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Reproduction status of the angelwing clam, *Pholas orientalis* (Gmelin, 1791), obtained from Selangor, Malaysia

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Abstract
To understand the habitat status of *Pholas orientalis* offshore of Selangor, which is an important fishing ground in Malaysia, *P. orientalis* was obtained from the region in August and November 2014. The shell sizes and weights of the specimens were measured, and tissue sections of various organs were prepared, stained with hematoxylin and eosin solution, and observed under a light microscope. The results obtained for the gonads, digestive tube, and midgut gland were summarised, and the results showed that many individuals of both sexes had already reached the mature stage in August, and the maturation was almost synchronised in both sexes. In November, more than 40% of both sexes entered the spawning stage, suggesting that the spawning peak in the region occurred during the rainy season. From observations of the presence or absence of food in the digestive tube and epithelium condition estimation of the midgut gland, food presence in the clams was confirmed in more than 80% of individuals in both months, and the epithelium condition was also good. In addition, from the Global Eutrophication Watch internet homepage, chlorophyll-a concentration data on the fishing ground area were shown as the stable phytoplankton occurrence area. Therefore, these data suggest that suitable food availability is an important factor for the habitation of this species.

Keywords
Angelwing clam, Histological observation, Sexual maturation, Chlorophyll-a, Peninsular Malaysia

Introduction

Pholas orientalis normally grows to a shell length of approximately 12 cm (Carpenter and Niem, 1998), with some large individuals measuring greater than 20 cm (Ronquillo and Mckinley, 2006). The shell is thin and fragile, the top is tilted forward, the anterior dorsal edge is curved, and the surface of the shell is clearly divided into a granular part consisting of growth veins, radial intercostal contacts, and a smooth part. This species inhabits tidal flats in tropical coastal areas such as northern Australia, Singapore, Malaysia, Indonesia, the Philippines, and southern China, as well as mud and sandy bottoms at depths of 5–10 m (Beewah, 2009Carpenter and Niem, 1998; Ronquillo and Mckinley, 2006). This species digs a hole in the bottom soil with its own foot, blows soil from the ground to the surface through a long water pipe, and inhabits depths of approximately greater than 30 cm (Beewah, 2009; Carpenter and Niem, 1998; Laureta and Marasigan, 2000; Ronquillo and McKinley, 2006). In recent years, angelwing clams have become a rare species in Southeast Asian countries because of tidal flat reclamation and overfishing (Beewah *et al.*, 2009;

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Laureta *et al.*, 2014; Ronquillo and Mckinley, 2006). In Malaysia, this species is called "Mentarang" (Golez *et al.*, 2011) and is distributed on the west coast of Peninsular Malaysia (Laureta and Marasigan, 2000; Ramli and Yusop, 2016; Yap *et al.*, 2009; Yusop and Ramli, 2017) and the coast of Kuching, Sarawak, Borneo Island, Malaysia (Hamli *et al.*, 2012). It is highly regarded as an edible species in the country and is used as an ingredient in a wide range of dishes, such as BBQ, fried food, stir-fried food, soup, and coconut milk stew. Therefore, it is considered a target species for aquaculture (Ramli and Yusop, 2016; Yusop and Ramli, 2017), and basic research on artificial seedling production is also proceeding (Beewah *et al.*, 2009; Ronquillo and McKinley, 2006). As of 2014, angelwing clams were sold on the street and provided in dishes at local restaurants in Selangor. However, according to the local newspaper (BH-online: New Straits Times Press, July 2015), the catch of this species is declining, and the price is soaring (more than 15 Malaysian ringgit/kg). Therefore, this study was conducted as a basis for *P. orientalis* fishing management in Selangor, Malaysia. The purpose of this study was to understand the habitat status of *P. orientalis* in the main fishing grounds off Sekinchan, Selangor. Tissue sections were prepared from the soft body tissue of *P. orientalis*, stained with hematoxylin and eosin (H&E) solution, and observed under a light microscope. The tissue sections were then evaluated for sexual maturation and feeding status. In addition, we collected information relevant to this study, such as meteorological and fishing ground environment data from the internet and evaluated the characteristics of the fishing ground area of the Selangor coast.

