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Reproductive Biology of Pacific Oyster (*Crassostrea gigas*): A Decade after the Tsunami Disaster in Aceh, Indonesia

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Abstract

The reproductive biology of pacific oysters (*Crassostrea gigas*) was studied in the coastal area of Banda Aceh City, Aceh Province, Indonesia. This study was conducted over six months from July to December, 2017. The samples were collected following the seasons. July to August represented the dry season, while September to October represented the transition season, and November to December had been within the rainy season. Surveys and observations were conducted at two locations, Tibang and Ulee Lheue, where samples were collected with a minimum of 150 samples per location per month. Data collection is done by the line transect method. The results of the analysis showed that the male oyster gonad first matured at a total length of 26.40 mm in Tibang and at the total length of 25.45 mm in Ulee Lheue. Furthermore, the female oyster gonad first matured at 20.46 mm of total length in Tibang and at a total length of 25.24 mm in Ulee Lheue. The range of oyster fecundity in Tibang is between 7,487,888-34,511,625 eggs/ind with an average fecundity reaching 17,360,821 eggs/ind, whereas the range of oyster fecundity in Ulee Lheue was between 9,237,258-40,575,863 eggs/ind with an average fecundity reaching 17,108,206 eggs/ind. The total number of the collected oyster samples was 1800 specimen, and all the samples were in the adult category. In addition to sex determination of the oysters into males and females, the oyster hermaphrodite sex is also found. The results of gonadal observation show that oysters are hermaphrodite synchronous (male and female gonads mature at the same time).

Keywords: Oyster, C*rassostrea*, Gonad, Reproductive, Fecundity, Hermaphrodite

1. Introduction

The tsunami in Aceh on December 26, 2004 occurred more than a decade ago (14 years ago). The earthquake in the Andaman Sea caused a tsunami in Aceh and claimed 130,000 human lives (Frankenberg *et al.,* 2008). In addition to causing human casualties, the tsunami also damaged the coastal environment which previously served as a habitat for both living flora and fauna organisms. It is known that the height of the tsunami waves in Aceh reached 30 m due to the 9.3SR earthquake which has damaged the coastal environment (Suppasri *et al.*, 2015).

Damage caused by tsunamis causes changes in coastal geomorphology, topography, and soil cover (Bayas *et al.*, 2011). Based on previous research, the effects of tsunami waves caused damage to corals in Aceh reaching up to 31 % in the low to medium category and 15 % in the heavily-damaged category (Hagan *et al.*, 2007). Furthermore, the tsunami also damaged 32,004 hectares of mangrove forests and coastal vegetation (Wibisono and Suryadiputra, 2006).

Tsunami waves that occurred in Aceh traveled up to a radius of 3-4 km from the coastline (Hagan *et al.*, 2007). This caused mudflows from the seabed to cover the surface of the land that was passed by the tsunami waves. Tsunami mud residues allowed changes in the ecology of the estuary area after the disaster occurred. One of the organisms that live in the estuary area is oysters.

Oysters are organisms that are very sensitive to environmental changes and can be indicators of pollution in an area (Sarong *et al.*, 2015; Astuti *et al.*, 2016). Based on previous studies that have been carried out in Aceh Province, oysters which are most commonly found consist of five species, included in two genera, namely the genus Ostrea and the genus Crassostrea, consisting of *C. virginica*, *C. gigas*, *C. iridescens*, C.*angulata*, and *O. edulis* (Octavina *et al.*, 2014). One of the mostly common species found is *C. gigas*. The *C. gigas* are reported to contribute to 80 % of the world's oyster trade and have been cultivated in sixty-six countries (Keightley *et al.*, 2015).

Research on oysters in Aceh, includes the analysis of heavy metal content in oysters (Sarong *et al.*, 2015; Astuti *et al.*, 2016), community structure of meat oysters (Fadli *et al.*, 2012; Octavina *et al.*, 2014), length-weight relationships, and oyster condition factors (Octavina *et al.*, 2015; Kasmini *et al.*, 2018). However, research on the reproductive biology of oysters after the tsunami phenomenon that struck in 2004 has not been carried out; thus this study would be an important contribution to the

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development of Aceh oysters in the future, especially for oyster conservation or cultivation.

2. Methods

2.1. Time and Location

The current research was carried out in the coastal area of Banda Aceh City over the period of six months from July to December, 2017. The samples were collected following the seasons where July to August represented the dry season, while September to October represented the transition season, and November to December had been within the rainy season.

