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Short Note

Semen Quality and Electron Microscopy of Captive Irrawaddy Dolphin (*Orcaella brevirostris*) Sperm

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The Irrawaddy dolphin (*Orcaella brevirostris*), a marine mammal in the family Delphinidae, is classified as a critically endangered species under the International Union for Conservation of Nature (IUCN) and the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). The adult female length is approximately 2.1 to 2.2 m, while an adult male can reach up to 2.8 m. They have a round head with a small dorsal fin and a greyish body. Irrawaddy dolphins are estimated to reach sexual maturity at 7 to 9 years of age, and the mating season is observed from December to June in the Northern Hemisphere (Smith, 2009). They feed on fish, shrimp, and small aquatic animals, and they can be found in marine, brackish, and freshwater environments throughout the Indo-Pacific region, including the Irrawaddy River, Maekong River, and Bengal Bay (Smith, 2009). The number of Irrawaddy dolphins is declining in the wild, but some are kept in captivity in Japan, Indonesia, Cambodia, and Thailand (Curry et al., 2013).

Assisted reproductive techniques can help maintain animal numbers in aquaria without impacting *ex situ* populations and can even be used to assist in re-introductions to the wild. Such techniques have been used for captive bottlenose dolphins (*Tursiops truncatus*; Yuen et al., 2009), Pacific white-sided dolphins (*Lagenorhynchus obliquidens*; Robeck et al., 2003, 2009), killer whales (*Orcinus orca*; Robeck et al., 2004), and beluga whales (*Delphinapterus leucas*; O'Brien et al., 2008). Semen collection has been successfully performed by manual stimulation of the genital area combined with positive reinforcement (Schroeder & Keller, 1989). Semen collection and quality assessment is the first step to initiating an artificial insemination program. Nonetheless, the method has never been investigated in Irrawaddy dolphins, an endangered species in urgent need of conservation. The present study develops a semen collection method for Irrawaddy dolphins, followed by a quality evaluation and an examination of sperm ultrastructure using an electron microscope.

Three healthy Irrawaddy dolphins from a dolphinarium in Chonburi Province, Thailand, at the approximate ages of 25 (Irrawaddy 1), 28 (Irrawaddy 2), and 35 (Irrawaddy 3) years old, and with a history of siring calves in captivity, were trained for manual semen collection 15 min per day for 6 months. They were kept in the same oval pool, 45 × 25 m in diameter and 6 m deep. The training began with ordering the dolphins to remain still above the water for routine health checks. They were then instructed to lay in a dorsal recumbency position and were stimulated at the area of the penile opening to expose the penis, ejaculating using positive reinforcement (Schroeder & Keller, 1989; Figure 1). Baitfish was given when successful penis exposure or ejaculation was achieved.

After the 6-month training period was complete, the semen collection for this study was conducted for another 6 months, two times per month. Therefore, a total of 36 attempts (12 attempts per dolphin) were performed. Semen was collected into a 50-ml sterile polyethylene tube with a light