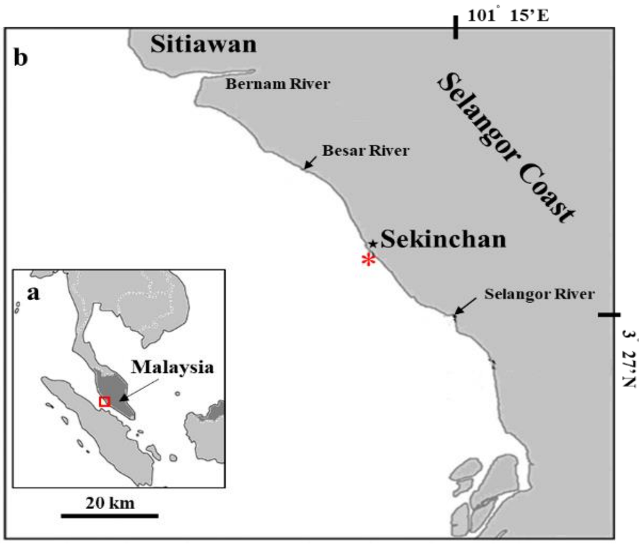

Figure 1. *Locations of the angelwing clam fishing ground in Sekinchan, Selangor, Malaysia. a: Location of Selangor Coast in Peninsular Malaysia (red square means location of map b). b: Locations of the angelwing clam fishing ground (red color asterisk) and the clam dealer in Sekinchan (solid star).*

Materials and Methods

Living angelwing clams, which were caught off the Sekinchan coast, Selangor (3.49 ° N, 101.09 ° E) (Ramli and Yusop, 2016; Yusop and Ramli, 2017), were purchased from a local fisherman in August and November 2014 (Fig. 1). The clams were transported to a laboratory, and the shell length, shell height, shell width, and weight were measured. The mean values were compiled, and the shell length data were summarised into a histogram. These samples were immersed in 10% seawater formalin solution for tissue fixation. At a later date, tissue sites containing gonad tissue, gastrointestinal tract, and midgut gland were dissected from these samples as tissue blocks, dehydrated with 70–100% ethanol, paraffin-impregnated through lemosol substitution, and embedded in paraffin to make a tissue paraffin block. In addition, tissue sections with a thickness of 5–7 μm were prepared from the tissue paraffin block using a microtome and H&E staining by a common method, and the various tissues were observed under a light microscope. The gonad condition stages, presence or absence of food in the digestive tube, and condition of the epithelium in the digestive gland were classified with reference to previous research (Maeno *et al.*, 2009; Yurimoto *et al.*, 2008; Yurimoto *et al.*, 2014; Yurimoto *et al.*, 2019; Author *et al.*, 2021a). Gonad development was classified into five stages: I, immature stage; II, development stage; III, mature stage; IV, partial spawning stage; and V, spent stage. I: The immature stage is filled with connective tissues, and it is difficult to identify males or females from the tissue condition; II: development stage, immature or developing germ cells appear along the follicular wall in both males and females, and even in observed mature cells, they are less in number. III: The mature stage includes follicles that are extended in both males and females, sperm in the testis are arranged radially, or mature germ cells in the ovary are located away from the follicular wall. IV: partial pawning period, sperm leak from the follicle, or eggs are already released, and conspicuous vacant spaces are observed in the follicle. Additionally, many follicles have already folded, although some germ cells remain in both sexes. The status of the gonads in each individual was classi-

