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Reproductive biology and histological profiling of maturity stages in Hilsa shad, *Tenualosa ilisha* (Teleostei: Clupeidae) in the mouth of Perak waters, Malaysia

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Abstract
This study was the first study that was conducted to evaluate the reproductive biology and histological profiling of this *Tenualosa ilisha* off Bagan Datoh, Perak waters. 1,174 samples have been collected at Bagan Datoh jetty since March 2017 to December 2019. Information on length-weight and ovary weight were recorded. Ovaries were grouped into four stages morphologically and histologically. The stage IV ovaries from 21 samples were subjected to fecundity assay whereas another 200 ovaries were subjected to histological assay. The ration of male to female sample was 2.3: 1. Only 21.5% of female and 11% of male sample reached the length at first maturity (Lm) size. Length weight relationship of female and male were given by equation BW = $0.00002FL^{3.0162}$ (isometric) and BW = $0.00005FL^{2.8066}$ (negative allometric). Results revealed that spawning season for Hilsa shad is in March - May with the fecundity of 172,951 to 1,219,040 oocytes.

Keywords: Reproductive biology, histological, spawning season, fecundity

1. Introduction
Terubok is a Malaysia local name for Tenualosa spp. Terubok was classified under order Clupeiformes and family Clupeidae. There are five species under Tenualosa spp which are *Tenualosa macrura* (Bleeker, 1852), *Tenualosa toli* (Valenciennes, 1847), *Tenualosa ilisha* (Hamilton, 1822), *Tenualosa reevesii* (Richardson, 1846) and *Tenualosa thibaudeaui* (Durand, 1940) [1]. Tenualosa spp. have protandrous hermaphrodite characteristic [2] where they were born as male and was transform to female after certain stage of maturity. They also show anadramous characteristics [2, 3] where they migrating up to the rivers from sea to spawn. However, there is a record where they also demonstrate amphidromy characteristics (migration of immature fish for non-spawning purposes) [4]. Tenualosa spp. have been discovered as one of the highly commercial fish species in Malaysia due to their unique taste. According to landing statistics by Department of Fisheries Malaysia [5], Tenualosa spp. landings were recorded only in Perak, Selangor, Sarawak and Sabah. Statistics since 2013 to 2018 shows that the highest landing was in 2015 with 2,837 metric tonne per year. However, the landing was declined in 2016 (1,846 metric tonne) and continuously declined in 2017 (1,334 metric tonne) before slightly increase in 2018 (1,580 metric tonne) (Fig. 1). The declination of Tenualosa spp. resources in Malaysian water show the need of proper fisheries management plan for this species, which require adequate information on this species.
In Malaysian waters, two species; *T. macrura* and *T. toli* have been recorded in Borneo [6]. In 2014, a study by Arai and Amalina [7] recorded the first occurrence of *T. ilisha* in Malaysia waters, specifically in Perak area. This finding have been proved by a study by Arjunaidi *et al*., [8] that terubok in Perak waters is *T. ilisha* after examining the morphometric, meristic and molecular genetic of terubok, which is the same species that were found in India, Bangladesh, Myanmar, [9], Oman, Pakistan. Vietnam, Thailand, United Arab Emirates, Pakistan, Oman, Saudi Arabia, Qatar and Sri Lanka [10].
Since *T. ilisha* or commonly known as Hilsa shad recently discovered in Perak area and there is no information on resources status, biological and reproductive characteristics of this species

thus, a comprehensive study on this species are required. Study will provide information that are crucial for estimating *T. ilisha* population and assessing the risks associated with its exploitation rate for implementation of proper fisheries management plan. Thus, a study was conducted to evaluate the reproductive biology and histological profiling of Hilsa shad, *T. ilisha* in the mouth of Perak waters.

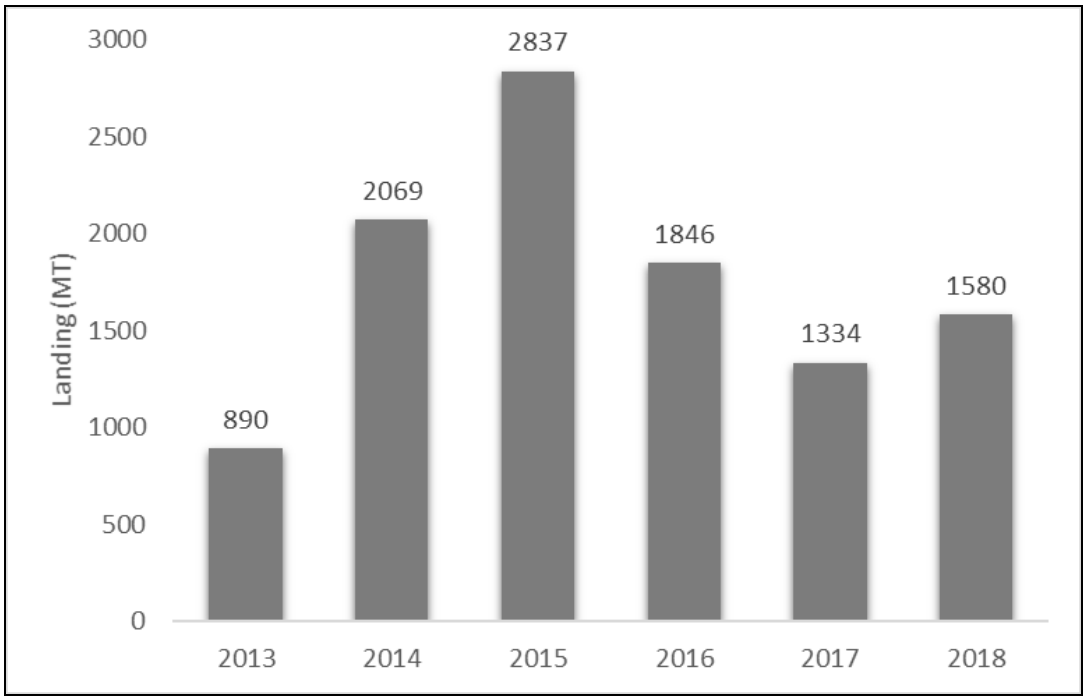


Fig 1: Annual landing of Tenualosa spp. in Malaysia water (state of Perak, Selangor, Sarawak and Sabah) from 2013 to 2018 [5].