2.2. Data Collection

The samples were taken from two research locations, Tibang (5^{0}33’36.7” N, 95^{0}17’22.8” E; 5^{0}33’21.0” N, 95^{0}17’11.7” E; 5^{0}33’01.6” N, 95^{0}17’09.5” E) and Ulee Lheue (5^{0}35’25.1” N, 95^{0}21’03.5” E; 5^{0}35’47.0” N, 95^{0}20’50.8” E; 5^{0}35’36.2” N, 95^{0}20’44.4” E). Each location was divided into three research stations. The collection of the samples was carried out using a purposive sampling method, and a line transect method. Every month, 150 samples were collected randomly from each location consisting of fifty samples per station. The samples that have been obtained were taken to the Marine and Fisheries Faculty of the Marine Biology Laboratory for further analysis. The oyster samples were then identified based on Batista *et al.* (2008). The collected samples were then separated from each other to facilitate the measurements of length and weight. Length measurements were performed using a digital caliper (Precision Measuring Error = 0.01 mm), and weight was taken for each individual using a digital scale (Pocket Scale, MH-Series, Error = 0.01 g).

The preservation of the oyster's body was performed using 10 % NBF (Neutral Buffer Formalin). The analyses of oyster reproduction biology, including histology examination, gonad maturity level, and fecundity were carried out at the Pathology Laboratory, Brackishwater Aquaculture Station (BBAP) Ujong Batee, Ministry of Maritime Affairs and Fisheries, Indonesia.

2.3. Data Analysis

2.3.1. Gonad Maturity Level

The observation of the level of gonad maturity was done visually according to Fabioux *et al.* (2005). This observation aims at determining the maturity and seeing the female and male reproductive organs, so that the size of the oyster body when the gonad is mature can be estimated. For this purpose, oysters with various sizes have been distributed in several groups or classes based on body size. Data on gonadal maturity are then correlated with the length and weight of the oysters in each class. The stomach of the oyster, the gonad shape, size, color and texture were all observed in addition to the presence of sperms or oocytes. Then the gonads were preserved and examined histologically according to the procedure described earlier (Roberts, 2012). The maturity level of the gonads was also determined based on gonadal anatomy and morphology, weighing of gonads, determination of gonadosomatic index, and based on observations of histological preparations according to Roberts (2012).

2.3.2 Gonadosomatic Index

The measurement of the gonadosomatic index (GSI) was done by taking samples of the oysters that have been weighed and measured in length, on which surgery has been done to separate the gonads. They were finally weighed using a digital scale. The GSI is calculated according to Gaughan and Mitchell (2000) by the formula:

$$\mathbf{GSI}\ (\%) = \frac{\mathbf{Wg}}{\mathbf{Wt}}\ \mathbf{x\ 100}$$

Where the GSI = gonadosomatic index (%), Wg = weight of the gonad (g), and Wt = weight of the body (g).

2.3.3 Fecundity

Fecundity was measured with the formula by the gravimetrics method based on Adenike (2013) as follows:

$$F = \frac{\mathbf{Wg}}{\mathbf{Ws}} \times \mathbf{Fs}$$

Where F = fecundity/number of eggs (grains), Wg = weight of the gonad (g), Wt = partial gonad weight (g), and Fs = number of eggs in parts of gonad (grains).

2.3.4 Sex Dimorphism

The observation of sex dimorphism was done to differentiate between the males and females of the oysters morphologically or by physical appearance. For this reason, the sampled oysters were observed visually regarding their body color and shape. The oysters taken were recorded morphologically, and the appearance of the shape and color of the gonads were observed. They were then grouped according to the same morphological appearance and sex that were confirmed on the basis of the histological examination under a microscope.

2.3.5 Histological Analysis and Reproductive Cycle

The sex and gonad maturity of each oyster was determined based on a histological examination. The procedures for making histology preparations using the slice method were done according to Roberts (2012).

2.3.6 Sex Ratio

The sex ratio analysis aims at comparing the numbers of male to the number of female individuals in a population. The observation of the level of gonad maturity and histology examination can help determine the sex of the oysters, thus the ratio of the numbers of females and males can be determined. Sex ratio is calculated by the formula of Adenike (2013) as follows:

$$\textbf{Sex ratio} = \frac{\textbf{Number of male oyster}}{\textbf{Number of female oyster}}$$

2.4. Statistical Analysis

The statistic data analysis was subjected to the chi-square test followed by the Duncans multi-range test applied for cheking the oyster sex ratio and gonadosomatic index between the same and different locations using SPSS ver. 22.0.