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fied based on the tissue classification. On the other hand, the presence or absence of food in the digestive tube was determined by food observation in the tube. Additionally, even if a small amount of food was found in the tube, it was classified as positive. The epithelium condition in the digestive gland was classified into three stages as follows: I, normal condition of epithelial cells; II, partial flattening of epithelial cells; and III, extensively flattening of epithelial cells. Next, to understand the environment along the coast of Sekinchan, Selangor, where *P. orientalis* was collected, meteorological information of Sitiawan (latitude: 4.22°N/ longitude:100.70°E, altitude: 7 m, a little further north of Fig. 1b) from January to December 2014 was obtained from the Japan Meteorological Agency website (https://www.data.jma.go.jp/gmd/cpd/monitor/), and the monthly changes in average temperature and rainfall are summarised in a graph (acquired in July 2021). In addition, the average value for each month measured from 1991 to 2020 is also shown in the graph. Moreover, from the homepage of the Global Eutrophication Watch (Maúre *et al.*, 2021), an online evaluation tool for eutrophication based on satellite data, coastal changes in the chlorophyll-a concentration map shown as a cutoff level of 5 mg m^{-3}, and the chlorophyll-a concentration value for each year is shown as a graph on the *P. orientalis* fishing ground (3.49 °N, 101.09 °E).

Results

Table, 1 shows the shell size and weight of *P. orientalis* collected in August and November of 2014. In August, 32 individuals were obtained with shell lengths, widths, and heights of 69 ± 6 mm (mean ± SD), 22 ± 2 mm, and 21 ± 2 mm, respectively. The whole and soft tissue weights were 15 ± 2 g and 5 ± 1 g, respectively. In November, 36 individuals were obtained with shell lengths, widths, and heights of 73 ± 10 mm, 25 ± 3 mm, and 23 ± 3 mm, respectively. The whole and soft tissue weights were 19 ± 7 g and 6 ± 2 g, respectively. Figure 2 shows the shell length distribution of *P. orientalis* caught in August and November. Both histograms showed unimodality, but their modes (minimum and maximum) and histogram shapes were 70–74 mm (59 mm; 79 mm) in the left-skewed histogram in August and 70-74 mm (52 mm; 91 mm) in the normally shaped histogram in November.

Month	n	Shell sizes (mm)			Weights (g)	
		SL	SW	SH	WW	TW
August	32	69 ±6	22 ±2	21 ±2	15 ±2	5 ±1
November	36	73 ±10	25 ±3	23 ±3	19 ±7	6 ±2

n = the number of samples, SL = shell length; SW = shell width; SH = shell height; WW = whole weight; TW = tissue weight.

Table 1 *Shell sizes and weights of the angelwing clam, Pholas orientalisis, obtained in this study*

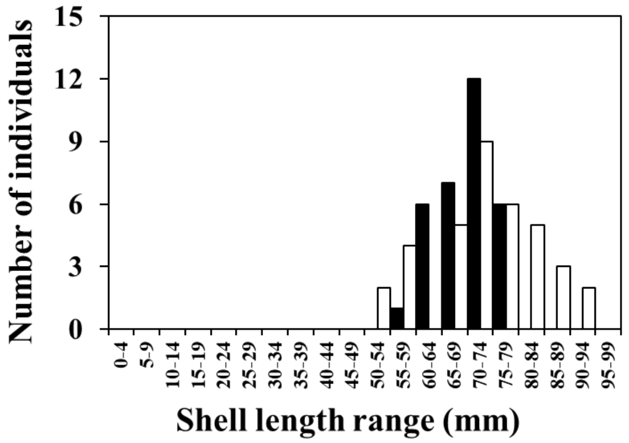

Figure 2. *Shell length distributions of the angelwing clam, Pholas orientalisis, obtained from Sekinchan. Solid bar (■): specimens (n=32) obtained in August 2014, open bar (□): specimens (n = 36) obtained in November 2014.*

Figure 3 shows the gonad tissue of *P. orientalis*. Sexual maturation had begun in all samples used for observation, and germ cells were observed, making it possible to distinguish between males and females. Here, the typical gonad tissue observation results for the August and November samples are shown separately for males and females. In males, testis tissues were observed in the development, maturation, and partial spawning stages. In females, ovary tissues were observed in the development, maturation, and partial spawning stages. Therefore, the results of male–female identification in the samples obtained in August and November and the developmental stages of the gonads of males and females are summarised in Table, 2.