2. Materials and Methods

2.1 Sampling activities

The sampling was carried out every month since March 2017 to December 2019, which covered Bagan Datoh area, which is the area at the mouth of Perak waters. A total of 1,174 samples were purchased at jetty and were brought back to laboratory to perform biological study. The total length lengths (TL) and body weight (BW) of each sample was measured to the nearest mm and g. Samples were then dissected and the maturity stage were determined according to the gonad size. Gonad weight were measured and recorded. The stage IV ovaries from 21 mature female *T. ilisha* samples were removed and weighed prior preservation in 10% formalin until used for fecundity assay. A total of 200 gonads were collected for histology assay where a piece of fish gonad tissue was removed and place in formalin for 48 hours and then stored in 70% ethanol.

2.2 Length-weight Relationship

Samples that comprises of 799 males and 349 males were then subjected to length-weight relationship analysis. The relationship analysis between fork length (FL) and the body weight (BW) of fishes was estimated using the equation as described by Flura *et al*., [11].

$BW = aTL^b$

Where,

BW = Body weight of fish in gram (g)

FL = Fork length of fish in centimetre (cm)

a = Constant (intercept)

b = An exponent indicating isometric growth when equal to 3

2.3 Gonadosomatic Index (GSI)

Gonad somatic index (GSI) were calculated to determine the reproductive cycle of Hilsa species for the year round with monthly intervals. The GSI can be calculated using formula; GSI (%) = Gonad weight (g) x 100/ Body weight (g). According to Zhang *et al*., [12], the spawning seasons could be determined based on the monthly changes of GSI indices and proportions for determine maturity stage.

2.4 Fecundity assay

Eggs from 21 Hilsa shad samples which reach maturity stage IV were preserved into 10% formalin. For the fecundity assay, a sub sample of 0.1 mg ripe ovary was weight using an electronic balance. The sub-sample was then taken from three different parts of ovary including anterior, middle and posterior part and were counted physically on glass plate. The average was computed and the fecundity was calculated by the formula given by Sinha (1995) [13].

F = OW x S/ ws

F = Fecundity

OW =Weight of ovary

S = Number of ova in the sub sample

ws = weight of sub sample

The relation between fecundity and total length, body weight and weight of ovaries were calculated and was presented in a scatter plot.

2.5 Histological study of gonad

A total of 200 samples of gonad were subjected to histological assay. The histological profiling was performed according to method described in Shihab *et al*., [14]. The sample tissues were washed and cut into smaller pieces. Three sub-sample of tissues were taken from anterior, posterior and middle part of tissues. The sample tissues were then inserted into histology

cassette and were labelled. The cassette containing tissues were washed in tap water for about 6-8 hours before load to an automatic tissues processor machine and passed through different grades of isopropyl alcohol, xylene and finally embedded in paraffin wax. The embedded tissues were moulded into blocks for sectioning. The blocks were trimmed and transverse sections of 3-5 µm were obtained using a semi-automatic rotary microtome. The sections were mounted on slides and were stained with hematoxylin and eosin. The prepared slides were then observed under a compound microscope. Histological sections were used to confirm maturity stages of Hilsa shad.

3. Results and Discussion

3.1 Sample Distribution

Over all sex-ratio of collected samples was in the favour of males with the ration of 1:2.3. The male to female ratio with favour to male also have been reported in a study of *T. ilisha* in Godavari River and Indus River, Pakistan with ratio of 1:9 and 1:3, respectively [15, 16]. The mean length for male sample was 246.20 mm whereas in female sample was 274.75 mm. This result clearly shows that the mean length of female was higher than male. This finding is consistent with the results that were reported by Roomiani *et al*., [17] & Ahmad and Saha [18].

3.2 Size Distribution of collected samples

Figure 1 and 2 reflect the position of *T. ilisha* for female and male sample before or after the length at first maturity captured through drift net, trammel net and trawlers. Length at first maturity was calculated using Spearman-Karbar formula as given by Udupe [19]. From the analysis, the length at first maturity (Lm) for female sample is 300 mm whereas for male sample is 273 mm. Approximately 79.5% female sample were not reach length at first maturity. The same condition also can be observed in male sample where only 14.5% sample reach length at first maturity. The mean length of female sample is 274.75 mm whereas the mean length for male sample is 246.20 mm.

The present result of Lm is close to the result that was reported by Hossain *et al*., [9] where the maturity size of male Hilsa shad is at size range of 260-290 mm whereas the female is at size range of 310-330 mm. Out of total collected samples, approximately 79.5% female sample were not reach length at first maturity. The same condition also can be observed in male sample where only 14.5% sample reach length at first maturity.

