

A preliminary study on semen collection, its evaluation, and testicular and sperm morphometries in the wild proboscis monkey (*Nasalis larvatus*)

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Abstract

Introduction: The proboscis monkey (*Nasalis larvatus*) is an endangered species with a declining population. This article describes the first successful attempt at sperm collection and evaluation, and the testicular and sperm morphometries of the wild proboscis monkey in Sabah, Malaysia. **Material and Methods:** Eight semen collection procedures using electro-ejaculation and digital manipulation were conducted in three wild adult male proboscis monkeys. A total of 21 ejaculates were collected. The testicular biometry was measured with the aid of ultrasonography. Sample evaluation included semen volume and pH and sperm concentration, viability, and abnormality. The sperm morphometry was undertaken using phase contrast microscopy. **Results:** The mean (\pm SD) total testicular volume of these animals was 5.77 cm³ (\pm 1.58). Semen collection by electro-ejaculation resulted in an 84% success rate, while digital manipulation did not result in any ejaculation. Each animal showed different semen characteristics, where the volume was 5–540 μ L, pH 8–9, and sperm concentration 0.041–83.00 $\times 10^6$ /mL. The percentage of abnormal sperm was high at 76.8% (\pm 89.60), largely due to midpiece abnormality. Normal sperm had a spherical head and long tail with a head:midpiece:tail length ratio of 1:2:8. **Conclusion:** The social status of these animals may contribute to the generally low quality of the semen. The techniques and data from this study are useful for future conservation and application of assisted reproductive technology in this species.

Keywords: proboscis monkey, *Nasalis larvatus*, semen collection, sperm morphometry, testicular morphometry.

Introduction

The proboscis monkey (*Nasalis larvatus*) is a species of non-human primate that is endemic to Borneo and is distributed along lowland rivers, near-shore islands, and coastal mangrove swamps (24). It is currently listed as an endangered species (3) and is showing a decreasing population trend (14), largely due to continuous hunting and habitat destruction (3). A recent estimation of the total population of the species in Sabah is 5,907 (23). *Nasalis larvatus* is included in the Wildlife Conservation Enactment 1997 passed in Sabah and classified as a Totally Protected Animal in Schedule I, Section I (19).

Previous studies on the proboscis monkey focused on the population, conservation, feeding ecology, and

social dynamics of this species (4, 12, 15). Studies pertaining to its reproduction mainly shed light on the behaviour, breeding ecology, and breeding seasonality (17), and no literature was found on the spermatology of *N. larvatus*. Sperm morphology, motility and concentration are considered the three most important parameters when assessing semen quality. Despite the decreasing proboscis monkey population, there is no study in the field of reproduction and theriogenology, including research on how assisted reproductive technology (ART) might be implemented.

In macaques, numerous studies have been carried out as they are considered a potential animal model for human and other endangered species of primates (29). It is important to evaluate various semen collection methods in wild animal populations as it aids

determination of the least risk-prone method that could yield good-quality semen. In primates including macaques, commonly used methods were electro-ejaculation, digital manipulation, and penile vibratory stimulation (10, 21).

Basic theriogenology studies of male animals usually comprise the techniques of semen collection, semen evaluation to obtain the baseline value of the collected semen, and description of the morphology of the sperm. Understanding the morphology of sperm could aid in understanding the evolutionary background of a certain species (31). This article is the first to report successful semen collection using electro-ejaculation and to evaluate semen and testes and sperm morphometry of the wild proboscis monkey.

Material and Methods

Animals. The study was conducted on three wild adult male proboscis monkeys between November 2017 and December 2019. One monkey inhabited Labuk Bay Proboscis Monkey Sanctuary, one Lok Kawi Wildlife Park, and the third Kinabatangan. All the locations are located in the state of Sabah, Malaysia. The selected proboscis monkeys were adults and weighed between 15 kg and 22 kg over the study duration, having overall mean body weight (\pm SD) of 19.79 kg (\pm 1.96). Individually, the mean body weight for animal ID1 was 19.67 kg (\pm 0.47), for ID2 was 20.00 kg (\pm 0.32), and for ID3 was 19.83 kg (\pm 2.74). During the first anaesthesia, the weights of the proboscis monkeys were estimated based on observation. In subsequent anaesthesia, the weights from previous occasions were used as reference. All three proboscis monkeys were from all-male groups. The study was approved by Sabah Wildlife Department.