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In August, 20 males and 12 females were identified among the 32 individuals, and many males and females had gonads at the mature stage (male n = 14, female n = 6). Partial spawning stage tissues were also found in some individuals (male, n = 4; female, n = 5). In November, 17 males and 19 females were identified among the 36 individuals, and many gonads of both males and females were in the partial spawning stage (male, n = 12; female, n = 15). In addition, when the samples from August and November were combined, a total of 68 individuals were identified, including 37 males (54.4%) and 31 females (45.6%). The sex ratio obtained using the formula 100 (sex ratio = number of males / number of females x 100) was male: female = 119:100.

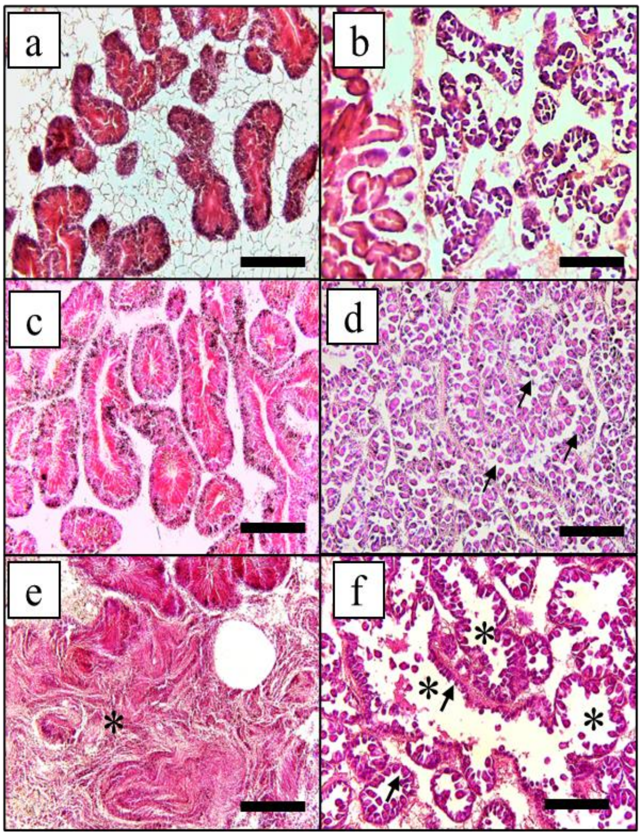

Figure 3. *Histological observation of H&E staining gonad tissue in the angelwing clam, Pholas orientalisis, obtained from Sekinchan. a: developing stage (II) in male, b: developing stage (II) in female, c: mature stage (III) in male, d: mature stage (III) in female, e: spawning stage (IV) in Male and f: spawning stage (IV) in female. The arrow in d shows the mature oocyte and in f shows the remaining immature oocyte. The asterisk in e shows spermatozoa leaked from the follicle and in f shows blank in the follicle after spawning. Scale bars = 200 µm*

Table 2 *Sexual discrimination and sexual maturation stage in the angelwing clam, Pholas orientalisis, with the gonad tissue observation*

Month	Sex	n	Stages I	II	III	IV	V
Aug.	Male	20		2	14	4	
	Female	12		1	6	5	
	Both total	32		3	20	9	
Nov.	Male	17			5	12	
	Female	19			4	15	
	Both total	36			9	27	

n = the number of observed samples, I = immature stage; II = developing stage; III = mature stage; IV = spawning stage; V = spent stage.

Figure 4 shows H&E staining images of the digestive tube tissue of *P. orientalis*. The ingested clam was filled with fragment particles, such as diatoms, in the tube (marked with *in Figure 4a). In contrast, non-ingested clams were observed to have no such particles in the tube (marked with * in Figure 4b). Among the tissue specimens prepared in this study, the conditions in the digestive tract are summarised in Table 3 for 30 individuals in August and 33 individuals in November, in which the condition of the digestive gland could be observed. As a result, it was found that 83% of the individuals ingested food in August and 97% in November, and even when the results were compared between sexes, no clear bias due to sex was observed.