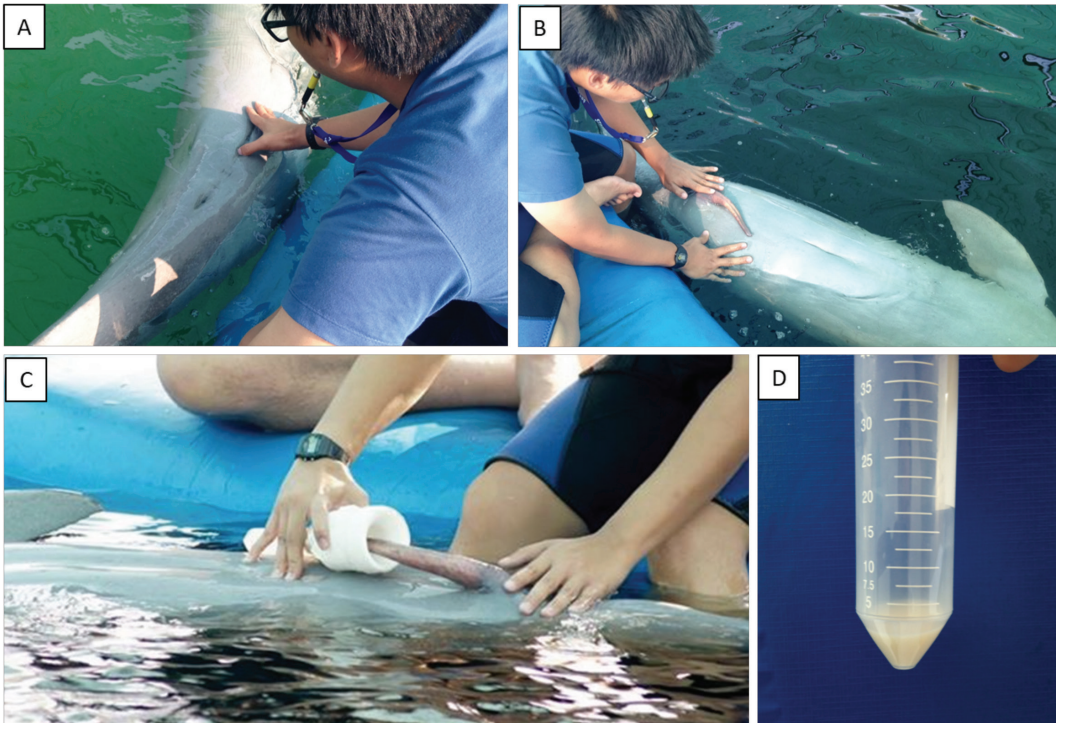

Figure 1. Training Irrawaddy dolphins (*Orcaella brevirostris*) for semen collection: (A) manual stimulation on the penile opening with whistle signaling, (B) protrusion of the penis, (C) ejaculation by dolphin into collection tube, and (D) collection tube with semen.

protection cover. The collection process from genital opening stimulation to ejaculation took less than 5 min per dolphin. During the procedure, the penis was always maintained above the water to avoid seawater contamination, and the first fraction of the semen was discarded. The procedure adhered to the animal use ethic protocol of the Chulalongkorn University Animal Care and Use Committee (CU-ACUC: 1431106).

A standard semen evaluation procedure was conducted, including volume, pH (using pH paper), concentration, osmolarity, and sperm progressive motility. Sperm viability, DNA integrity, and morphology were assessed by eosin-nigrosin (Zilli et al., 2004), toluidine blue (Aksoy et al., 2012), and Papanicolaou staining (World Health Organization [WHO], 2021), respectively. A total of 500 sperms per semen sample were evaluated, and they reported as mean percentages of live sperm, DNA intact sperm, and abnormality in head, midpiece, and tail of the sperm.

Scanning and transmission electron microscopy (SEM & TEM) were used to analyze ultrastructure and to estimate size of the sperm. Semen samples were fixed in 2.5% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.4). For SEM, samples were smeared on a glass slide, fixed in glutaraldehyde, and kept at 0 to 4°C for 8 hours. Then, the slide was washed twice with buffer solution and submerged in 0.1% osmium tetroxide. The slide was dehydrated using methanol before being processed with a Field Scanning Electron Microscope (FESEM; JSM-7610F; JEOL, Tokyo, Japan). For TEM, semen samples were embedded in agar, and 2.5% glutaraldehyde was added when the agar was set. The sample was sectioned to 60 to 90 nm thickness and then stained with uranyl acetate and lead citrate before observing under TEM (JEM-2100, JEOL). Average sperm size was calculated via TEM by measuring 200 individual sperms per sample. The mean percentage of each parameter was analyzed for statistical differences between the dolphins using analysis of variance (ANOVA) with Tukey post hoc tests (*R* software; www.r-project.org).

