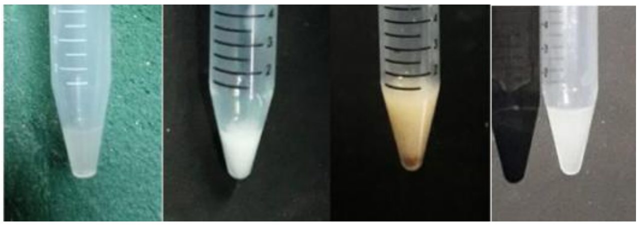

Figure 2. Semen collected by transrectal massage

Table 1. Abnormal morphology proportion of banteng spermatozoa collected by transrectal massage

Spermatozoa abnormalities	Proportion (%)
Abnormal contour (amorphous)	0.51±0.10
Detached head	1.97±0.23
Double heads	0.03±0.02
Knobbed acrosome	3.58±0.49
Macrocephalic	0.19±0.05
Microcephalic	0.86±0.18
Narrow at the base	5.94±0.62
Pyriformis	4.30±0.53
Round head	0.28±0.06
Tapered	5.51±0.50
Abaxial tail	1.64±0.30
Abnormal midpiece	3.52±0.51
Accessory tail	0.69±0.13
Bent principal piece	1.28±0.45
Coiled tail	4.46±1.04
Cytoplasmic droplet	9.24±2.00
Dag-like defect	1.45±0.34
Double tails	0.05±0.03
Tail stump	7.88±0.74
Teratoid form	1.33±0.22

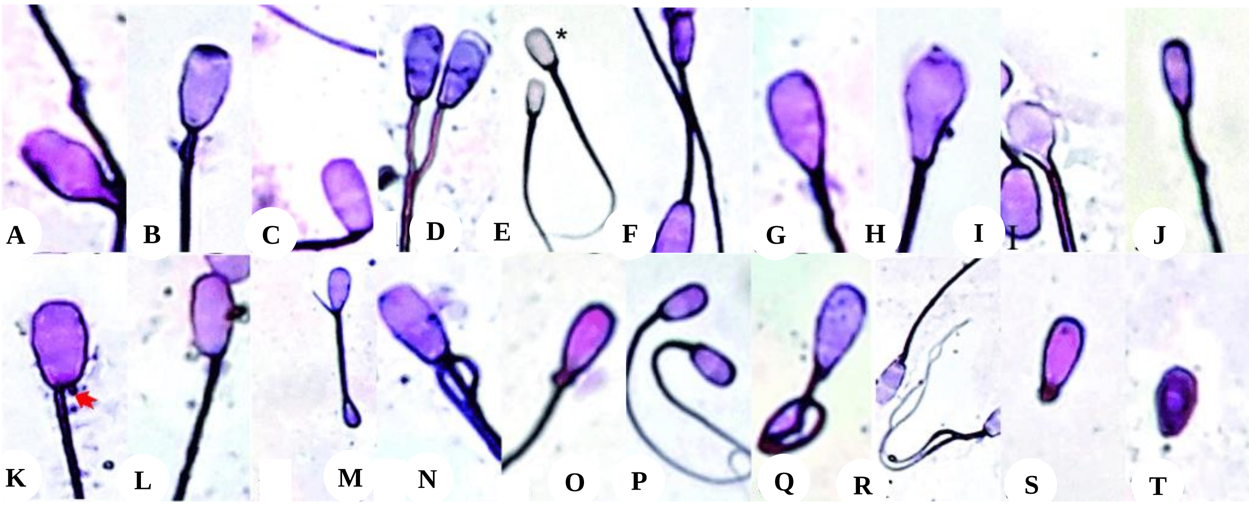

Figure 3. Abnormal spermatozoa in banteng bull's semen collected by transrectal massage method. A. Amorphous, B. Detached head, C. Double heads, D. Knobbed acrosome, E. Macrocephalic, F. Microcephalic, G. Narrow at the base, H. Pyriform, I. Tapered, J. Round head, K. Abaxial tail, L. Accessory tail, M. Abnormal midpiece, N. Coiled tail, O. Bent principal piece, P. Cytoplasmic droplet, Q. Dag-like defect, R. Double tails, S. Tail stump, T. Teratoid form