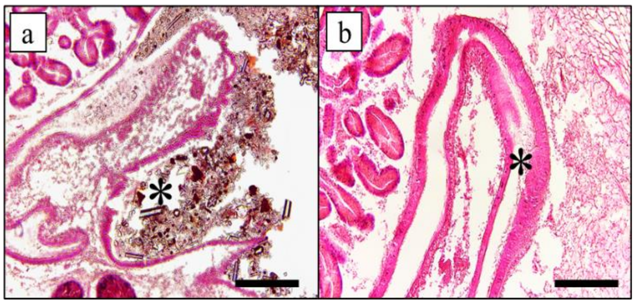

Figure 4. *Histological observation of H&E staining digestive tube tissue in the angelwing clam, Pholas orientalisis, obtained from Sekinchan. a: presence of ingested food (asterisk) in the digestive tube, b: empty of inside of the digestive tube (asterisk). Scale bars = 200 µm*

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Table 3 *Food presence in digestive tube in the angelwing clam, Pholas orientalisis*

Month	Sex	n	Food presence	
			Food	Empty
Aug.	Male	18	16	2
	Female	12	9	3
	Both total	30	25	5
Nov.	Male	16	16	
	Female	17	16	1
	Both total	33	32	1

n = the number of observed samples, Food = the sample number of food presences in the digestive tube tissue; Empty = the sample number of non-presences of food in the digestive tube tissue.

Figure 5 shows H&E staining pictures of the midgut gland tissue in *P. orientalis*. The midgut gland was roughly divided into two types: midgut glands with thick epithelium (Fig. 5a) and midgut glands with thick and thin epitheliums (Fig. 5b). However, in this study, no histological condition was observed in which the thin epithelium spread over a wide area in the midgut gland tissue. Therefore, among the tissue specimens prepared in this study, the state of the epithelium in the midgut gland tissue is summarized in Table, 4 for 26 individuals in August and 25 individuals in November, which is the suitable condition for the midgut gland tissue observation. As a result, normal condition individuals of the epithelium among the observed specimens were identified at high rates at 96% in August and at 95% in November, respectively. In addition, only one individual had glands with partially thin epithelium in August and November, respectively (Fig. 5b).

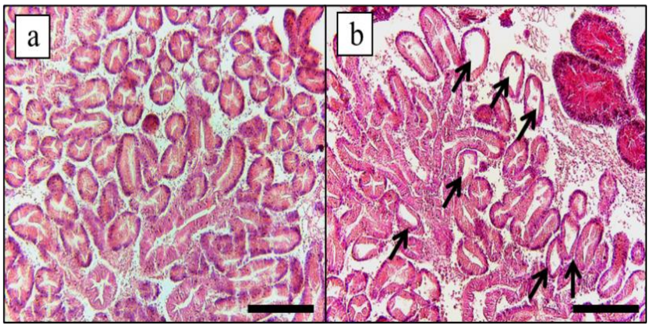

Figure 5. *Histological observation of H&E staining midgut gland tissue in the angelwing clam, Pholas orientalisis, obtained from Sekinchan. a: Normal condition of epithelial cells (I). b: Partial flattening of epithelial cells (II). Individual of extensively flattening of epithelial cells (III) was not observed in this study. Arrows in figure b mean flattening of epithelium. Scale bars = 200 µm*

Table 4 *State of epithelium of midgut gland tissue in the angelwing clam, Pholas orientalisis*

Month	Sex	n	Stages		
			I	II	III
Aug.	Male	17	16	1	
	Female	9	9		
	Both total	26	25	1	
Nov.	Male	9	8	1	
	Female	11	11		
	Both total	20	19	1	

n = the number of observed samples, I = Normal condition of epithelial cells; II = Partial flattening of epithelial cells; III = Substantial flattening of epithelial cells.

Figure 6 shows the changes in the monthly average air temperature (●) and monthly rainfall (■) from January to December 2014. In addition, the monthly changes in the average air temperature (○) and rainfall amount (□) for 30 years are also shown as normal average values. The average air temperature in June-July 2014 was 28.9–28.5 °C, which is approxi-