A total of 15 successful ejaculations were obtained from the 36 attempts—five ejaculations per dolphin. The semen was milky white, the volume was between 3 to 5 ml (mean 3.8 ± 0.8 ml), and the pH was 7.5. Semen quality parameters are presented in Table 1. Sperm concentration was significantly higher in Irrawaddy 1

and Irrawaddy 3, while progressive motility was significantly higher in Irrawaddy 1. Irrawaddy 3 had significantly lower sperm viability and DNA integrity which corresponded to the higher percentage of structure abnormalities.

The sperm ultrastructure observed by SEM revealed that the head was oval and thin. The anterior part of the sperm was tapered with concave edges. The concave edge was smooth up to the midpiece of the body, with the curved crest on the dorsal side of the sperm. The sperm head tapered sharply when observed from the side. The anterior was exceedingly thin with progressive thickening upon the posterior side of the sperm head to the convex edge at the posterior end of the head, which is the connection between the head and the body of the sperm. Mitochondria were observed on the sperm midpiece (Figure 2).

Internal ultrastructure was investigated by TEM. The longitudinal cutting of the head showed a thin and smooth acrosome covering the posterior side of the nucleus. The cross-section of the body revealed the midpiece to be consisting of two microtubules surrounded by nine microtubules. The outer layer was surrounded by dense fiber and mitochondria. The tail consisted of dense fiber, surrounded by exoneme—similar to the main body portion (Figure 3). The average sperm size observed by the electron microscope was not significantly different among individual Irrawaddy dolphins as illustrated in Table 2.

Data on sperm characteristics and semen quality are important for understanding the reproductive biology of a species and evaluating fertilization capabilities. Herein, we used manual stimulation to collect semen from Irrawaddy dolphins—the sixth species in captivity for which this type of collection has been successful. Sperm concentrations varied among the three dolphins (10^8 to 10^9 sperm/ml) and were higher when compared to the reports from other Delphinidae (10^6 to 10^8 sperm/ml), even while other parameters were comparable (Robeck et al., 2003, 2004, 2005, 2009; O'Brien et al., 2008; Yuen et al., 2009; van der Horst et al., 2018). The variation in sperm quality may be due to the differences in species, age, breeding status, and captive management of individual dolphins (van der Horst et al., 2018). In the present study, Irrawaddy 3 had the lowest sperm quality, and this may have been due to the

Table 1. Semen quality of Irrawaddy dolphin (*Orcaella brevirostris*)

	Mean ± SD			
Parameters	Irrawaddy 1	Irrawaddy 2	Irrawaddy 3	Min.-Max.
Sperm concentration (ml)	1.4 ± 0.5 × 109	6.6 ± 1.1 × 108	2.4 ± 1.2 × 109	5.5 × 108 – 3.6 ×109
Progressive motility (%)	76.2 ± 1.7	71.6 ± 1.8	70.4 ± 2.8	67-78
Osmolarity (mOsmol/kg)	355 ± 3.3	357.4 ± 3.7	357.4 ± 3.4	351-361
Viability (%)	98.77 ± 1.13	97.13 ± 0.55	85.97 ± 6.77	79-99
DNA integrity (%)	98.33 ± 0.52	97.47 ± 0.90	89.49 ± 3.29	86-99
Head abnormality (%)	0.33 ± 0.32	2.50 ± 1.05	2.10 ± 1.54	0.5-3
Midpiece abnormality (%)	0.67 ± 0.52	1.67 ± 1.21	5.97 ± 3.41	1-10
Tail abnormality (%)	1.33 ± 1.27	2.50 ± 2.35	5.43 ± 2.88	1-8
Total sperm abnormality (%)	2.33 ± 1.97	6.67 ± 3.33	13.83 ± 5.18	1-18