Anaesthesia and semen collection. The anaesthesia and semen collection were conducted based on a previous description of a protocol used with pig-tailed macaques (*Macaca nemestrina*) (29). The proboscis monkeys were baited with a small amount of food and intramuscularly administered (26) the anaesthetic in a pelvic limb using a 3 mL two-chambered dart with a 1.5 \times 20 mm plain needle and Vario 1V CO₂-powered blowpipe system (Telinject, Dudenhofen, Germany). The preparations used were 350 μ g/kg medetomidine (Medetomidine 40 mg/mL, Kyron Laboratories, Johannesburg, South Africa) and 80 mg/kg ketamine HCl (Ilium Ketamil 100 mg/mL, Troy Laboratories, Glendenning, Australia).

Upon induction of anaesthesia, the proboscis monkeys were intubated with an ID 6.0 cuffed endotracheal tube guided by a laryngoscope. Their body weights were measured using electronic scales. Blood oxygen saturation was measured using VE-H1008 Veterinary Pulse Oximeter (EDAN Instruments, Shenzhen, China) with a probe placed on the nares. End tidal CO₂ was measured using an EMMA Mainstream capnometer (Masimo, Irvine, CA, USA). The anaesthetic depth was assessed by jaw tone, anal tone,

palpebral reflex, pupillary light reflex and response to noxious stimulus. Supplemental doses of ketamine were administered intravenously to maintain anaesthesia if required. Semen was collected from anaesthetised monkeys by electro-ejaculation or digital manipulation of the penis.

Anaesthetised proboscis monkeys were subjected to rectal emptying by cleansing enema using water. Once the anaesthetic depth was stable, the penis was placed inside a conical tube and a Seager Model 14 electro-ejaculator probe 1.5 cm in size (Dalzell USA Medical Systems, The Plains, VA, USA) was inserted into the rectum. The voltage used was between 2V and 7V, and 10 stimulations were given for each volt. Stimulation was given for 2–3 seconds with 1-second intervals before the next stimulation. Each ejaculation was collected, placed on a rack in a water bath at 37°C, and liquefied for 30 min.

Testicular assessment. Following the anaesthetic induction, the dimensions of each testis were measured for length (L) and width (W) using an ultrasound. The volume (V) of each testis was calculated using Hansen's formula $V = L \times W^2 \times 0.524$ (18) and the total volume was determined by combining the volume of the left and right testes (11). After the completion of the procedures, the proboscis monkeys were moved to a holding cage and administered a reversal drug, Atipamezole (Alzane 5 mg/mL, Zoetis, Madison, NJ, USA), intramuscularly (26, 29) at a dose of five times the dose of medetomidine. They were allowed to recover undisturbed before release.

Semen evaluation. Following ejaculation and liquefaction of the coagulated semen, evaluation was conducted. Each ejaculation was denoted as n, signifying a single ejaculate. The semen pH was measured using pH indicator strips (Whatman, Cytiva, Global Life Science Solutions, Marlborough, MA, USA). The semen motility was assessed using a CX43-32P01 phase contrast microscope (Olympus, Tokyo, Japan) based on semen criteria previously detailed (30). In brief, a volume of 5 μ L of each ejaculate was placed on a 37°C prewarmed glass slide with a coverslip and 200 spermatozoa cells were counted. The general motility percentage, *i.e.* the percentage of moving spermatozoa cells, was calculated. The progressive sperm percentage was obtained from the observation of the proportion of spermatozoa cells which were swimming forwards. Good quality semen from all ejaculates was pooled and transferred into a 2 mL vial float on an expanded polystyrene rack in a water bath at 37°C.