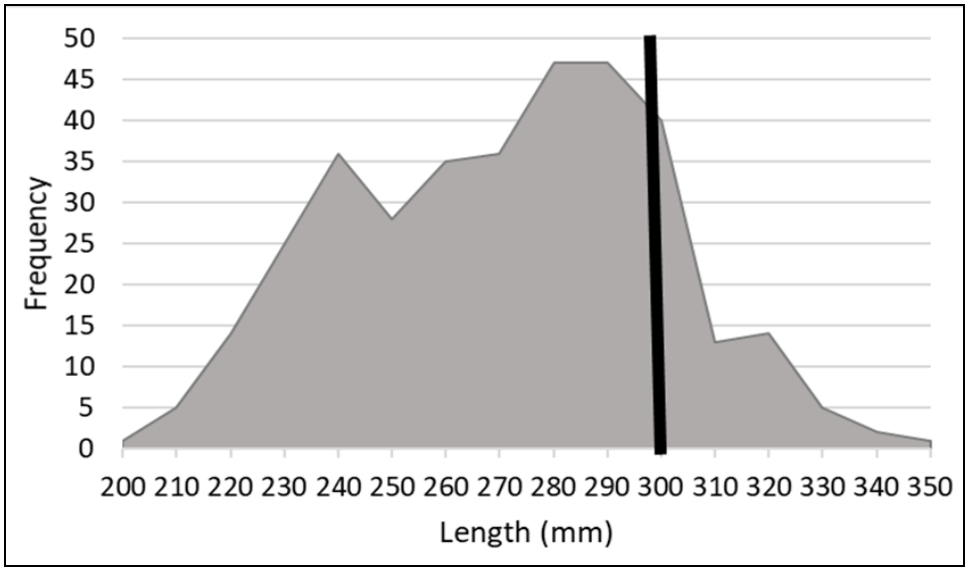


Fig 1: Length frequency distribution for female sample of *T. ilisha*. The size at maturity (Lm = 300 mm) throughout study period (2017-2019).

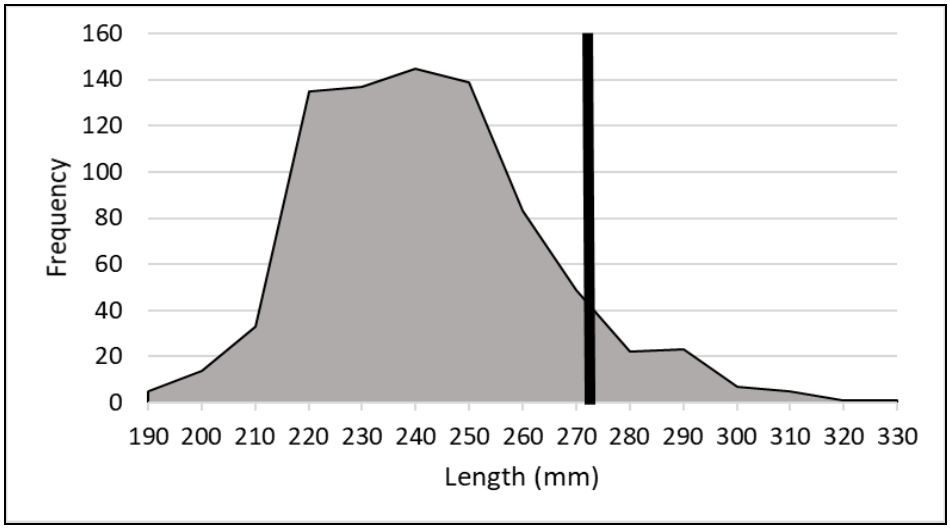


Fig 2: Length frequency distribution for male sample of *T. ilisha*. The size at maturity (Lm = 273 cm) throughout study period (2017-2019).

3.3 Length-weight Relationship

A total of 1,174 (799 males, 349 females and 26 juvenile) sample were collected throughout the study period (2017-2019) with the range size from 180-356 mm in the fork length (FL) and 97-787 g in total body weight (BW) were collected and analysed. A fork length and total body weight relationship of hilsa fish collected during the study periods were given by power relationship of $BW= 0.00002TL^{3.0162}$ ($R^2 = 0.9369$) for female sample (Figure 3) and $BW= 0.00005TL^{2.8066}$ ($R^2 = 0.9001$) for male sample (Figure 4).

Length-weight relationship analysis shows that for both sexes, there is a strong relationship between fork length and total body weight in the samples and the positive value of the constant reflects that the slope is positive. This result suggests that the increasing of fork length will enhance the increment in body weight, vice versa. The parameters 'b' in the power curve equation of female is 3.016, indicates that the growth type of Hilsa female sample is isometric. However, the growth in male sample indicates the negative allometric growth pattern with the parameter 'b' value = 2.8066. These results are in agreement with study in Pakistan that was reported by Pahnwar *et al*., [20]. The relationship of fork length and body weight was statistically significant with confidence interval of 95% with R^2=0.9001 in male and 0.9369 in female (p<0.05). The value of coefficient of determination explained that 90.01% and 93.69% of variation in body weight was due to the variation in total length in the sample of hilsa shad that was collected throughout the study periods.