Table 2. Morphometric dimensions of banteng spermatozoa collected by transrectal massage

Variable	Mean±SEM
Head area	38.12±0.05 µm^2
Head length	9.88±0.01 µm
Head width	4.91 µm
Tail length	60.48±0.03 µm

The volume of banteng semen in this study was less than the semen volume successfully collected by electro-ejaculation reported in previous studies (Yoelinda et al. 2018). This finding was in agreement with studies (Abril-Sánchez et al. 2017) that also showed that semen collected by transrectal massage was lower compared to the electro-ejaculation method. Thus, the semen collection method may act as a factor influencing semen volume. Semen volume is also affected by the amount of liquid or seminal plasma produced by the bull accessory glands. Therefore, stimulation during semen collection may be relatively inconsistent, especially if compared with electro-ejaculation and artificial vaginas. That, in turn, caused the semen to be emitted heterogeneously and was not physiologically optimum.

Spermatozoa motility from all semen collected in this study was lower than banteng semen collected by electro-ejaculation in previous studies (Yoelinda et al. 2018). In our study, banteng penile erection was scarcely observed, and only a small portion of the banteng penis protruded, and it took a while until the semen was emitted. Spermatozoa collected with transrectal massage tended to drip down from the prepuce and may be exposed to the fluid around the prepuce. Furthermore, spermatozoa might undergo prolonged exposure to the environment temperature during semen collection. Thus, spermatozoa motility might be compromised. Variability of results from various species showed a possible influence of species or even individuals in terms of the response given to the transrectal massage method that affected the characteristics of the semen produced. Other factors such as genetics, breed, age, stress, and environmental factors have also been reported to affect the quality of the semen of bulls (Enciso et al. 2011; Sitali et al. 2016).

In this study, massage stimulation of the ampulla on ductus deferens and touch on the banteng accessory gland triggered the movement of spermatozoa from the cauda epididymis to the urethra. Reserved spermatozoa movement may vary due to the stimulation of transrectal massage that is not as optimum as in the normal process of ejaculation (Sylla et al. 2015). Those may cause variations in spermatozoa quality. Other factors contributing to the high proportion of spermatozoa abnormalities are breed, body condition score, age, scrotal circumference, feed quality, and stress (Sitali et al. 2016). Since we could only use one banteng in this study, specific factors cannot be identified.

As shown in Table 1, banteng spermatozoa in this study showed primary and secondary abnormalities. Primary abnormalities occurred in seminiferous tubules due to disruption during spermatogenesis (Kaya et al. 2014). Meanwhile, secondary abnormalities may indicate maturation disruption within the epididymis and may also be caused by semen handling or processing. The type of head abnormalities most frequently observed in this study was narrow at the base (Figure 3.G). This abnormality was characterized by a narrowing in the basal region of the head of spermatozoa and remaining normal in the anterior region. Enciso et al. (2011). showed a correlation between narrow at-the-base abnormalities and DNA fragmentation.

Another abnormality in this study was abnormal contour (amorphous head) (Figure 3.A) characterized by the irregular shape of the spermatozoa head. Abnormal chromatin condensation is thought to be able to trigger the elasticity of the cell nucleus in spermatozoa. Those could be causing the irregular shape of the spermatozoa head (Ma et al. 2019).

Abnormal spermatozoa with detached heads (Figure 3.B), characterized by the separated head from the tail, were observed in this study. One known factor that influences the proportion of this abnormality in cattle is the sexual activity of individuals. Barth and Oko (1989) showed that bulls in a sexually inactive status for several weeks have an increased proportion of detached heads. It is interesting to note since the banteng used in this study has not been sexually active. Therefore, future semen collection and observation are needed to elucidate this phenomenon. Furthermore, Ito et al. (2019) showed that the decrease in a specific protein related to the Outer Dense Fiber (ODF) bind might cause the weakening of its binding ability and lead to the detached head from the tail.