Aliquots of 5 μ L were used for semen concentration count and morphology. For a sperm count, 2 μ L of semen was diluted with 38 μ L of formal saline to give dilution of 20 \times . A 10 μ L volume of the mixture was pipetted into haemocytometer chambers and examined under a light microscope at 400 \times magnification to determine the sperm concentration. For morphological examination, thin smears on glass slides were prepared from droplets of diluted semen. If the sperm

concentration was moderate or below, a dilution ratio of 1:2 (aliquot of semen:stain) was used (30) and if the sperm concentration was high, a dilution ratio of 1:3 (aliquot of semen:stain) was used (30). Two types of stains were utilised: Diff-Quik for observation of abnormalities and eosin-nigrosin for evaluation of live or dead status of spermatozoa. Sperm abnormalities were divided by site and categorised as in the head, midpiece, or tail region. The percentages of normal, abnormal, live, and dead sperm were subsequently calculated.

Sperm morphometry. The morphology of the sperm head was evaluated after staining a thin smear using Diff-Quik staining procedures. Sperm morphology was evaluated with a DME phase contrast microscope (Leica, Wetzlar, Germany) at 400× magnification. The percentage of abnormalities of the head, midpiece, and tail was assessed by inspecting at least 200 spermatozoa per sample. Morphometric measurements were taken manually using NIS-Elements imaging software (Nikon, Tokyo, Japan) and in accordance with systems proposed by the World Health Organization (28) and a previous report (2). The length, width, perimeter and area of the head and length of the midpiece, tail, and whole spermatozoon were the measured dimensions. From these, the indices of head elongation, ellipticity, roughness, and regularity were derived using the formulae presented in Table 1. This morphometric inspection was carried out on a total of 200 spermatozoa from various ejaculates collected from the three proboscis monkeys.

Table 1. Variables and formulae used to calculate the sperm morphometric indices of proboscis monkeys

Parameter	Variable/Formula
Head length (μm)	L
Head width (μm)	W
Head perimeter (μm)	P
Head area (μm ²)	A
Head ellipticity	L/W
Head elongation	(L-W)/(L+W)
Head roughness	4π(A/P ²)

Data analysis. The data of semen evaluation, sperm and testicular morphometry were analysed descriptively for each proboscis monkey. When statistical analysis was possible, individual animals' parameters were analysed using one-way ANOVA. Pearson's correlation test was used to determine the correlation between the various parts of the spermatozoon. The results were presented as mean (±SD) and range. All data were analysed using SPSS version 25 (IBM, Armonk, NY, USA).

Results

Testicular biometry. Testicular size and scrotal circumference during the collection period are shown in Table 2. All three male proboscis monkeys had a firm

scrotum. The mean lengths of the left and right testes were 2.04 cm (±0.16) and 2.24 cm (±0.24), respectively and the mean widths were 1.58 cm (±0.21) and 1.55 cm (±0.21). These dimensions showed the testes to be slightly oval-shaped with a width:length ratio ranging from 1:1.29 to 1:1.45. The mean volume of the left testes was 2.77 cm³ (±0.84), while that of the right testes was 2.91 cm³ (±0.90). The total volume calculated was 5.77 cm³ (±1.58).

Semen collection. Semen was collected successfully by electro-ejaculation from all three male proboscis monkeys. The overall success of semen collection using this technique was 84%. Digital manipulation was undertaken only twice, which did not result in ejaculation. In proboscis monkey ID2, no semen was ejaculated during the first attempt with electrostimulation on November 18, 2017, while a subsequent attempt on May 23, 2018 resulted in only expulsion of urine. A subsequent attempt on June 20, 2019 obtained only a few dead spermatozoa. These semen samples were discarded and not included in the data analysis. The other two attempts on proboscis monkey ID2 and all attempts on the other two proboscis monkeys ID1 and ID3 were successful and yielded semen samples useable for analysis. Table 3 summarises the semen collection from the proboscis monkeys. The ejaculated semen was either pale white or light creamy yellow in colour.