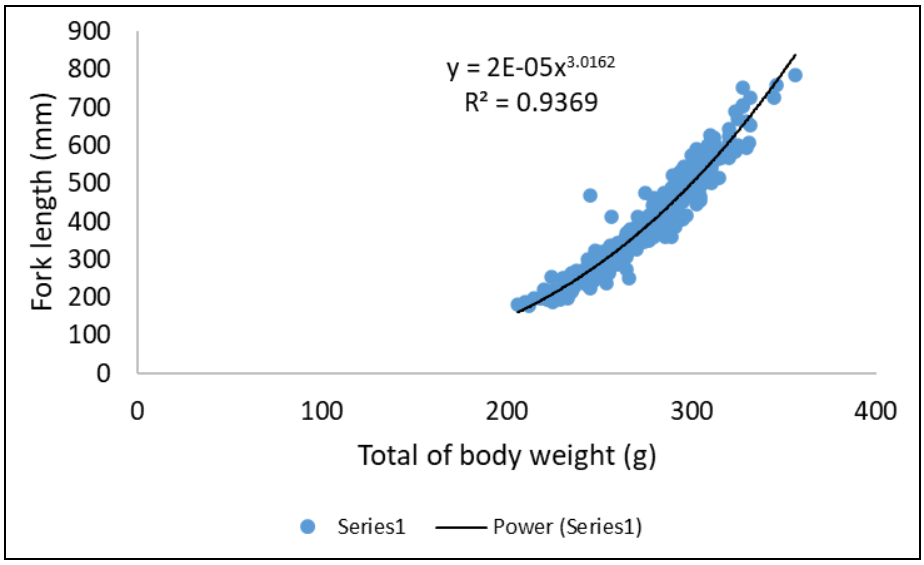


Fig 3: The generalized relationship between total body weight (g) and total fork length (mm) in the female *T. ilisha* in Bagan Datoh, Perak

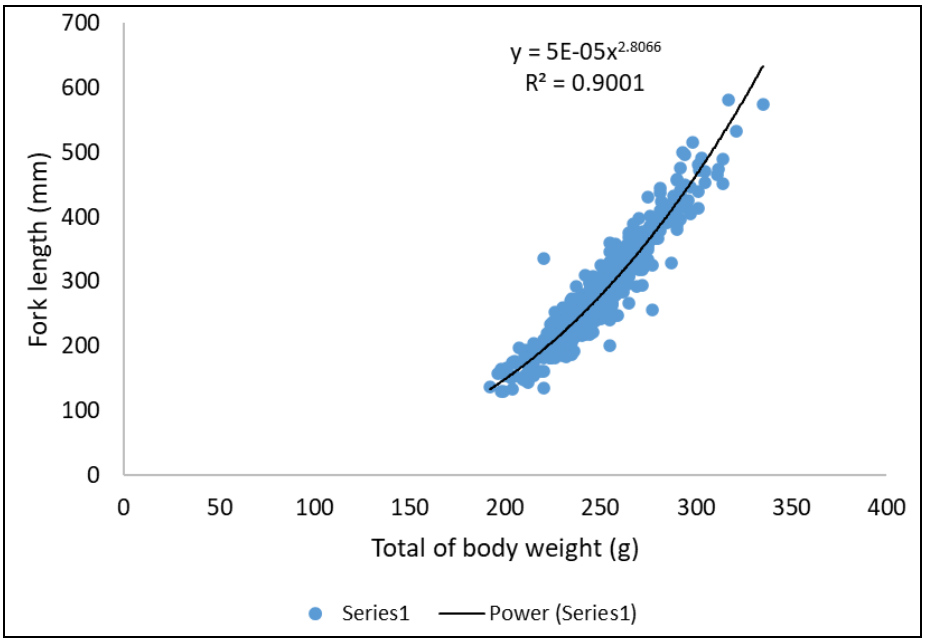


Fig 4: The generalized relationship between total body weight (g) and total fork length (mm) in the male *T. ilisha* in Bagan Datoh, Perak

3.4 Gonadosomatic (GSI) indices

The GSI value of male samples ranged from 0.51 to 1.31 whereas the GSI value for female samples ranged from 0.45 to 10.15. The male GSI value recorded the highest value in Jun with the GSI of 1.31 in May 2017 while for female the highest value of GSI in December 2018, 10.15. From the results, female gonads are relatively larger compared to male and the size of gonad varies among female individuals. This result is supported by a study by Bladon *et al.* [2], which reported that this condition happened according to maturity and reproductive cycle. Our result shows that the GSI of female samples consistently higher than male GSI throughout

the study period except for Oct 2017 and Jun 2018. Analysis of GSI revealed that the spawning peak is in Mar- May (Figure 5). The analysis on GSI of the present study are almost similar with the result of the previous study in Myanmar that was reported by Bladon *et al.*, [2] which revealed that the spawning season for female Hilsa shad is from April to May.