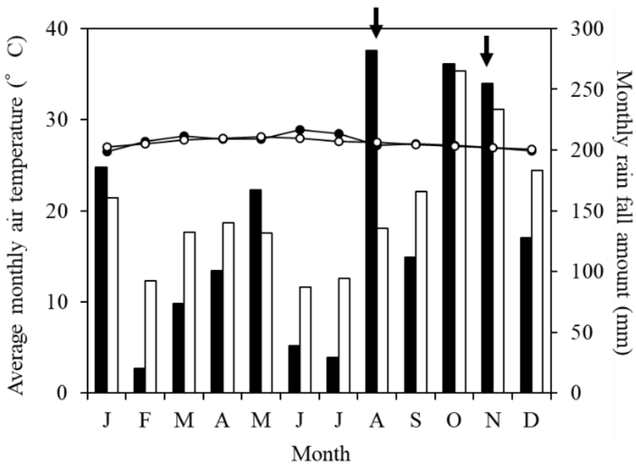

Figure 6. *Monthly average air temperature and monthly rainfall amount in Sitiawan, Malaysia, 2014. The statistical data were obtained from the meteorological agency homepage. Solid circles (●) and solid bars (■) represent average air temperature and rainfall amount observed in 2014, respectively. Open circles (○) and open bars (□) represent the 30-year average of values from 1991 to 2020, respectively.*

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mately 1 °C higher than the normal average value, and the value in August 2014, when the samples were obtained, was 27.2 °C. similar to the normal average air temperature. The monthly rainfall was 282 mm in August 2014, which was approximately twice the normal value (135 mm). In addition, the monthly average air temperature and rainfall amount in October–November 2014 were 27.2–26.9 ° C and 271–255 mm, respectively. Both were almost the same as the normal values (27.1–26.9 °C, 265–233 mm). Figure 7 shows the distribution map of changes in chlorophyll-a concentration around the coast of Selangor (Fig. 7a) using the global eutrophication watch and the annual changes in chlorophyll-a around the fishing grounds (*) along the coast of Sekinchan (Fig. 7b). According to a colour-coded map with a cut-off level of chlorophyll-a of 5 mg.m^{-3}, the fishing ground area was identified as High Neutral (HN). This indicates that the chlorophyll-a concentration was higher than the cut-off level in this area, and has remained stable over the years. In addition, the chlorophyll-a concentration of each year in the fishing ground area showed a value of 7.4 mg.m^{-3} or more in 2007, 2009, and 2011, which was particularly high; however, after this period, it was 4.0 to 5.9 mg.m^{-3} and the trend of the annual change from 2005 to 2019 remained stable at approximately 5.5 mg.m^{-3}.