The prevalence of spermatozoa with double heads (Figure 3.c) in this study was lower compared to anoa (Yudi et al. 2010). Meiosis disorders cause the appearance of two heads in one spermatozoon in spermatogenesis. Disturbances in the process of cytogenesis and/or the formation of intracellular bridges during the division of meiosis cause imperfect separation in spermatozoa and are associated with DNA damage (Enciso et al. 2011). A study by Boe-Hansen et al. (2018) showed a positive correlation between the DNA fragmentation index and high-density stainability with this abnormality.

This study also found spermatozoa with knobbed acrosome (Figure 3.D). This abnormality can be found in the form of protrusion at the acrosome apex or in the form of flat (flattened/ indented) at the apex of the spermatozoa head. Genetic factors and environmental factors are known to cause this abnormality. In addition, spermatozoa with a flat acrosome form are known to have decreased oolemma-penetrating ability. However, those spermatozoa could still be used for natural mating and artificial insemination (Meyer and Barth 2001).

This study also found abnormalities in the form of varying sizes in spermatozoa (Figure 3.E-F). Spermatozoa with a smaller-sized head than normal (microcephalic) and a larger-sized head than normal (macrocephalic) were found in a low proportion. Barth and Oko (1989) stated that microcephalic spermatozoa could normally be found with a proportion of less than 1% in cattle with good fertility. Macrocephalic spermatozoa can be caused by genetic factors, such as a mutation in a particular gene (Chianese et al. 2015; Gatimel et al. 2017). Microcephalic and macrocephalic spermatozoa are categorized as minor abnormalities Blom (1973). This abnormality does not interfere with fertility if it occurs in a low proportion (Barth and Oko 1989).

Narrow at the base abnormality is characterized by a narrowing at the base of the spermatozoa head, while the anterior part remains normal. This abnormality has been linked with DNA damage that may be caused by oxidative stress (Enciso et al. 2011). Pear-shaped or pyriform head (Figure 3.H) is characterized by narrowing in the post-acrosomal region. Meanwhile, the tapered head (Figure 3.I) is characterized by a narrowing starting from the acrosomal until the post-acrosomal region Barth and Oko 1989. The pyriform head is usually correlated with testis degeneration. On the other hand, the tapered head has been linked with ectoplasmic specialization in the Sertoli cell and acroplaxome, which is involved in nucleus formation (Tang et al. 2018). Semen containing a high proportion of pyriform and tapered head spermatozoon cannot initiate fertilization. However, recovery from this abnormality is still possible depending on the severity and duration of occurrence (Barth and Oko 1989)

Spermatozoa with a round head (Figure 3.J) were found in this study due to the absence of an acrosome boundary. This condition can cause spermatozoa to fail to penetrate the ovum. Case reports in humans showed this abnormality without certain causative factors and other hormonal disorders (Chenoweth 2005). The cause of this abnormality is still unknown, but it might be correlated with DNA fragmentation (Nguyen et al. 2022).

Normal spermatozoa tail is also important, particularly for spermatozoa movement to reach the ovum. Morphology evaluation on the spermatozoa also identifies the presence of pseudo droplets and distal midpiece reflex. Pseudodroplet is characterized by thickening at the midpiece Barth and Oko (1989) and was correlated with genetics (Chenoweth 2005). Reflex at the distal spermatozoa midpiece was one of the commonly found abnormalities in bull spermiogram. This abnormality is categorized as a minor abnormality, according to Blom (1973), and thus does not significantly affect fertility. Spermatozoa with a bent principal piece found in this study were characterized by folding or bending at the principal piece of the tail.

