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Spermiogram of Wild and Captive Malaysian Horseshoe Crab (*Tachypleus gigas*) from Pantai Balok, Kuantan, Pahang, Malaysia

(Spermiogram Belangkas Liar dan Kurungan Malaysia (*Tachypleus gigas*) dari Pantai Balok, Kuantan, Pahang, Malaysia)

HASSAN I. SHEIKH, KAMARUZZAMAN, B.Y.*, AKBAR JOHN, B., ZALEHA, K. & SOLACHUDDIN J.A. ICHWAN

ABSTRACT

Horseshoe crab (HSC) populations around the world are declining in recent decades mainly due to destruction of breeding grounds and habitats. A short-term solution for this problem is captive breeding and artificial rearing. This experiment aimed at looking for a source of good sperm donors for in-vitro *fertilization and captive breeding.* Tachypleus gigas *were collected from Balok Beach, Kuantan, Pahang, Malaysia. Crabs were divided to wild and captive groups. Wild* T. gigas *sperm was collected on the same day, while sperm of captive* T. gigas *was collected after 5 months of captivity to assess captivity effect on sperm traits. Sperm density and sperm viability were studied and correlated to morphometric measurements. The results indicated that* T. gigas *from Balok is a good sperm donor. Captivity was found to affect sperm traits where sperm density dropped significantly and sperm viability increased slightly. Intraocular width to carapace width ratio (IO-Car) of the HSC body correlated to sperm density, hence can be used as an indicator for donor selection during sampling. In conclusion,* T. gigas *from Balok can be used as a sperm donor for* in-vitro *fertilization for* T. gigas *propagation.*

Keywords: Captive rearing; morphometric; sperm traits; Tachypleus gigas

ABSTRAK

Populasi belangkas di seluruh dunia semakin menurun terutamanya akibat daripada pemusnahan kawasan pembiakan dan habitat semula jadinya. Satu langkah jangka pendek untuk mengatasi masalah ini ialah melalui pembiakbakaan kurungan dan pemeliharaan tiruan. Uji kaji ini bertujuan untuk mencari sumber penderma sperma yang baik untuk persenyawaan in-vitro *dan pembiakbakaan kurungan.* Tachypleus gigas *telah diambil dari pantai Balok, Kuantan, Pahang, Malaysia. Belangkas telah dibahagikan kepada kumpulan liar dan dalam kurungan. Sperma* T. gigas *liar telah dikumpulkan pada hari yang sama ia ditemui, sementara sperma* T. gigas *kurungan dikumpul selepas 5 bulan pemeliharaan dalam kurungan untuk menilai kesan pemeliharaan kurungan ke atas trait sperma. Kepadatan dan kedayahidupan sperma dikaji dan dihubungkaitkan dengan ukuran morfometri. Keputusan kajian menunjukkan* T. gigas *dari Balok adalah penderma sperma yang baik. Pemeliharaan kurungan dilihat memberikan kesan negatif kepada trait sperma kerana kepadatan sperma didapati menurun secara signifikan walaupun ada peningkatan minimum kedayahidupan sperma. Nisbah panjang introkular kepada karapas belangkas adalah berhubungkait dengan kepadatan sperma dan boleh digunakan sebagai penunjuk untuk pemilihan haiwan penderma di lapangan. Kesimpulannya,* T. gigas *dari Balok berpotensi digunakan sebagai penderma sperma untuk persenyawaan* in-vitro *bagi tujuan pembiakan* T. gigas.

Kata kunci: Morfometri; pemeliharaan kurungan; Tachypleus gigas*; trait sperma*

INTRODUCTION

Habitat degradation due to anthropogenic activities led to depletion of HSC populations around the world (Carmichael & Brush 2012; Nelson et al. 2016). In Malaysia, HSCs are facing unmonitored harvesting, exposure to pollution and habitat destruction (John et al.2012; Kamaruzzaman et al. 2011). *T. tridentatus* is exported to Thailand for human consumption, while the habitat of *T. gigas* and *Carcinoscorpius rotundicauda* along the east coast of Peninsular Malaysia are severely damaged. *T. gigas*is known to spawn in east coast of Malaysia, specifically at Tanjung Selongor and Pantai Balok, Pahang. Both of these two spawning sites has undergone destruction, for instances, roads and bridge construction at Tanjung Selongor and jetty construction at Pantai Balok has severely limited *T. gigas* spawning activities (Nelson et al. 2016). Captive rearing and releasing of HSC has been established to be a key approach in HSC conservation (Carmichael & Brush 2012; Hong et al. 2009). Captivity has been linked to a number of infectious and non-infectious diseases of HSCs, which cause mortality (Nolan & Smith 2009). Coates et al. (2012) demonstrated how a single parameter such as temperature could cause immunodeficiency in captive HSCs. However, there is a lack of studies on the effect of captivity on sperm traits of HSCs. If captivity is found to have no effect on sperm traits such as density and viability,

then the crabs can be used for induced and artificial fertilization. This study aimed at determining the effect of captivity on the quality and quantity of sperm produced by HSCs and whether there is a correlation between size and morphometric measurements on these sperm traits.