3. Results

3.1. Sex Ratio and Gonadosomatic Index

Observations during the study showed that the size of the oysters was between the total lengths of 20.40-136.22 mm with an average size of 46.29 mm concerning the 1800 samples at both locations of the study (Tibang and Ulee Lheue). The results of male sex ratio (SR) analysis show that the highest value was in October (31.33 %) at the Tibang location and during September (36 %) at the Ulee Lheue location. The the highest value of female SR was found in December (75.33 %) at the Tibang location and in August (66.67 %) at the Ulee Lheue location. Furthermore, this study found oysters with hermaphrodite genitals, and their highest SR values were found in September (28.00 %) at Tibang and in October (28.67 %) at Ulee Lheue locations (Tables 1 and 2).

The results show that the sex ratio (SR) studied at two research sites (Tibang and Ulee Lheue) was dominated by the female ratio with a mean value of 65.11 % in Tibang area and 59.55 % at Ulee Lheue location. Male oysters have an SR value of 20.22 % at Tibang location and 28.11 % at Ulee Lheue location. This study also detected hermaphrodite oysters, where the average ratios were 14.67 % at Tibang location and 12.33 % at Ulee Lheue location. Based on the average SR results, female and hermaphroditic sexes at Tibang sites were higher than those at Ulee Lheue, but lower than the mean values of male SR.

Oyster SR values fluctuate every month, which is most significantly seen through the genitals of hermaphrodites. At the Tibang location, the value of hermaphrodite SR increased in July to September and decreased from October to December, whereas at the location of Ulee Lheue the value of hermaphrodite SR continued to decline in July to September and increased in October, but decreased again between November to December (Figure 2a and b).

An analysis of gonadosomatic index (GSI) of female oysters shows that the highest average value was found in October (4.53 %) in Tibang and in November (4.80 %) in Ulee Lheue. The highest mean male GSI score was found in October (3.81 %) in Tibang and in November (5.11 %) in Ulee Lheue. Furthermore, the highest mean hermaphrodite GSI values were found in October in Tibang (4.81 %) and in December (5.05 %) in Ulee Lheue (Tables 5 and 6).

3.2. Gonad Maturity Level

In this study, male oysters first gained gonad at the total length of 26.40 mm in Tibang and at 25.45 mm in Ulee Lheue. This suggests that the mature size of male oyster gonads does not significantly occur between the two different sites. Furthermore, over the period of six months, the study has found oysters with the highest gonadal maturity in two locations occurring in August 96.77 % (Tibang) and 92.31 % (Ulee Lheue) respectively (Table 3 and Figure 3).

Also, this study found female oysters' gonads that first matured at the size of 20.46 mm in Tibang and at 25.24 mm in Ulee Lheue. Furthermore, over the period of six months, the study found oysters in the highest mature gonad state in two locations (Tibang and Ulee Lheue) occurring in August with the same value reaching up to 96 % (Table 4 and Figure 4).

3.3. Gonad Histology and Fecundity

The histologic results of the gonads indicate that reproductive oysters are hermaphrodite (Figure 6). Furthermore, the gonad of mature female oysters has a maximum egg size of ± 50 μm which means that eggs cannot be seen visually without the aid of tools. The size of oysters with mature gonads in Tibang ranged from 20.46 to 94.30 mm with a fecundity range between 7,487,888 and 34,511,625 eggs/ind with an average of fecundity of 17,360,821 eggs/ind.

The size of oysters with ripe gonads in Ulee Lheue ranged from 25.24 to 110.87 mm with a fecundity range between 9,237,258 and 40,575,863 eggs/ind and a fecundity average of 17,108,206 eggs/ind. Regression results showed a very close relationship (Tibang r = 0.95% and Ulee Lheue r = 0.953%), in which the number of eggs increases with the increase of oyster size (Figure 5).

4. Discussion

Based on sex identification, hermaphrodite oysters were found in addition to male and female oysters at the time of the study. The sex ratio (SR) during the study found oysters dominated by the presence of females in two locations, where the mean value was 65.11 % in the Tibang area and 59.55 % in the Ulee Lheue location (Table 5). The SR values of oysters fluctuate each month, with the most significant fluctuation occurring in the hermaphrodites. At the Tibang site, the hermaphrodite SR value experienced an increase in July to September and decreased in October to December, while at the Ulee Lheue location, the SR hermaphrodite value continued to decline in July to September, and experienced an increase in October, but there was a further decline in November to December (Figure 2).