Semen characteristics. During semen collection, it was noticed that ejaculation usually yielded semen and some amount of coagulum. Proboscis monkey ID1 produced the highest mean volume of semen at 159.44 μL (±162.59). This is significantly ($P < 0.05$) higher than the volumes from the other two proboscis monkeys. The semen pH values were generally basic, ranging between 8 and 9. Although statistical analysis of sperm counts to elicit any significant differences between the individual monkeys was not possible, the counts were noted to range broadly, from a low of 41,000/μL to a high of 83,000,000/μL. Sperm motility ranged between 5% and 90%, with no statistically significant difference ($P > 0.05$) between the three proboscis monkeys. For sperm progression, superiority by a statistically significant ($P < 0.05$) margin was observed in proboscis monkey ID1.

The percentage of normal sperm (Fig. 1a) was low in all proboscis monkeys, ranging between 10.4% and 23.2%. In general, sperm abnormalities were observed at the head, midpiece, and tail. In the head, abnormalities detected included macrocephaly, microcephaly (Fig. 1b), bicephaly (Fig. 1c), and detachment. Only one type of abnormality was found in the midpiece: it could be bent (Fig. 1d). In the tail, bending (Fig. 1e), coiling (Fig. 1f), tight coiling (Fig. 1g), and duplication (Fig. 1h) were observed. The percentage of abnormal sperm was consistently high. Midpiece bending, which was between 26.1% and 46.4% prevalent (mean = 35.40% ± 8.71, median = 34.55%), comprised a large part of this percentage. Abnormalities of the head and tail ranged between 0.8% and 6.4% (mean = 2.75% ± 2.62, median = 1.90%) and between 37.7% and 51% (mean = 44.70% ± 5.86, median = 45.05%), respectively. A comparison of semen

quality among the three proboscis monkeys is shown in Table 4.

Semen morphometry. The mean (\pm SD) head width was 4.50 (\pm 0.38) and the mean head length was 5.53 (\pm 0.41). The dimensions showed the heads to be nearly spherical with a width:length ratio of 1:1.23, a low ellipticity index of 1.12 (\pm 0.07), and a very low elongation index of 0.05 (\pm 0.03). The roughness and regularity indices were 0.96 (\pm 0.03) and 1.01 (\pm 0.07), respectively. The mean midpiece and tail lengths were 11.62 (\pm 2.31) and 44.27 (\pm 3.60), respectively. In general, the proboscis monkey spermatozoon has a long

tail compared to the head and midpiece, with a head:midpiece:tail ratio of about 1:2:8. Determination of correlations between a part of the spermatozoon and its total length showed that the head and total length had a weak positive correlation at R value of 0.2120. On the other hand, the midpiece and total length revealed a weak negative correlation at R value of -0.2105. These correlations were not significant ($P > 0.05$). Between tail length and total length a moderate positive correlation was shown with R value of 0.7214 and this was significant ($P < 0.05$). A summary of the semen morphometry is presented in Table 5.

Table 2. Summary of testicular biometry for the three male proboscis monkeys

End point	Left		Right		Total	
	Mean	Range	Mean	Range	Mean	Range
Length (cm)	2.04 (\pm 0.16)	1.86–2.22	2.24 (\pm 0.24)	1.94–2.64	-	-
Width (cm)	1.58 (\pm 0.21)	1.49–1.96	1.55 (\pm 0.21)	1.18–1.84	-	-
Volume (cm ³)	2.77 (\pm 0.84)	1.40–4.47	2.91 (\pm 0.90)	1.47–4.19	5.77 (\pm 1.58)	3.61–8.33

Table 3. Dates of anaesthesia and semen collection, status, and number of ejaculates