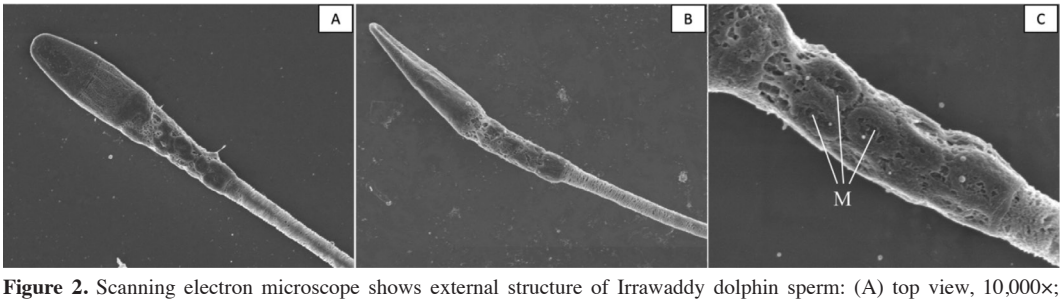

Figure 2. Scanning electron microscope shows external structure of Irrawaddy dolphin sperm: (A) top view, 10,000×; (B) side view reveals thin anterior part with progressive thickening upon the posterior part, 10,000×; and (C) sperm midpiece connecting head and tail containing round-shape mitochondria (M), 30,000×.