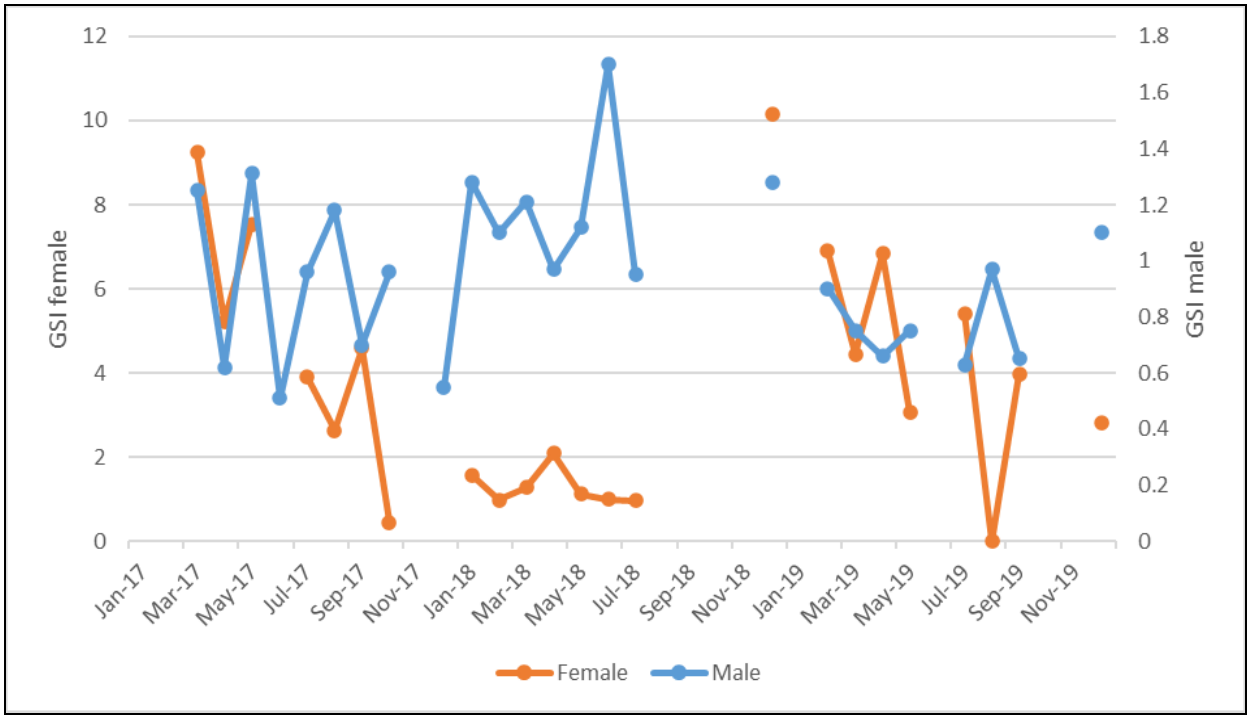


Fig 5: Gonadosomatic index of both female and male samples throughout study period (2017-2019)

3.5 Fecundity

Fecundity is one of the important aspects of reproductive biology of fish species which provide information on the number of mature ova laid by a female in one spawning season [21, 22]. Only the ripen ova were considered for estimation of fecundity. Thus in this study, only ova from stage IV onwards was taken into account. The absolute fecundity of *T. ilisha* in Perak water ranged from 172,951 (295 mm, 477. 1 g) to 1,219,040 (328 mm, 704 g). This result showed that this fish species is a high fecund fish which can reach millions of eggs per female. The minimum body weight of mature *T. ilisha* was 439 g and the maximum weight was 704 g with body length ranged from 280 to 332 mm.

The fecundity of *T. ilisha* varies among studies where previous study in 1958 reported the fecundity of fish with length of 253 to 481mm from Hooghly estuary was in a range of 250,000 to 1,600,000 ova [23]. Another study that was reported by De [24] showed that the fecundity of *T. ilisha* are in a range of 373, 120 to 1,475, 676 ova. Study in 1959 and 1964 at Ganga river system recorded the fecundity *T. ilisha* was in a range of 289,000 to 1,168, 622 (length= 315 to 506 mm) and 316, 316 to 1,849,179 (length= 310 to 436 mm) ova, respectively [23; 24]. Pahnwar *et al.*, [20] reported that the average fecundity of *T. ilisha* is 113,483, 382,105 and 572,709 eggs for the respective size class of 200-250 mm, 251-300 mm and 301-350 mm. The recent study on *T. ilisha* that was reported in Southern Al Hammar Marsh Basra, Iraq revealed that the fecundity of this species was in a range of 223,750- 1,477,780 oocytes [27]. Comparing the fecundity values that reported by present study with previous study, the fecundity was strongly influenced by size of fish.

Table 1: Relationship of fecundity with length and weight of *T. ilisha*

Total length (cm) Size group	Average Body weight (g)	Average fecundity
28-28.9	464.5	408,568
29-29.9	499.0	373,747
30-30.9	552.5	482,576
31-31.9	566.4	711,380
32-32.9	649.8	667,644
33-33.9	654.0	441,000

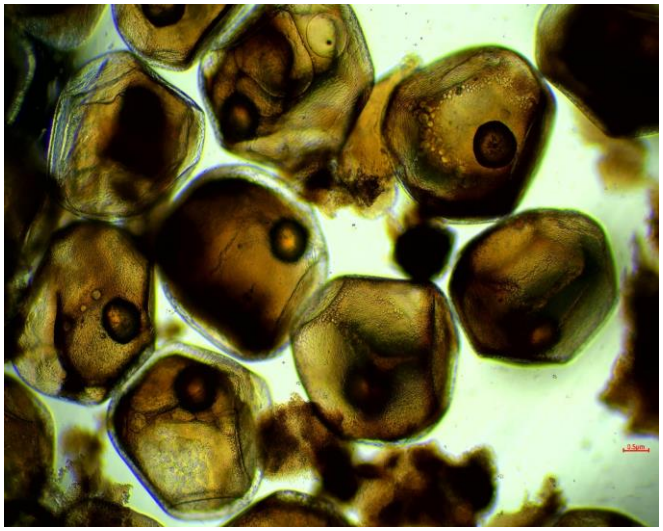


Fig 6: Microscopic image of Hilsa shad oocyte under 4X magnification

3.5.1 Relation between fecundity and weight of *T. ilisha*

The weight and fecundity relationship of *T. ilisha* showed that the number of ova is directly proportional with body weight (Fig.7). However, slight variations in fecundity with respect to weight were detected. Analysis of logarithmic values of fecundity against logarithmic values of weight showed a linear relationship between these two variables with the relationship presented by equation y= 2.0569x + 0.0118, where y= log total fecundity and x= log body weight of *T. ilisha*. The correlation co-efficient "r" of these two variables was at 0.2887.