Collection	Proboscis monkey ID1	Proboscis monkey ID2	Proboscis monkey ID3
1 st	July 19, 2018 n = 5	November 18, 2017 Discarded	September 29, 2018 n = 7
2 nd	August 26, 2018 n = 4	May 23, 2018 Discarded	
3 rd		June 20, 2019 Discarded	
4 th		November 13, 2019 n = 3	
5 th		December 3, 2019 n = 2	

n – number of ejaculates per animal

Table 4. Semen characteristics of the three proboscis monkeys ID1, ID2 and ID3

Characteristic	ID1 (n = 9)	ID2 (n = 5)	ID3 (n = 7)	Mean (\pm SD)	Range
Volume (μ L)	159.44 (\pm 162.59)*	56.00 (\pm 40.30)	52.43 (\pm 32.92)	99.14 (\pm 121.68)	5.00–540.00
pH	8.25 (\pm 0.25)	8.50 (\pm 0.5)	8.75 (\pm 0.25)	8.50 (\pm 0.43)	8.00–9.00
Concentration ($\times 10^6$ sperm/mL)	9.35 (\pm 7.65)	0.04	83.00	25.44 (\pm 33.89)	0.04–83.00
Sperm motility (%)	56.67 (\pm 26.67)	42.00 (\pm 9.80)	26.67 (\pm 26.09)	44.00 (\pm 26.67)	5.00–90.00
Sperm progression (%)	42.22 (\pm 29.36)*	8.00 (\pm 2.45)	4.17 (\pm 4.49)	22.25 (\pm 26.90)	0.00–80.00
Sperm viability (%)	65.74 (\pm 0.94)	51.90	69.10	63.12 (\pm 6.65)	51.90–69.10
Normal sperm (%)	13.50 (\pm 3.10)	18.30	23.20	17.13 (\pm 5.29)	10.40–23.20
Abnormal sperm (%)	86.50 (\pm 3.10)	81.70	76.80	82.88 (\pm 5.29)	76.80–89.60
Total head abnormality (%)	0.85 (\pm 0.05)	6.40	2.90	2.75 (\pm 2.62)	0.80–6.40
Macrocephaly (%)	0.45 (\pm 0.45)	0.00	0.00	0.23 (\pm 0.45)	0.00–0.90
Microcephaly (%)	0.20 (\pm 0.20)	0.00	2.90	0.83 (\pm 1.40)	0.00–2.90
Bicephaly (%)	0.20 (\pm 0.20)	0.50	0.00	0.23 (\pm 0.26)	0.00–0.50
Detached head (%)	0.00 (\pm 0.00)	5.90	0.00	1.48 (\pm 2.95)	0.00–5.90
Bent midpiece (%)	38.95 (\pm 7.45)	37.60	26.10	35.40 (\pm 8.71)	26.10–46.40
Total tail abnormality (%)	46.7 (\pm 6.08)	37.70	47.70	44.70 (\pm 5.86)	37.70–51.00
Bent (%)	36.65 (\pm 1.65)	23.80	44.80	35.48 (\pm 8.78)	23.80–44.80
Tightly coiled (%)	7.05 (\pm 0.92)	0.00	0.00	3.50 (\pm 4.11)	0.00–7.70
Coiled (%)	3.00 (\pm 2.83)	13.90	1.20	5.28 (\pm 6.04)	1.00–13.90
Double-tailed (%)	0.00 (\pm 0.00)	0.00	1.70	0.43 (\pm 0.85)	0.00–1.70

Results are presented as means unless otherwise indicated. Statistical analysis was performed for the volume, pH, concentration, sperm motility, and sperm progression only. * – statistically significant difference between animals; n – number of ejaculates per animal

Table 5. Semen morphometry of proboscis monkeys

Spermatozoon part	Parameter	Mean (\pm SD)	Range
Head	Length (μ m)	5.53 (\pm 0.41)	4.68–6.35
	Width (μ m)	4.50 (\pm 0.38)	3.86–5.46
	Perimeter (μ m)	15.31 (\pm 0.81)	13.63–16.91
	Area (μ m ²)	17.95 (\pm 2.05)	12.97–23.26
	Elongation index	0.05 (\pm 0.03)	0.02–0.11
	Ellipticity index	1.12 (\pm 0.07)	1.01–1.25
	Roughness index	0.96 (\pm 0.03)	0.88–0.98
	Regularity index	1.01 (\pm 0.07)	0.89–1.13
Midpiece	Length (μ m)	11.62 (\pm 2.31)	8.50–16.58
Tail	Length (μ m)	44.27 (\pm 3.60)	38.03–49.20
Total length (μ m)		61.42 (\pm 2.22)	57.92–66.80