Materials and Methods

A total of 38 *T. gigas* were captured at Balok (latitude 3°58.194‟ N, longitude 103°22.608‟ E), Kuantan, Pahang, Malaysia. The HSCs were carefully inspected for bodily injury of which six injured crabs were returned to sea and the rest of crabs that were considered in healthy state, was transported to INOCM Research Station (IRS), Pahang, Malaysia. The size of remaining 32 samples ranged between 185 and 210 mm (average = 198 mm) perosomal width (PW) and total length (TL) of 295-382 mm (average = 338 mm). Ten crabs were randomly selected andlabelled as 'wild' and used as the control group, while the rest of 22 crabs were labelled as 'captive', tagged with black plastic cable ties on their telson and reared in captivity for five months. Crabs were divided to tanks at density of 2 pairs/square meter. Sperm was collected from wild group on the day they were sampled, while sperm of captive group were collected at the end of the five-month captivity period. The parameters of the rearing conditions were: salinity: 32 ± 1ppt, dissolved oxygen: 7.0±1 mg/L; temperature: 26 ± 2°C; water ammonia concentration: below 0.05 mg/L. These parameters were checked 2 or 3 times a week. The water was changed every day, while sand bedding was used to mimic the natural environment of the HSC and was cleaned/replaced every 2-3 days. The aquarium diameters were 92" × 48" × 30" (L × W × D). HSCs were fed bivalves (*Meretrix meretrix*) at 3% body weight which was equivalent to 2 clams/crab/day (Akbar John et al. 2017).

Sperm Collection

HSCs were placed with dorsal side down and ventral side facing up. The sperm collection was carried out via lifting the opercular flap and massaging the two gonophores. The ejaculated semen (at least 10 µL) was collected from the gonopores using micropipette. The semen was pipetted into Eppendorf micro-centrifuge tubes containing HEPES buffer in a ratio of 1:49 (Sasson et al. 2012).

Spermiogram

Sperm viability and density of wild and captive crabs were investigated according to Fatihah et al. (2016) method with slight modification. For determining sperm viability, sperm in the semen-HEPES solution (100 µL) was stained using 10 µL eosin (5%). Serial dilution was carried out using the HEPES buffer. Sperm density was recorded as a measure of sperm concentration in 1 mL of ejaculate. Sperm viability was determined as the ratio of live to dead spermatozoa. The viable (live) spermatozoa appeared as unstained against red-stained background, whereas dead spermatozoa appeared dark/black. Stained semen sample (10 µL) was counted using an improved Neubauer haemocytometer. Microscopic examination was done using Nikon ECLIPSE DS-Ri1 microscope (NIS Element imaging software V 4.13) bright field settings at 40× magnification.

Morphometric Analysis

Total length (TL), carapace width (CW), inter-ocular width (IO) and intraocular width to carapace width ratio (IO-Car) were recorded using Vernier calliper. Morphological measurements of *T. gigas* according to Chen et al. (2010) method.

Data Analysis

Shapiro-Wilk normality test showed that sperm density of captive group (df= 10; p=0.353) as well as sperm viability of wild (df= 10; p=0.067) and captive (df= 10; p=0.094) groups were not normally distributed, hence, non-parametric tests were used. Mann-Whitney test was used to compare captive and wild groups. Spearman correlation was used to analyse the relationship between groups, morphological parameters and sperm traits. All data were expressed in mean ± SD and statistical significance between captive and wild HSCs were expressed as 95% confidence level (p<0.05) or 99.9% confidence level (p<0.001).

Results and Discussion

Wild and captive HSCs showed significant difference in terms of sperm density (F = 1559.035, df = 1; p<0.001) and viability (F = 53.950, df = 1; p<0.01). Moreover, there was an inverse correlation between sperm density and viability. Wild crabs had approximately 10 times more spermatozoa concentration (10.68×10^6 ± 2.41 sperm/mL) compared to captive group (1.649×10^6 ± 0.03 sperm/mL), while the sperm viability of captive group (87 ± 2.45%) was higher than wild group (68.99 ± 5.38%).

A recent study on American HSC (*Limulus polyphemus*) reported sperm density of 0.3-1×10^{10} sperm/mL (Sasson & Brockmann 2016). This was expected as the size of American HSC which is much bigger than Asian counterparts affects sperm traits. Asian HSC such as *C. rotundicauda* was reported to have 3-23×10^6 sperm/mL (Hajeb et al. 2009), compared to wild *T. gigas* in this study which ranged from 4 to 13 $\times10^6$ sperm/mL). Sasson et al. (2012) had also reported that body size affects sperm quantity. Sperm viability of wild crab though was relatively low at 68.99% compared to sperm viability of American HSC (86-89%) reported by Sasson et al. (2012). However, sperm traits of wild *T. gigas* from Balok indicated that these males could be a good sperm donor for artificial and induced fertilization.