The results of gonadal observation show that oysters are hermaphrodite synchronous (male and female gonads mature at the same time) (Figure 6). However, other studies mention that in the reproductive cycle, oysters are protandrous hermaphrodites whose life begins with the male genitals turning into females several years later if the environment is good and when sufficient nutrition is available (Westphal *et al.*, 2015). According to Dheilly *et al*. (2012) oysters are generally protandrous hermaphrodites, but there are several cases of synchronous hermaphrodite oysters found because the gonads are labile. Thus, there are changes in the physiological properties of the oyster bodies in the study locations caused by environmental factors. Changes in physiological properties may occur due to tsunami disasters or other environmental factors such as global warming or pollution. However, the anthropogenic pressure of seawater can be a problem in the development of aquatic organisms (Hamdani and Soltani-Mazouni, 2011).

Analysis of the reproductive aspects of oysters showed spawning peaks occurring in August (Table 3 and Table 4). This is due to the fact that both male and female oysters are dominated by mature categories. In August, especially in Banda Aceh, the peak of the dry season occurred, when temperatures were relatively high and rain did not occur. The dry season that occurs in August causes an increase in water salinity which then induces the spawning season of oysters. This is consistent with the results of research conducted by Dheilly *et al*. (2012) which states that oyster spawning peaks occur in the summer because the gametogenesis process occurs when the water temperature increases. This finding was also strengthened by the results of research conducted by Fabioux *et al*. (2005) which state that the gametogenesis process in *C. gigas* develops during summer with water temperatures being above 19 ^{0}C and salinity>30 ppt. In other studies, it was also mentioned that first-time oyster gonads develop in April and continue to grow until they become matured in August when the initial spawning process occurs and continues until September (Li *et al*., 2000).

In this study, the male oyster gonad was first ripe at 26.40 mm in Tibang and at 25.45 mm in Ulee Lheue. This shows that the size of first-time mature gonad of male oysters did not differ significantly between the two different locations. The gonads were first mature in female oysters at 20.46 mm in Tibang and at 25.24 mm in Ulee Lheue. According to Westphal *et al*. (2015), the genus *Crassostrea* first reproduced (spawning) at a size of 20 mm. Thus all of the oysters collected from the Tibang and Ulee Lheue estuaries are in the adult category.

Based on the results of the present study, it appears that the ability of oyster recruitment is very high. This is indicated by the high number of eggs (fecundity), at an average of 17,360,821eggs/ind in Tibang and 17,108,206 eggs/ind in Ulee Lheue. The study found a positive correlation (r) between the addition of length and egg production in oysters with a value reaching 95 % in both study locations, where the size of 110.87 mm (the highest size of female genital oysters) can produce more than 40,000,000 eggs (Figure 5). If an organism has a high recruitment ability, it can maintain the stability of its population in nature (Bakun and Broad, 2003). Furthermore, there is no dispensatory dynamics in oysters similar to the phenomenon that often occurs in fish, where the recruitment ability is low due to the difficulty of finding a partner or allee effect (Myers *et al*., 1995). In addition to that, the environment that often experiences eutrophication has a positive influence on oysters for the development of reproductive organs more often than the environment which is poor in organic content (Fabioux *et al*., 2005). It is known that oyster reproductive activity (recruitment) is done by random removing of eggs and sperm into the waters when stimuli for spawning are detected (Westphal *et al*., 2015).

The maximum size of *C. gigas* eggs with mature gonads during the study only reached ± 50 µm and that is why the eggs could not be seen visually. This is consistent with the results of research conducted by Lango-Reynoso *et al.* (2000) stating that the size of *C. gigas* eggs matured in gonads ranging from 31 to 60 µm. Furthermore, in other studies, it was stated that the maximum size of *C. gigas* eggs in mature conditions reached 48.7 µm (Li *et al*., 2000).

Thus, the size of the oyster is very important as an indicator of egg-laying productivity. The measurement shows reproductive performance and its association with the process of recruiting new offspring of oysters. In male oysters the number is much less than in female oysters, so the ability of oyster regeneration will be very fast due to the fact that the role of female oysters are very significant because of the feature of egg production. Oysters with mature gonads can be found every month, which means that oysters can spawn throughout the year.