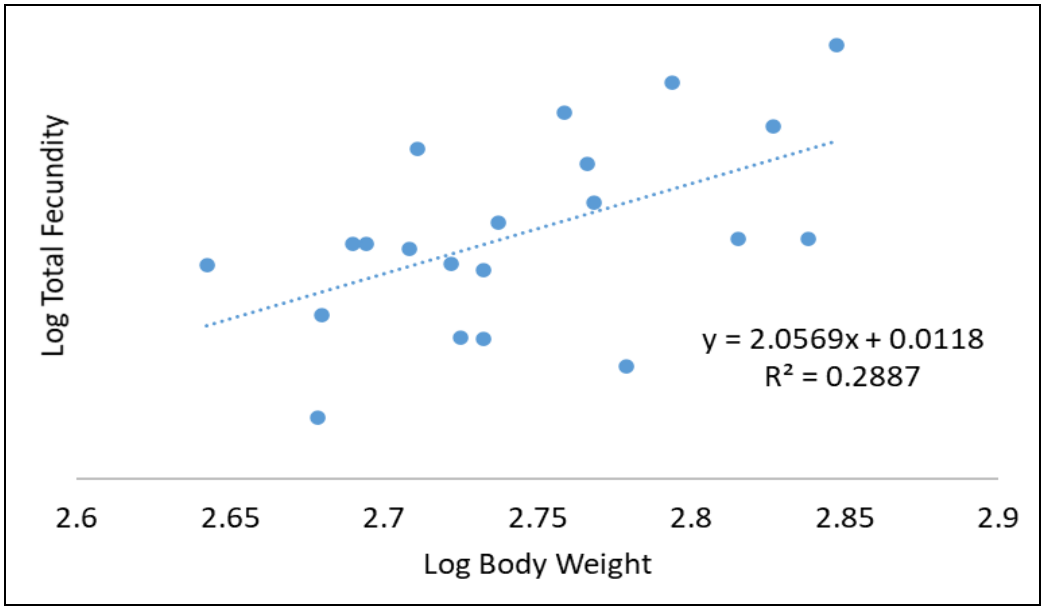


Fig 7: Logarithmic weight and fecundity relationship of *T. ilisha*

3.5.2 Relation between fecundity and length of *T. ilisha*

Analysis of relationship between fork length and fecundity also showed the same pattern where the number of ova increases with the increase in length (Fig.8). Logarithmic values of fecundity against logarithmic values of length also showed a linear relationship with the relationship equation of y= 3.7456x + 3.6545, where y = log total fecundity and x = log fork length of fish. The correlation co-efficient "r" of these two variables was at 0.1265.

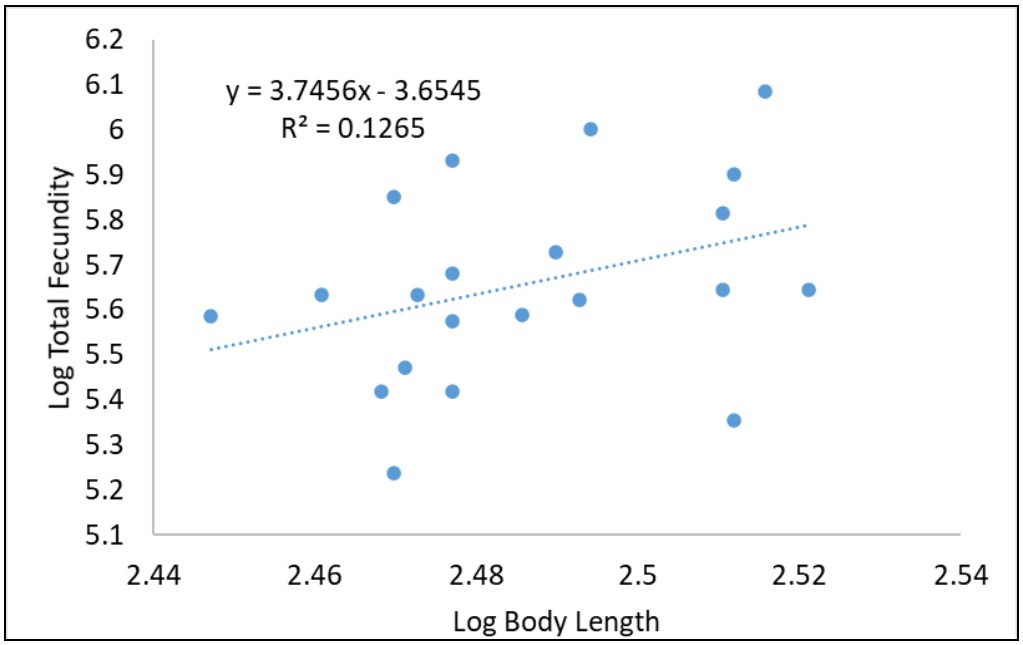


Fig 8: Logarithmic length and fecundity relationship of *T. ilisha*

3.5.3 Relation between fecundity and ovary weight of *T. ilisha*

Analysis of relationship between ovary weight and fecundity also showed that fecundity was directly proportional to ovary weight where the number of ova increases with the increase of ovary weight (Fig.9). Logarithmic values of fecundity against logarithmic values of ovary weight also showed a linear relationship with the relationship equation of y= 1.0138x + 3.9719, where y = log total fecundity and x = log total ovary weight of *T. ilisha*. The correlation co-efficient "r" of these two variables was at 0.6462.