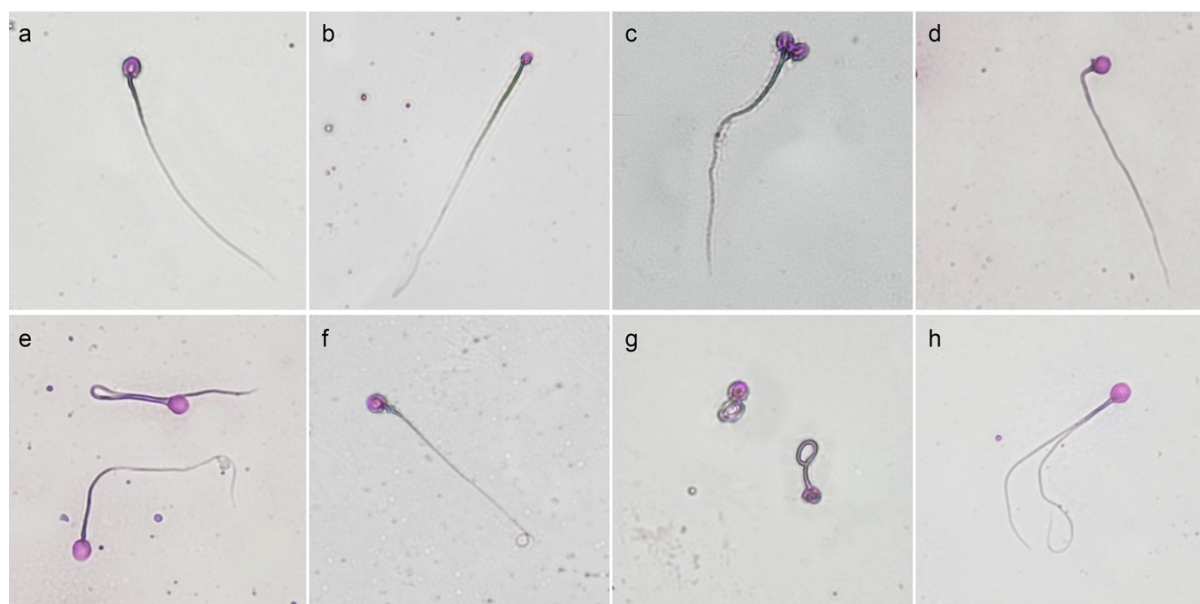


Fig. 1. Morphology of normal and abnormal sperm of proboscis monkeys.

a – normal sperm; b – microcephalic; c – bicephalic; d – bent midpiece; e – bent tail; f – coiled tail; g – tightly coiled tail; h – tail duplication

Discussion

This is the first report to describe a successful semen collection attempt on proboscis monkeys using electro-ejaculation. The high success rate suggests that this technique is reliable and safe to use with the species. The intermediate viability, motility, and concentration and minute volume of the proboscis monkey's sperm indicates low potential for ART to be effective if the technique requires long storage. Sperm motility and viability implied that the semen would not fare well if subjected to cryopreservation. It was found that >70% motility is the viability threshold for cryopreservation processes (8). The semen quality necessitates a different ART strategy in this species using semen shortly after obtainment. The most practical and viable option is temporary storage and utilisation of the semen under chilled conditions. A study in primates found artificial insemination with fresh semen to be successful in a rhesus species (6). Nevertheless, with advanced ART such as intracytoplasmic injection (ICSI), storage and cryopreservation of gametes should still be considered despite the low-quality sperm of a given species. This is particularly important to conserve the genetic diversity of valuable, endangered, and small population non-human primates such as the proboscis monkey.