Table 1. Sex ratio (SR) and gonadosomatic index (GSI) *C. gigas* at Tibang

Month	Ind	Male Oyster		Female Oyster		Hermaprodite	
		SR (%)	GSI (%)	SR (%)	GSI (%)	SR (%)	GSI (%)
July	150	21.33	0.53-8.51 2.73±1.45	68.67	0.08-14.32 2.57±1.83	10.00	0.40-6.89 3.24±1.93
August	150	20.67	1.22-8.60 3.71±1.62	66.67	0.50-17.67 3.85±2.31	12.67	0.54-7.87 3.44±1.80
September	150	14.67	0.82-5.93 2.82±1.37	57.33	1.08-12.27 3.78±1.83	28.00	0.88-11.85 3.80±1.85
October	150	31.33	0.41-11.57 3.81±2.77	48.00	0.31-11.97 4.53±2.46	20.67	0.80-18.60 4.81±4.26
November	150	14.67	1.43-7.33 3.81±1.64	74.67	0.48-11.25 4.18±1.98	10.67	0.75-6.88 3.89±1.75
December	150	18.67	0.39-6.38 3.25±1.70	75.33	0.58-9.91 3.53±1.98	6.00	0.75-5.17 2.84±1.73

Table 2. Sex ratio (SR) and gonadosomatic index (GSI) *C. gigas* at Ulee Lheue

Month	Ind	Male Oyster		Female Oyster		Hermaprodite	
		SR (%)	GSI (%)	SR (%)	GSI (%)	SR (%)	GSI (%)
July	150	32.67	0.04-6.97 0.78±1.40	57.33	0.11-5.58 1.47±1.37	10.00	0.35-4.81 1.72±1.45
August	150	26	0.04-6.26 1.85±1.33	66.67	0.01-12.17 2.64±2.13	7.33	0.57-6.99 3.50±1.76
September	150	36	0.77-13.85 4.06±3.05	60	0.30-16.73 3.61±2.66	4	0.39-2.97 1.77±1.04
October	150	25.33	0.42-12.36 3.25±2.23	46.00	0.60-10.26 3.87±1.92	28.67	0.77-10.24 3.81±2.03
November	150	20.67	1.02-19.06 5.11±3.35	63.33	0.34-16.56 4.80±2.44	16.00	0.35-9.54 4.54±2.33
December	150	28.00	0.95-8.10 3.00±1.56	64.00	0.05-16.56 3.56±2.42	8.00	1.58-9.55 5.05±2.62

Table 3. Gonad maturity level of male oyster at Tibang and Ulee Lheue

Month	Tibang						Ulee Lheue					
	Ind	Gonad maturity level (%)					Ind	Gonad maturity level (%)				
		I	II	III	IV	V		I	II	III	IV	V
July	32	3.13	0.00	3.13	90.63	3.13	49	73.47	6.12	4.08	16.33	0.00
August	31	0.00	0.00	3.23	96.77	0.00	39	7.69	0.00	0.00	92.31	0.00
September	22	0.00	50.00	27.27	18.18	4.55	54	20.37	59.26	9.26	9.26	1.85
October	47	0.00	14.89	14.89	44.68	25.53	38	0.00	15.79	21.05	28.95	34.21
November	22	0.00	9.09	63.64	27.27	0.00	31	3.23	16.13	35.48	45.16	0.00
December	28	0.00	0.00	53.57	42.86	3.57	42	0.00	4.76	21.43	73.81	0.00

Table 4. Gonad maturity level of female oyster at Tibang and Ulee Lheue

Month	Tibang						Ulee Lheue					
	Ind	Gonad maturity level (%)					Ind	Gonad maturity level (%)				
		I	II	III	IV	V		I	II	III	IV	V
July	103	2.91	2.91	9.71	77.67	6.80	86	5.81	5.81	33.72	48.84	5.81
August	100	0.00	0.00	2.00	96.00	2.00	100	3.00	0.00	1.00	96.00	0.00
September	86	3.49	13.95	24.42	24.42	33.72	90	18.89	18.89	23.33	25.56	13.33
October	72	0.00	30.56	20.83	33.33	15.28	69	1.45	17.39	27.54	47.83	5.80
November	112	0.00	0.89	27.68	71.43	0.00	95	0.00	16.84	32.63	50.53	0.00
December	113	0.00	2.65	35.40	61.95	0.00	96	3.13	8.33	33.33	55.21	0.00

Table 5. The statistical analysis of SR and GSI according to each sampling location. The values in the same row followed by different superscripts are significantly different (*P*<0.05).