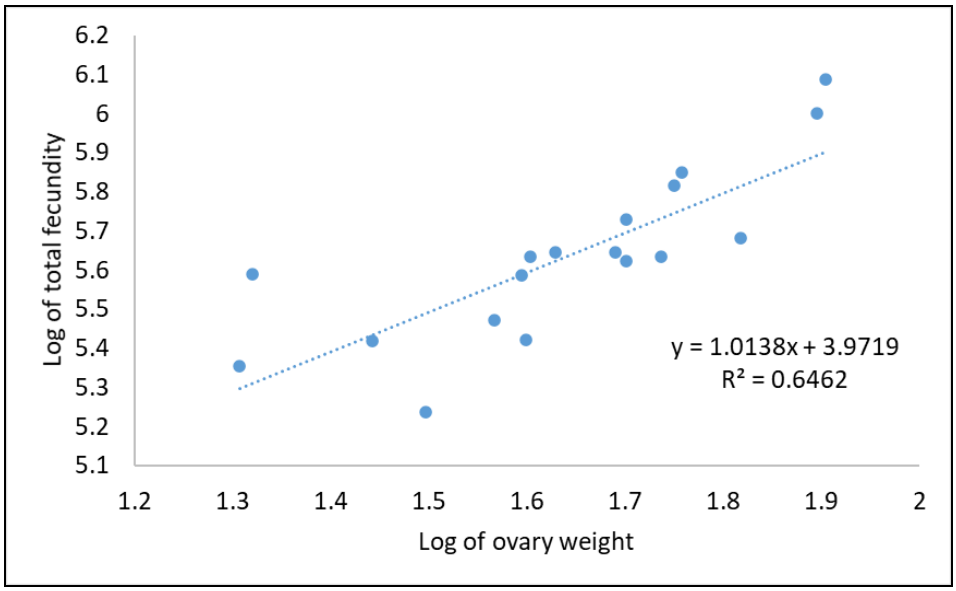


Fig 9: Logarithmic ovary weight and fecundity relationship of *T. ilisha*

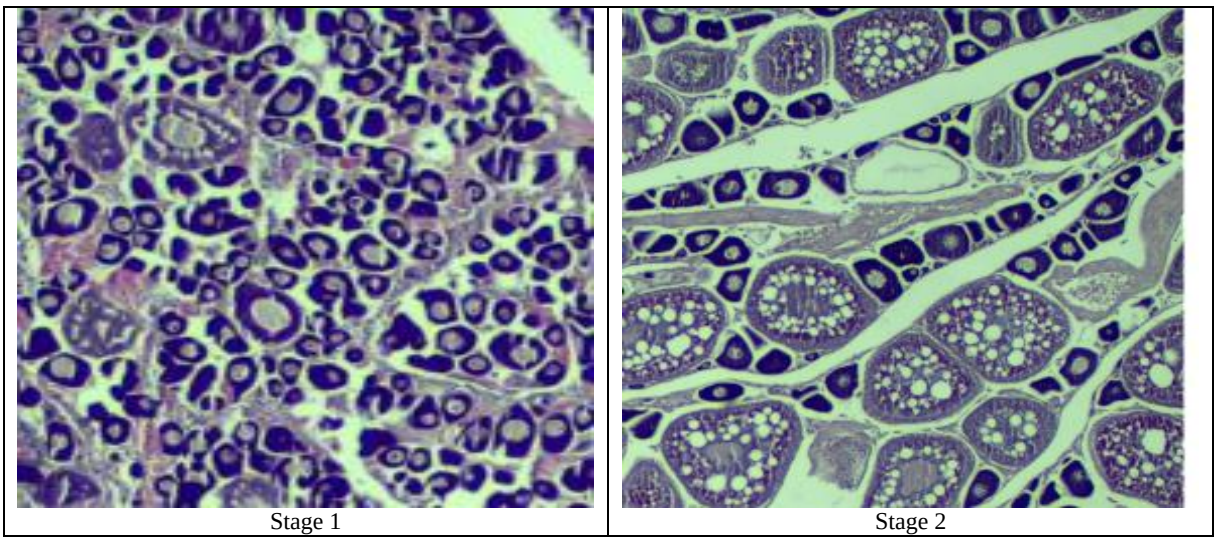
Relationship analysis show that among the three parameters, fecundity was observed to be most closely correlated with gonad weight (r = 0.6462) followed by body weight (r = 0.2887) and body length (r = 0.1265). Thus, this suggested that the gonad weight is a better index of fecundity rather than total weight and length. Bahuguna and Khatri [28] have reported similar result in a study on hill stream loach. However, in previous study, Jabeen *et al*., [29] reported that fecundity was more dependent on fish length compared to gonad weight, which did not correspond with these findings. Thus, there must be some factors that might have resulted in such variation. Hence, further study on another parameter such as food availability and geographical region need to be performed to further clarify the potential reproduction of this species

Despite of body weight, length and gonad weight, the variation of egg production among individual can be seen due to the age of individual as older conspecifics has tendency to spawn over longer periods of time than younger conspecifics [30]. Another factor that also affect the variation on fish fecundity include availability of space, food and geographical region [31].

3.6 Histological structure of *T. ilisha*

Histological studies give the most reliable and objective data in gonad staging and they are also significant in determining the maturation cycle. Histological, anatomical and physiological studies have been performed in order to expose the reproductive strategies of many fish species especially for anadromous species like *T. ilisha*. The aim of the histological study is to obtain more detailed information about the reproductive biology of *T. ilisha* and also to examine the seasonal gonad differentiation. Ovaries were divided into four stages morphologically and histologically which are: A. developing (Stage 1 & 2), B. maturating (Stage 3 & 4), C. spawning, D spent and redeveloping stage. In the present study, we managed to observed developing stage (stage 1 and 2) and maturating stage (Stage 3 & 4).