Captivity was shown to have significant effect on sperm traits, where very significant drop in sperm concentration and a slight increase in sperm viability were

TABLE 1. Sperm traits of wild and captive *T. gigas*

T. gigas Group	Density (Mean ±SD) (10^6)	Viability (Mean ±SD) (%)	IO-Car* ratio (Mean ±SD) (%)
Captive	1.65 ±0.03	87.00 ±2.45	39.75 ±1.7
Wild	10.68 ±2.41	68.99 ±5.38	39.98 ±1.31

*IO-Car: intraocular width to Carapace width ratio

recorded. Sasson and Brockmann (2016) had shown how different site significantly affected sperm traits. Sasson et al. (2012) found that season also have significant effect on sperm concentration, where HSCs collected in spring 2010 had much less sperm count compared to those sampled in autumn and spring of 2008 and 2009. Therefore, the drop in sperm count could be related to the change in diet and environmental parameters of captive *T. gigas* in this study. These results indicated that crabs kept in captivity could also be a sperm donor, however, other sperm traits must be studied such as sperm velocity and biochemical contents such as zinc that affects motility and attachment (Eisler 2009; Sasson et al. 2012).

Morphometric analysis of captive and wild *T. gigas* were carried out to try and correlate one or all morphometric parameters to sperm traits. Total length (TL), Carapace width (CW), inter-ocular width (IO) and intraocular width to Carapace width ratio (IO-Car) were analysed. Previous study (Sasson et al. 2012) reported that ejaculate size had weak positive correlation to carapace width ($r = 0.594$, $p = 0.07$) which might be significant at slightly higher sample number. In this study, despite the small sample number (10/group), sperm density wild *T. gigas* was significantly and negatively correlated to IO-Car ratio (Spearman Correlation: -0.705, $p<0.05$). This might be due to fact that larger animal might be much older. Older horseshoe crabs were reported to have less ejaculate size, less sperm concentration; lower sperm/ejaculate, lower sperm velocity and viability compared to younger ones. This was attributed to their body diverting most of the energy and resources towards maintaining cell function and survival, rather than producing sperm (Sasson et al. 2012). Previous studied established that PW of 154-194 (mm) have survived between 10 and 11 years and reached 14th instar, 194-244 (mm) are 11-12 years old and in their 15th instar, while male *T. gigas* that exceeded 244 mm are in their 16th instar and survived for more than 12 years (Hajeb et al 2005; Tan et al. 2012). Hajeb et al. (2005) reported that 70% of male *T. gigas* at Balok are in their 14th instar. Seven years later, Tan et al. (2012) reported that 64% only were in their 14th instar, while 32% already reached 15th instar. Tan et al. (2012) also reported that a total of 45% of the *T. gigas* males were already infested with epibiont, a sign of aging. In this study, only 30% of male *T. gigas* were in their 14th instar (belonged to the age group 194-244 mm), while 70% had reached 15th instar. Therefore, *T. gigas* males in this study can be considered as old.

Morphometric parameters could be used to select wild males to be sperm donors, which can reduce the number of crabs per sampling trip. Sperm count and IO-Car ratio can be correlated using this equation:

$$y = -0.0122x + 2.1338$$

where y is sperm count; and x is IO-Car ratio.

CONCLUSION

Wild *T. gigas* from Balok, Pahang were found to poses good sperm count (4 to 13 × 10^6 sperm/mL) and can be good sperm donors for *in-vitro* fertilization experiments. Captivity was found to affect sperm traits, while intraocular width to carapace width ratio (IO-Car) can be used to select individual sperm donors. Other morphometric parameters were not significantly correlated to sperm density of wild *T. gigas*. Younger males (14th instar, prosomal width = 194-244 mm) were also found to produce more sperm.

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Hassan I. Sheikh
School of Fisheries and Aquaculture Sciences
Universiti Malaysia Terengganu
21030 Kuala Nerus, Terengganu Darul Iman
Malaysia

Kamaruzzaman, B.Y.* & Zaleha, K.
Department of Marine Science
Kulliyyah of Science, International Islamic University Malaysia
Jalan Sultan Ahmad Shah, Bandar Indera Mahkota
25200 Kuantan, Pahang Darul Makmur
Malaysia

Akbar John, B.
INOCEM Research Station, (IRS)
Kulliyyah of Science, International Islamic University Malaysia
Jalan Sultan Ahmad Shah, Bandar Indera Mahkota
25200 Kuantan, Pahang Darul Makmur
Malaysia

Solachuddin J.A. Ichwan
Department of Basic Medical Sciences
Faculty of Dentistry
International Islamic University Malaysia
Jalan Sultan Ahmad Shah, Bandar Indera Mahkota
25200, Kuantan, Pahang Darul Makmur
Malaysia

*Corresponding author; email: kama@iium.edu.my

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