Location	Variable	Male	Female	Hermaprodite
Tibang	SR (%)	14.67-31.33 $20.22±6.15^{a}$	48-75.33 $65.11±10.62^{b}$	6-28 $14.67±8.13^{a}$
	GSI (%)	2.73-3.81 $3.35±0.49^{a}$	2.57-4.53 $3.74±0.67^{a}$	2.84-4.81 $3.67±0.68^{a}$
Ulee Lheue	SR (%)	20.67-36 $28.11±5.49^{b}$	46-66.67 $59.55±7.39^{c}$	4-28.67 $12.33±8.93^{a}$
	GSI (%)	0.78-5.11 $3.00±1.54^{a}$	1.47-4.8 $3.32±1.14^{a}$	1.72-5.05 $3.39±1.39^{a}$

Table 6. The comparative analysis of SR and GSI at the Tibang and Ulee Lheue locations. The values in the same row followed by different superscripts are significantly different (*P*<0.05).

Sex	SR (%)		GSI (%)	
	Tibang	Ulee Lheue	Tibang	Ulee Lheue
Male	14.67-31.33 $20.22±6.15^{a}$	20.67-36 $28.11±5.49^{a}$	2.73-3.81 $3.35±0.49^{a}$	0.78-5.11 $3.00±1.54^{a}$
Female	48-75.33 $65.11±10.62^{a}$	46-66.67 $59.55±7.39^{a}$	2.57-4.53 $3.74±0.67^{a}$	1.47-4.8 $3.32±1.14^{a}$
Hermaprodite	6-28 $14.67±8.13^{a}$	4-28.67 $12.33±8.93^{a}$	2.84-4.81 $3.67±0.68^{a}$	1.72-5.05 $3.39±1.39^{a}$

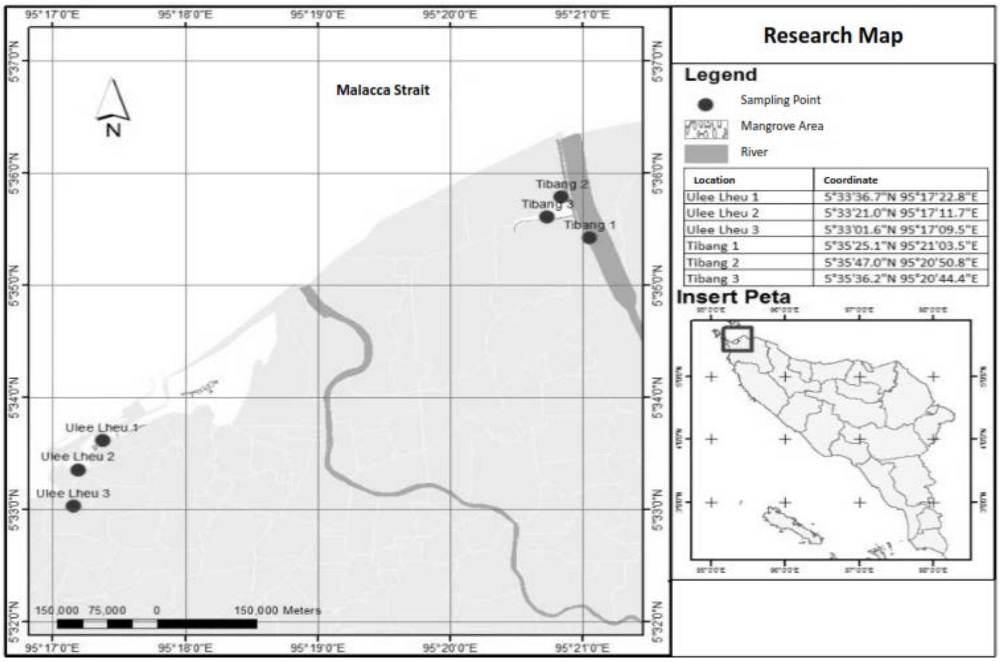

Figure 1. Research map. The study was conducted in Ulee Lheue and Tibang, each location consisted of 3 sampling points.

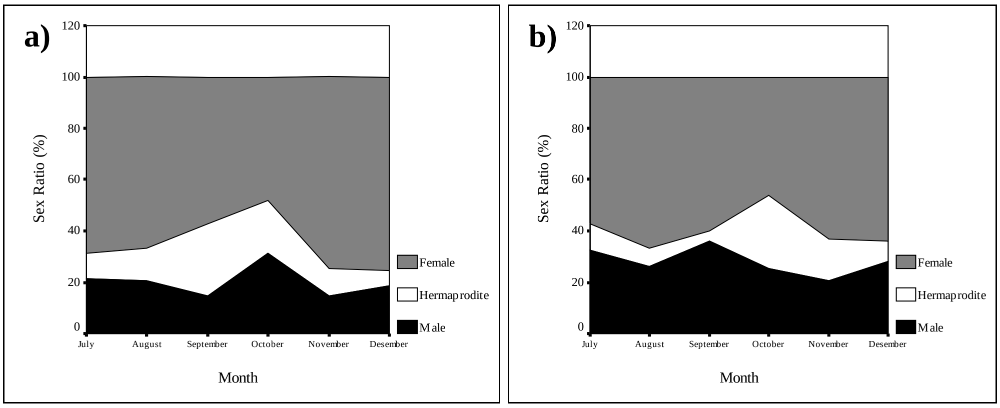

Figure 2. Sex ratio ratio (SR) oyster Crassostrea gigas, where a) Tibang location and b) the location of Ulee Lheue.