According to previous study by Rahman *et al*., [32], Hilsa have been grouped into four size groups; small (<30 cm), medium (30-39 cm), large (40-49 cm) and extra-large (>50 cm). In the present study, *T. ilisha* samples that were collected were fall in only two groups; small and medium which range between 28-33.9 cm in length. This result explained on the reason of only 2 stages out of 4 stages; developing and maturating stages were observed. This is due to the life stage of *T. ilisha* is dependent on the body size and oocyte size. Analysis that was performed showed that oocytes diameter increased significantly during gonadal development as highest value 360.11 um ± 38.72 was recorded in vetilogenesis stage.



Stage 1 Stage 2

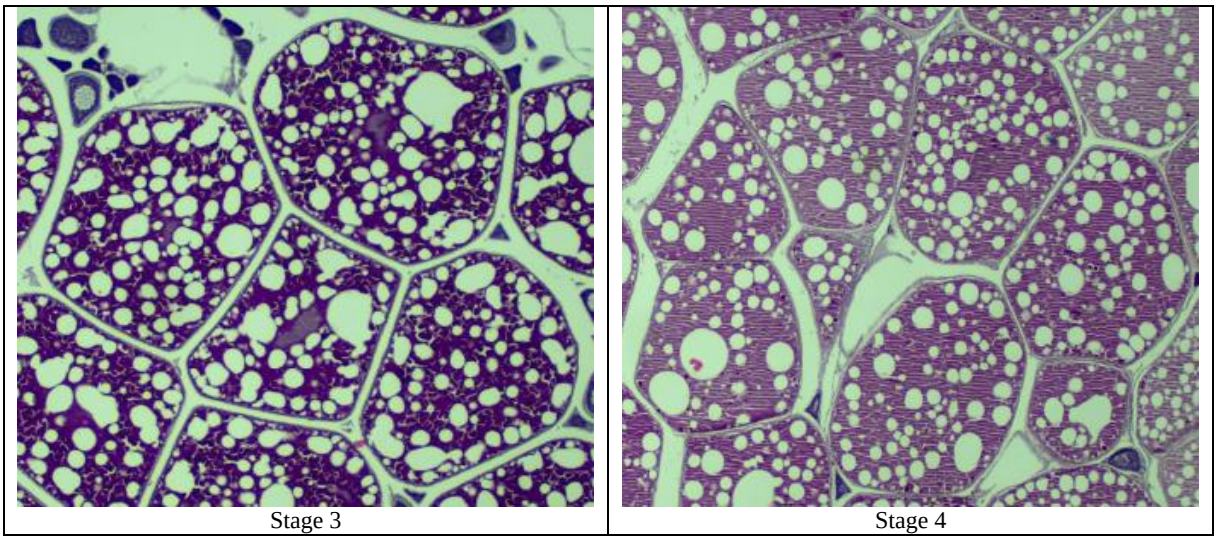


Fig 10: The histological observations of oocytes and determination of development stages of *T. ilisha* A) Developing, (stage 1 & 2) B) Maturating, (stage 3 & 4)

Significant differences can be seen in oocytes when grouped by stage as shown in the figure 11 below. Oocytes from the stage 4 group were the largest and the size recorded ranged from 522 to 752 um. For oocytes from the stage 3 ranged between 380-520 um and for stage 2 the oocytes range from 270 to 522 um. This result clearly shows that the oocyte size is highly dependent on the stage of maturity.

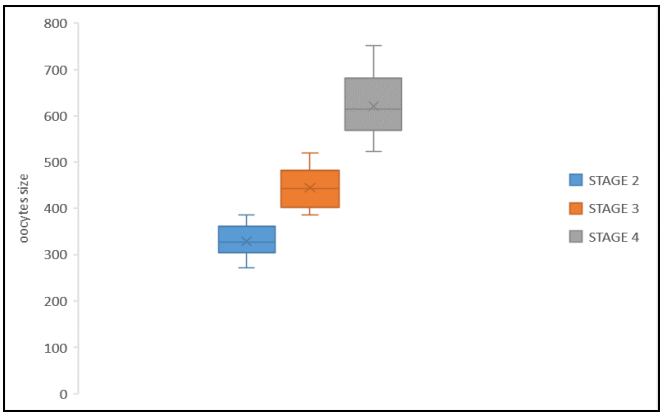


Fig 11: Oocytes size according to different stages

4. Conclusions

The present study revealed that most of the Hilsa shad that were landed are small in size, which is not reach the size of length at first maturity. Out of total collected samples, approximately 79.5% female sample were not reach length at first maturity. The same condition also can be observed in male sample where only 14.5% sample reach length at first maturity. This result is supported by histological study which reported that the samples that were collected in the present study are in developing and maturating stage. This results show that the samples that were landed at the mouth of Perak waters are mostly not matured and not yet have chance to spawn. This condition explains the declining landing of Hilsa spp. in Malaysian waters since 2016 (Department of Fisheries Malaysia, 2019). Thus, a proper fisheries management plan for Hilsa shad is required in order to sustain the Hilsa resources in Malaysia water especially in Perak area. One of the fisheries management plan action that should be taken by management is reducing the fishing effort to ensure the sustainability of Hilsa shad resources.

5. Acknowledgement

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