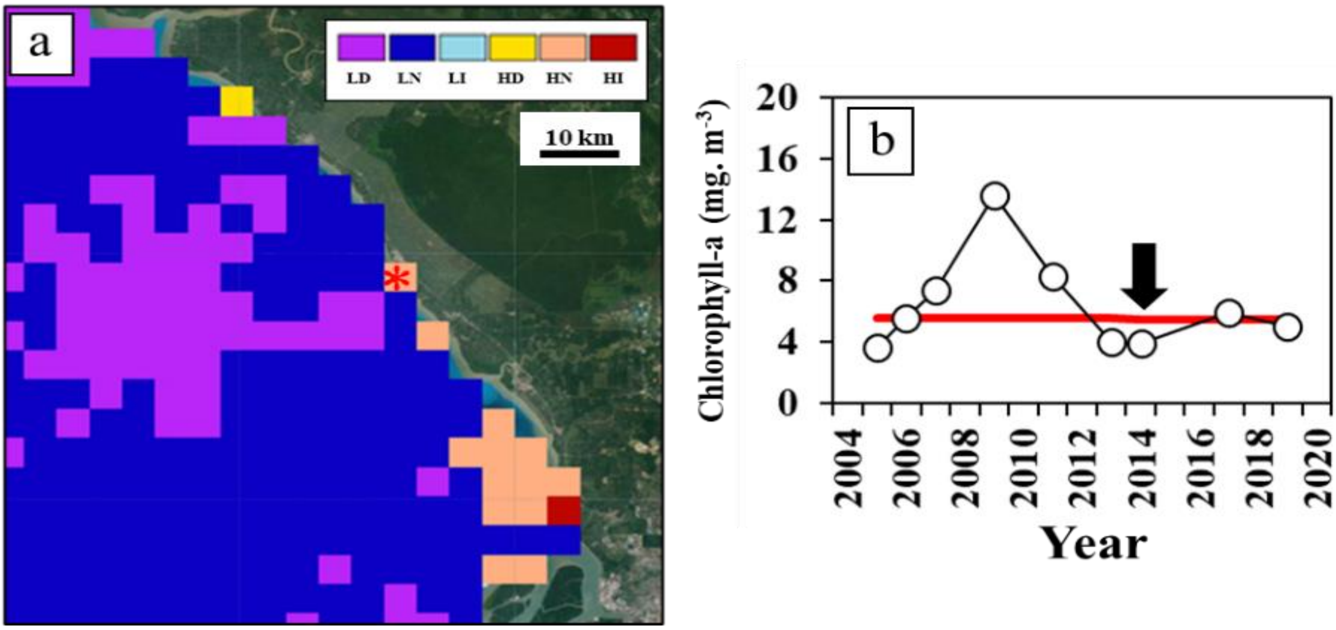

Figure 7. *Distribution map of chlorophyll-a along the coast of Selangor (a) and annual changes of chlorophyll-a around the clam fishing ground (red colour asterisk in figure a) along the coast of Sekinchan (b) by the Global Eutrophication Watch. a: annual change trend of chlorophyll-a level mapping result when the cut-off level was 5 mg.m^{-3}. LD (Low Down), LN (Low Neutral), LI (Low Increase), HD (High Down), HN (High Neutral), and HI (High Increase) with respect to the cut-off level on each grid. b: the red bar indicates the annual changing trend, and the black arrow indicates the study year.*

Discussion

From the average shell length of *P. orientalis* collected in August and November 2014, it was estimated that the shell length increased by 4 mm in approximately three months in the population inhabiting the Sekinchan coast, Selangor. In addition, it was estimated that the shell width and height also increased by 3 mm and 2 mm, respectively, from the difference between the average values of both months; the whole and soft body weights were also estimated to increase by 4 g and 1 g, respectively. The growth of *P. orientalis* cannot be compared among sea areas due to a lack of data, but various culture feeds (a mix of *Chaetoceros* sp. and *Isochrysis galbana* or a mix of *Tetraselmis* sp. and *Chaetoceros* sp.) are used for individuals with a shell length of approximately 100 mm. Considering the growth of the shell length of *P. orientalis* used in experimental feeding, the growth level was almost flat for two and a half months (Golez *et al*., 2011). Therefore, the population in this