After ejaculation, the semen of proboscis monkeys underwent coagulation, which was similarly reported in other primates (1, 29). This study showed the semen to be generally low quality with many abnormalities, and semen concentrations to be widely disparate between different individual proboscis monkeys. It should be noted that all of the proboscis monkeys used in this study were from all-male groups. This might have affected the quality of the animals' semen, as social hierarchy and conditions have long been known to affect the sexual behaviour of primates (20). It would be interesting and

pertinent to investigate the effect of the presence of females on semen quality. This could be effected by comparing the semen of the studied monkeys to the semen of male proboscis monkeys from mixed-sex groups. The positive effect of female presence on semen quality is known in many mammals (5, 7) but was not observed in the tufted capuchin monkey (*Sapajus apella*) (13). At this point in time, it is impossible to determine whether the presently investigated species of animal naturally has such semen parameters or the findings are related to the anaesthesia, semen collection techniques, and/or influence of their social group composition.

There appeared to be a difference in semen quality between individual proboscis monkeys. However, the current sample size is not adequate to investigate the factors causing the differences in parameters. The factors which could be the causes include the environment, health, social group and hierarchy position, age and feed (20). The effect of the season should be considered in this study since the sampling was conducted over three years in the same individual monkeys. In this study, ID1 would likely be the highest-ranking male of the three studied based on the observation that it was the largest in its group (25), and this individual had better semen quality in terms of volume and progressive motility. It is desirable to collect semen from this male individual for the purpose of ART. However, the success of semen recovery from other males in this study, which may not have been dominant, promises the wider achievability of gamete recovery in the species. Semen collection from males which may have starkly limited opportunities to mate ensures good genetic diversity. Male proboscis monkeys achieved sexual maturity between 5 and 7 years of age and at about 20 kg in previous studies (9, 25). Successful collection from one small and possibly young or sub-

adult proboscis monkey is important in indicating that semen collection can be performed earlier, at the time male proboscis monkeys reach a weight of approximately 19 kg. The success of semen collection in this study suggests that males mature earlier than the literature seemed to indicate and that they could be used as donors of genetic material sooner. Modern ART techniques have also allowed the usage of poorly performing sperm for genetic propagation and management. The newest advancements in ART auger well for the proboscis monkey as its population declines, because it is essential to be able to use semen from all male individuals to widen the genetic pool of the species.

In the wild, the breeding season for proboscis monkeys could be affected by the rainfall pattern altering the abundance of leaves which give nutrients, consequently affecting chewing efficiency and rumination (27). It is logical to investigate the environmental impact on the breeding season of the proboscis monkey.

The sperm of the proboscis monkey has an exceptionally long tail compared to the head and the midpiece. The significant correlation between the tail length and the total length of the spermatozoon indicates that a large part of the total length is comprised by the length of the tail. In primates, it was found that the length of the sperm tail indicates high competition between spermatozoa to fertilise an ovum (16).

Concerning sperm abnormalities, it was observed that a preponderance of them were in the midpiece where bends were seen. A previous study of vervet monkeys showed similar findings of a high number of bent midpieces (22).

Some data for semen evaluation were not available in this study, hindering complete statistical analysis between individual proboscis monkeys. This was due to the discarding of poor quality semen because it was of no value for cryopreservation for future conservation efforts. This is the first report of semen collection, semen quality, and basic spermatology in the proboscis monkey. The data and procedures presented serve as baseline parameters of semen quality collected using electro-ejaculation in anaesthetised wild proboscis monkeys and will be useful for the future conservation of the species.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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Animal Rights Statement: Samples and data were collected according to Sabah Wildlife Department (SWD) protocols, under the supervision of SWD employees. Samples and data were collected as part of

a routine conservation programme for wildlife in Sabah. Samples and data were non-experimental and regulated by the Wildlife Conservation Enactment 1997. The Malaysian Code of Practice for The Care and Use of Animals for Scientific Purposes does not apply to non-experimental procedures. Therefore, Animal Experiment Committee approval was not required.

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