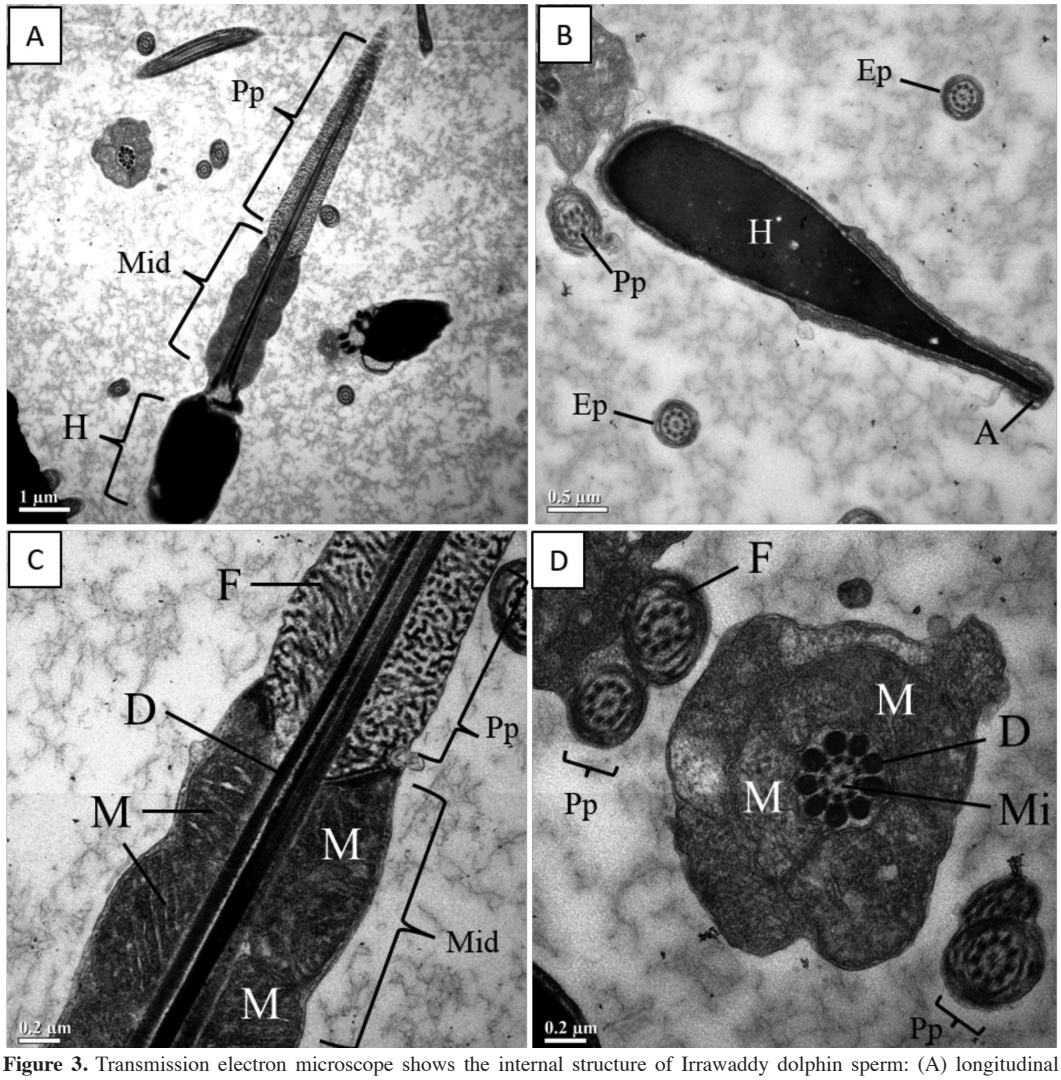

Figure 3. Transmission electron microscope shows the internal structure of Irrawaddy dolphin sperm: (A) longitudinal section of the sperm; (B) longitudinal section of the head with acrosome on the tip of the sperm; cross-sectional section of the sperm principal piece and end pieces are also presented; (C) longitudinal section of the midpiece and principal piece; and (D) cross-sectional section of the sperm midpiece and principal piece. The sperm midpiece contains 9 + 2 arrangement of microtubules surrounded by mitochondria. A = acrosome, D = dense fiber, F = fibrous sheet, H = sperm head, M, = mitochondria, Mid = sperm midpiece, Pp = sperm principal piece, and Ep = sperm end piece.

age of the dolphin. Thirty-five years old is relatively mature given the estimated life span for the species (30 to 50 y; Wildlife Conservation Society [WCS], 2007). Although there is no data for this species, the ages for sexual maturity for bottlenose dolphins are between 10 to 15 years (Cockcroft & Ross, 1990), and 6 to 8 years for Pacific white-sided dolphins (Dierauf & Gulland, 2001).

Like all cetacean spermatozoa, Irrawaddy dolphin sperm have small and short heads with a thicker posterior region than the anterior component (Meisner et al., 2005). The midpiece is one of the shortest midpieces measured in mammals. The mitochondria, however, are large and contain numerous mitochondrial cristae, which likely enable the spermatozoa to swim at a high speed, facilitating a multimale mating system in this species (Kita et al., 2001; van der Horst & Maree, 2014). For sperm size, Irrawaddy dolphin sperm are shorter than that of other Delphinidae (60.10 ± 3.45 µm compared to 69.26 to 74.44 µm), but closer to the sperm of the Indo-Pacific finless porpoise

Table 2. The average sperm size of the three Irrawaddy dolphins (5 samples per dolphin). A total of 200 sperms per sample were measured under transmission electron microscope.

Parameters	Size (μm) Mean ± SD
Head length	4.37 ± 0.18
Head width	1.61 ± 0.14
Tail length	55.73 ± 3.44
Total length	60.10 ± 3.45
Total length: Head length ratio	7.29 ± 0.48%
Total length: Tail length ratio	92.71 ± 0.48%

(*Neophocaena phocaenoides*) (62.72 μm) and Dall's porpoise (*Phocoenoides dalli*) (60.48 μm), both members of Phocoenidae. The head length of Irrawaddy sperm (4.37 ± 0.18 μm) is close to that of members of Delphinidae, including the common dolphin (*Delphinus delphis*) (4.29 μm) and bottlenose dolphin (4.41 μm), while the tail length of Irrawaddy sperm (55.73 ± 3.44 μm) is closer to members of Phocoenidae (56.50 to 59.14 μm) than to other Delphinidae (64.98 to 70.01 μm) (Kita et al., 2001; Plön & Bernard, 2006).

In conclusion, the present study provided comprehensive data on semen collection and assessment, as well as sperm characteristics of captive Irrawaddy dolphins. The method used for land mammals was applicable to this species. The semen quality is acceptable with high sperm concentrations, good motility, and few abnormal sperms. The semen collection and evaluation procedures presented in this study can be a part of a breeding program using artificial insemination of captive Irrawaddy dolphins. Further studies should investigate the preservation protocol for chilling and freezing semen to preserve the genetic material of this species.

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