This study also found the occurrence of the coiled tail. According to Barth and Oko (1989), a coiled tail is not correlated with fertility. The increased prevalence of coiled tails has been linked with various nongenetic etiology (Chenoweth 2005). The same incidence has been reported in wild felids such as clouded leopard (*Neofelis nebulosa*) (Tipkantha et al. 2017), Javan leopard (*Panthera pardus melas*) (Mulia et al. 2021), and mountain lion (*Puma concolor*) (Huffmeyer et al. 2022).

Cytoplasmic droplet is characterized by small-sized round-shaped mass approximately 2-3 μm and is commonly found in small proportion in the ejaculated spermatozoa. Normally, the cytoplasmic droplets are discarded when spermatozoa are ejaculated. The high proportion of abnormality in this study may be caused by the age of the individual, which was considerably young or at puberty. This circumstance agrees with Barth and Oko (1989) that young bulls at puberty commonly show cytoplasmic droplets within the semen.

Spermatozoa with abaxial tails also found that the tail was improperly implanted at the base of the spermatozoa head. According to du Plessis and Soley (2012), morphogenesis of this abnormality occurs in the testes, particularly at the early elongated spermatid stage. However, other factors, including genetic-related factors, may contribute to the abnormality occur (Chenoweth 2005). Accessory tail, another tail abnormality, which is usually associated with the abaxial tail, was also found in this study. Tail stump also occurred with a quite high proportion in this study, shown as rounded structure, or irregular short tail or short straight tail protruding at the base of spermatozoa head. In addition to these types of tail abnormality, abnormal midpiece, coiled tail, bent principal piece, double tails, and dag-like defects were also found in lower proportion.

The teratoid spermatozoa, also known as underdeveloped, were observed in the banteng bull's semen in this study. According to Blom (1973), teratoid spermatozoa abnormality was classified as a major abnormality, and which presence of this abnormality at a certain proportion causes fertility impairment. However, according to Perry (2021), the incidence of this abnormality in the semen of normal bulls was 1% and should be no more than 15% for a good prognosis. Thus, the result of the present study can be considered as low.

This study evaluated approximately 2400 morphologically normal spermatozoa and found that the morphometry value of banteng spermatozoa was higher than that of several other species. This study's head morphometry of banteng spermatozoa was comparable to the Bali cattle spermatozoa, bison plains, and wood bison (Pegge et al. 2011; Arifiantini et al. 2012). This study's morphometry of banteng spermatozoa was higher than that of swamp buffalo spermatozoa (Arifiantini and Ferdian 2006). The results of spermatozoa tail length measurements in this study were close to those reported by (Arifiantini et al. 2012) in Bali cattle but higher than anoa (Yudi et al. 2010). As reported by several studies, differences in measurement results can be due to species differences (Pegge et al. 2011; Armengol et al. 2015; Rossi et al. 2018; Andraszek et al. 2020) and even between different breeds of the same species (Soler et al. 2017; Felton-Taylor et al. 2020). The results of spermatozoa morphometry measurements can also be affected by the fixation techniques, solutions used to incubate specimens, and the staining methods, as found in many research (Banaszewska et al. 2015; Wysokińska et al. 2021).

Despite the limitation in animal numbers used in this study, the success in semen collection by transrectal massage implied its possibility for future use, which can be further used in genetic material collection and preservation. Moreover, this procedure was safely and practically performed on the unanesthetized banteng bull, meaning it was less invasive. The banteng bull used in this study was considerably young, and its reproductive status was still unknown. Therefore, early examination of the banteng semen will be a helpful tool to evaluate the individual's fertility.

This study is the first report on the banteng spermatozoa morphological and morphometrical evaluation. In terms of conservation measures, can be a good reference for further exploration of banteng reproductive biology and its difference from other species. Further investigation on factors affecting morphologically abnormal spermatozoa in banteng semen is needed, such as the DNA fragmentation evaluation. This study also demonstrated that banteng semen could be collected using the transrectal massage method, which is essential for developing the banteng breeding program as one of the conservation measures.

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