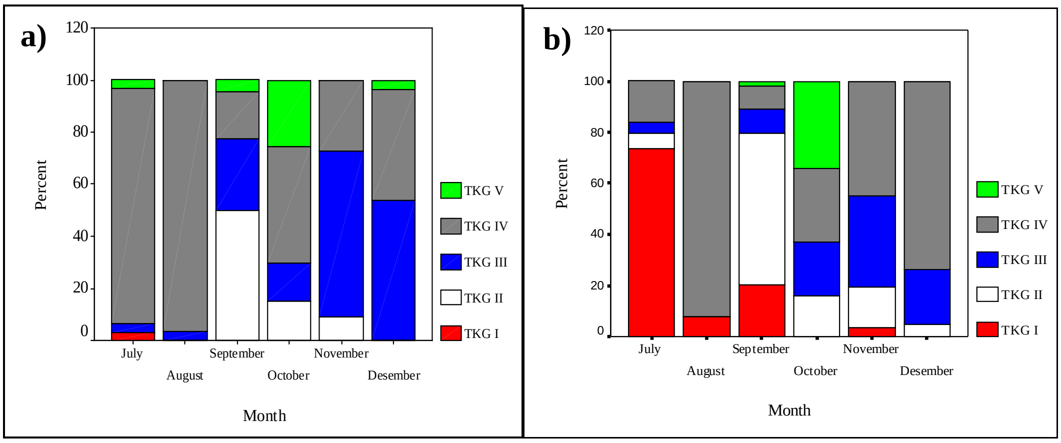

Figure 3. Percentage of mature male oyster gonad maturity levels (TKG) in a six-month study, where a) loaction of Tibang and b) Ulee Lheue.

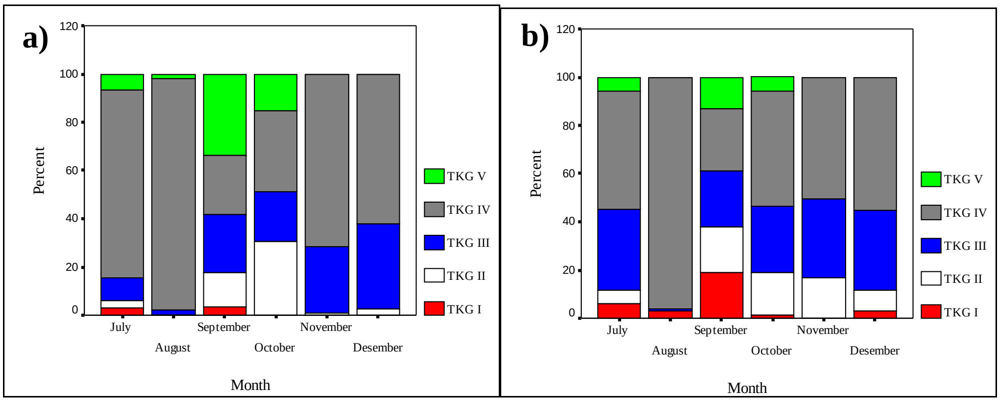

Figure 4. Percentage of mature female oyster gonad maturity levels (TKG) for 6 months of study, where a) loaction of Tibang and b) Ulee Lheue.