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study was under good feeding conditions, and the growth of the shell length was at an average level. In addition, the shell length of *P. orientalis* grows to approximately 120 mm ordinally (Carpenter and Niem, 1998), but the shell length of most *P. orientalis* is less than 90 mm in Sekinchan, Selangor. Based on the von Bertalanffy growth curve derived from the population of *P. orientalis* inhabiting the Kelang coast of Indonesia by Manullang *et al.* (2021), *P. orientalis* with a shell length of approximately 70 mm was obtained in this study. The population age was estimated to be 10–11 months after settlement to the bottom, and many of them were considered to have been caught before the emergence of the complete adult age group of *P. orientalis* off the Sekinchan coast, Selangor. Next, from the results of gonad tissue observation of *P. orientalis*, sexual maturation had already progressed in both males and females as of August, and many individuals were identified to be in the mature stage in both males and females. In November, more than 70% of both males and females were identified during the partial spawning stage, and the rainy season was thought to be the high spawning season for *P. orientalis*. It is said that *P. orientalis* larvae appear mainly in the fishing grounds in January–February of the Sekinchan coast, Selangor (Ramli and Yusop, 2016). Considering that the planktonic larvae stage period and the settled spat takes time to grow to a size easily confirmed by the naked eye, the spats occurring in January–February were suggested to spawn around November. In addition, Laureta and Marasigan (2000) investigated the reproduction cycle of *P. orientalis* inhabiting the coast of Iloilo City in the Philippines, with a sex ratio of 53% males and 47% females (n = 147); the minimum maturity size was 59 mm for males and 64 mm for females, their spawning season was almost year-round, and the peak season was from June to October during the rainy season in this region. On the other hand, the sex ratio was 54.4% for males and 45.6% for females (n = 68), and the minimum maturation size was 55.7 mm for males (Stage III; Nov.) and 51.7 mm for females (Stage III; Nov.) in this study. Compared with the results in the Philippines, the ratio of males was slightly higher, and sexual maturation was confirmed in males and females with shorter shell lengths. In addition, in this study, many individuals in the spawning stage were observed from August to November; the population along the Sekinchan coast continued to spawn for several months and maturation was observed to be synchronised in males and females. Therefore, it was concluded that the peak spawning period of *P. orientalis* is in the rainy season in this region, and it seems to be similar to the reproductive cycle characteristics of *P. orientalis* in the Philippines. Furthermore, focusing on the monthly rainfall in 2014, the rainfall in August of this year was 282 mm, which was approximately twice the volume of normal rainfall. This amount was comparable to that during the peak of the rainy season (October–November) in this region. For this reason, in the coastal areas in August, rainfall may have supplied nutrients to the coastal areas through rivers, caused the bloom of phytoplankton for feeding bivalves, and resulted in the development of the gonads of *P. orientalis* to be earlier than usual. The results of nutrient monitoring in the estuary along the coast of Selangor by Shimoda *et al.* (2020) show that nutrient levels increase when river water flows into the estuary, especially during the rainy season. In addition, Yurimoto *et al.* (2020, 2021b) conducted regular environmental monitoring along the coast of Selangor, often measuring chlorophyll-a level above 10 $\mu g.L^{-1}$ along the coastal area during the rainy season (September–November). Additionally, according to NASA's WORLDVIEW (https://worldview.earthdata.nasa.gov), satellite image data (Terra/Aqua-MODIS satellite image) on 21st August and 15th November 2014 showed the occurrence of phytoplankton in Selangor, Malaysia. It has been shown that the area around the *P. orientalis* fishing ground (3.49 °N, 101.09 °E) in Selangor had a chlorophyll-a concentration of more than 6 $mg.m^{-3}$ ($\mu g.L^{-1}$) on both days, suggesting that sufficient phytoplankton volume occurred for the clams at the time. Furthermore, the distribution map of changes in chlorophyll-a concentration along the coast of Selangor using the Global Eutrophication Watch also showed that the chlorophyll-a concentration in this area has remained high and stable for many years (Fig. 7). Additionally, considering the ingestion status of food in the digestive tube of *P. orientalis* and the condition of the epithelium of the midgut gland in August, more than 80% of the individuals ingested food and the epithelium of the midgut gland was thick. Therefore, there was no problem with the ingestion, digestion, and nutritional absorption status of food. In addition, *P. orientalis* obtained in November had digestive gland conditions as more

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than 90% of the individuals ingested food, and the epithelium of the midgut gland was thick. Therefore, it was concluded that this area had a good feeding environment, and that there were no problems with the feeding conditions and sexual maturation of *P. orientalis*. However, a decrease in the catch of *P. orientalis* has become a problem in recent years. In this study, the collected individuals were mainly small individuals (less than 90 mm), and there is concern about the effects of overfishing in the area. As a countermeasure, it is important to limit the catch size of *P. orientalis* so that large individuals maintain the reproductive population or to set a closed fishing area and/or period during the spawning season to promote their reproduction.

Conclusion

P. orientalis, which inhabits Sekinchan in Selangor, Malaysia, peaked their spawning in November during the rainy season. In addition, the presence or absence of food in the digestive tube of the clams and the condition of the epithelium in the midgut gland confirmed the presence of food in many individuals, and the condition was good. Therefore, there were no problems with *P. orientalis* sexual maturation and feeding conditions. In addition, from the chlorophyll-a data on the homepage of the Global Eutroohication Watch website, the coastal water off Sekinchan experienced a stable phytoplankton occurrence area of more than 5 $mg.m^{-3}$ ($\mu g.L^{-1}$) which is one of the reasons for the good fishing grounds for *P. orientalis*. However, the clams in this area are small, mainly with a shell length of less than 90 mm, and resource protection measures are important.

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