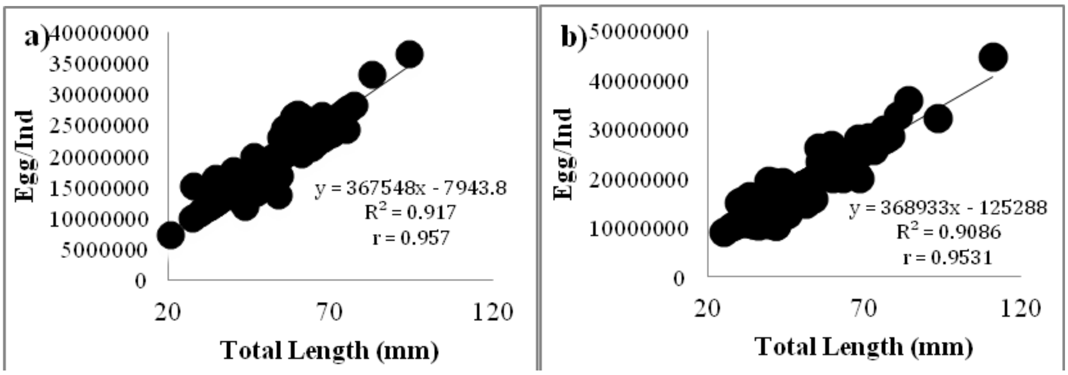

Figure 5. Regression of total length (mm) and oyster fecundity (egg/ind), where a) is Tibang and b) Ulee Lheue

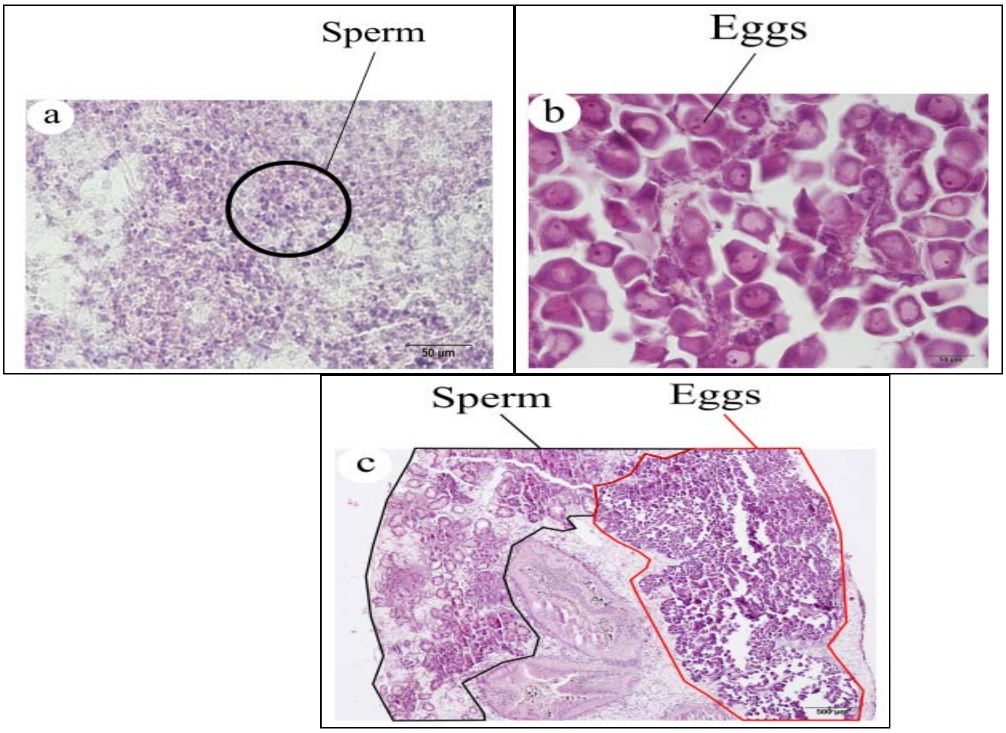

Figure 6. Histological appearance of oyster gonads, a) male, b) female, c) hermaphroditic.

5. Conclusion

The male oyster gonad first matured at 26.40 mm in Tibang and at 25.45 mm in Ulee Lheue. Furthermore, the female oyster gonad first matured at 20.46 mm in Tibang and at 25.24 mm in Ulee Lheue. The range of oyster fecundity in Tibang Village is between 7,487,888 and 34,511,625ggs/ind with an average fecundity reaching 17,360,821 eggs/ind; the range of oyster fecundity in Ulee Lheue was between 9,237,258 and 40,575,863eggs/ind with and average fecundity reaching 17,108,206 eggs/ind.

In addition to the male and female oysters, oysters of the hermaphrodite sex were also found.

Conflict of interest

The authors declare that there is no conflict of interest.

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Author contribution

L.K.: the author is responsible for data collection, sample maintaining, data analysis, and manuscript drafting; T.A.B.: the author is responsible for developing of the study design, supervision, data validation, review and editing of the draft manuscript; M.A.S.: developing of the study design, supervision, data validation, review and editing the draft manuscript; M.B.M.: research supervision, data validation, review and editing of the draft manuscript; A.S.B.: data collection, sample maintaining, data analysis and validation. All authors read and approved the final manuscript.

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