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Accumulation of Lipofuscin and Preliminary Estimation of Age-Structure in Wild Mud Crab (*Scylla paramamosain*) Population in Tropical Mangrove Swamps, Thailand

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Abstract: The age structure of wild mud crab (*Scylla paramamosain*) was explored using autofluorescent age pigment, lipofuscin. Samples were collected from the mangrove swamp area in Pak Phanang mangrove swamps, Thailand. The carapace width-frequency distribution did not show any distinct modes of the sample population, whereas lipofuscin concentration showed positive correlation with carapace width. Lipofuscin concentration in the Olfactory Lobe Cell Mass (OLCM) of the brain was measured using image analysis of fluorescent micrographs. The lipofuscin concentration (% of area fraction) ranged from 0.06 to 0.26 with the formation of three regularly-spaced modes developed by modal analysis that could be regarded as distinct age classes. Strong correlation was found between lipofuscin concentration and modes observed in the lipofuscin concentration histogram ($R^2 = 0.99$) and the lipofuscin accumulation rate was almost constant (0.08% of area fraction) in each year. Although, existence of wide size ranged population in a lipofuscin concentration mode, the analysis suggested that *S. paramamosain* live in the mangrove ecosystem at best of 2^+ year class.

Key words: *Scylla paramamosain*, lipofuscin quantification, age estimation

INTRODUCTION

Understanding the age structure of wild population of mud crabs is undoubtedly necessary for better stock management. This resource provide basic source of income for coastal fishing communities throughout the Indo-Pacific region, especially in Thailand (Moser *et al*., 2002). But difficulties in age determination in crustaceans are apparent due to high variability in growth rates and molting frequencies. It is also impossible to use permanent hard body parts as growth indicator, frequently used in other animals, because of the crustacean's molting properties.

Thus, growth parameters in crustaceans have been traditionally assessed either by tagging and recapture experiments (Moser *et al*., 2002; Le Vay *et al*., 2007) or using specimens cultured in captive condition (Plaut and Fishelson, 1991; Hill, 1992) or analysis of length-frequency data (Rothschild *et al*., 1992). However, each method has limitations to use for estimation of crustacean age. Such as many tags lost during molting (Van Montfrans *et al*., 1986; Fitz and Wiegert, 1991), time consuming for rearing aspects if the organisms are longevous as well as error due to artificial condition and limitation of using length-frequency data because growth difference in individuals (Prager *et al*., 1990; Ju *et al*., 2001; Moser *et al*., 2002). Recently, quantitative studies of lipofuscin have encouraged researchers to determine age on the basis of chronological deposition of lipofuscin in neuron cell masses.

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Lipofuscin is a lipopigment that is produced in secondary lysosomes as a result of cellular metabolism (Dowson and Harris, 1981). The universal property of lipofuscin is the emission of yellow to greenish autofluorescence when excited with ultraviolet or blue light (Sohal and Wolfe, 1986; Brunk *et al*., 1992). These characteristics have given the lead to measurements of the autofluorescence and to quantify the amount of lipofuscin accumulated by the cells for application in age determination (Dowson, 1982; Marzabadi *et al*., 1992). Although lipofuscin are likely to form in all postmitotic cells (Sheehy, 1989), most cells turnover at different rates, which is difficult to follow over the lifespan of an organism. Nervous tissues are special as they divide and are replaced very slowly in all organisms. Thus, nervous cells can accumulate lipofuscin for relatively longer periods, hence suggesting their usability for measuring age. The promising results in aging crustaceans were achieved by in situ quantification of lipofuscin granules on histological sections of nervous tissue using fluorescence microscope (Sheehy, 1989).

To date, the quantification of lipofuscin method were successfully applied in many studies of wild crustaceans population *Cherax cuspidatus* (Sheehy, 1989), *Notocrangon antarcticus* (Bluhm and Brey, 2001), *Waldeckia obesa* (Bluhm *et al*., 2001), *Oratosquilla oratoria* (Kodama *et al*., 2005) and in captive condition *Cherax quadricarinatus* (Sheehy, 1990a; Sheehy *et al*., 1994), *Euphausia superba* (Nicol *et al*., 1991), *Homarus gammarus* (Sheehy *et al*., 1996), *Marsupenaeus japonicus* (Vila *et al*., 2000), Dendrobranchiate shrimps (Medina *et al*., 2000), *Homarus gammarus* (Uglem *et al*., 2005).

In genus *Scylla*, although lipofuscin accumulation has been reported in nerve cell masses in the brain (Sheehy, 1990b) lipofuscin concentration was used first time as an age marker for *S. olivacea* (Islam *et al*., 2007) and no study yet in other mud crab species. The present study was conducted to gain a deeper knowledge in the existence of lipofuscin in *Scylla paramamosain* and to use the lipofuscin quantification technique to assess the age of wild population in the tropical mangrove forest, Thailand.

MATERIALS AND METHODS

Study Site

The Pak Phanang estuary is located in Nakhon Si Thamarat province, on the east coast of southern Thailand (8° 9'-11' N and 100° 9' -18' E; Fig. 1). The eastern half of the estuary is fringed by a wide mangrove forest (approximately 9,000 ha), which is associated with an extensive mud flat (1-3 km wide) that emerges at low tide. The present study was conducted within the eastern mangroves that cover approximately 7,000 ha, or 82% of the total Pak Phanang mangroves (Fig. 1). The average rainfall ranges about 2000-3000 mm and salinity fluctuates between 1-25 ppt (Boromthanarath *et al*., 1991). Crab fishing is conducted throughout the year within the mangrove channels as well as associated channels connected with the bay.

Samples

Samples were collected randomly seven times from the middlemen traders in the mangrove communities during June 2006 to January 2008. The live crabs were brought back to the laboratory where internal carapace width (ICW: the distance across the carapace between the eight and ninth anterolateral spines) were measured using digital caliper. The lipofuscin analysis focused on the samples of May 2007 in which *S. paramamosain* constitutes 42% in species composition. Mud crab recruitment is year round but since mature females were observed to migrate offshore mostly from June (fishermen's experience), samples from May is expected to contain various age classes. Crab samples were ice-shocked to anaesthetize the animal. The head part (containing the brain) was then dissected out and was fixed in 10% neutral buffered formalin. After 10 days of fixation, the brain was isolated and preserved in 70% ethanol for histological observation.

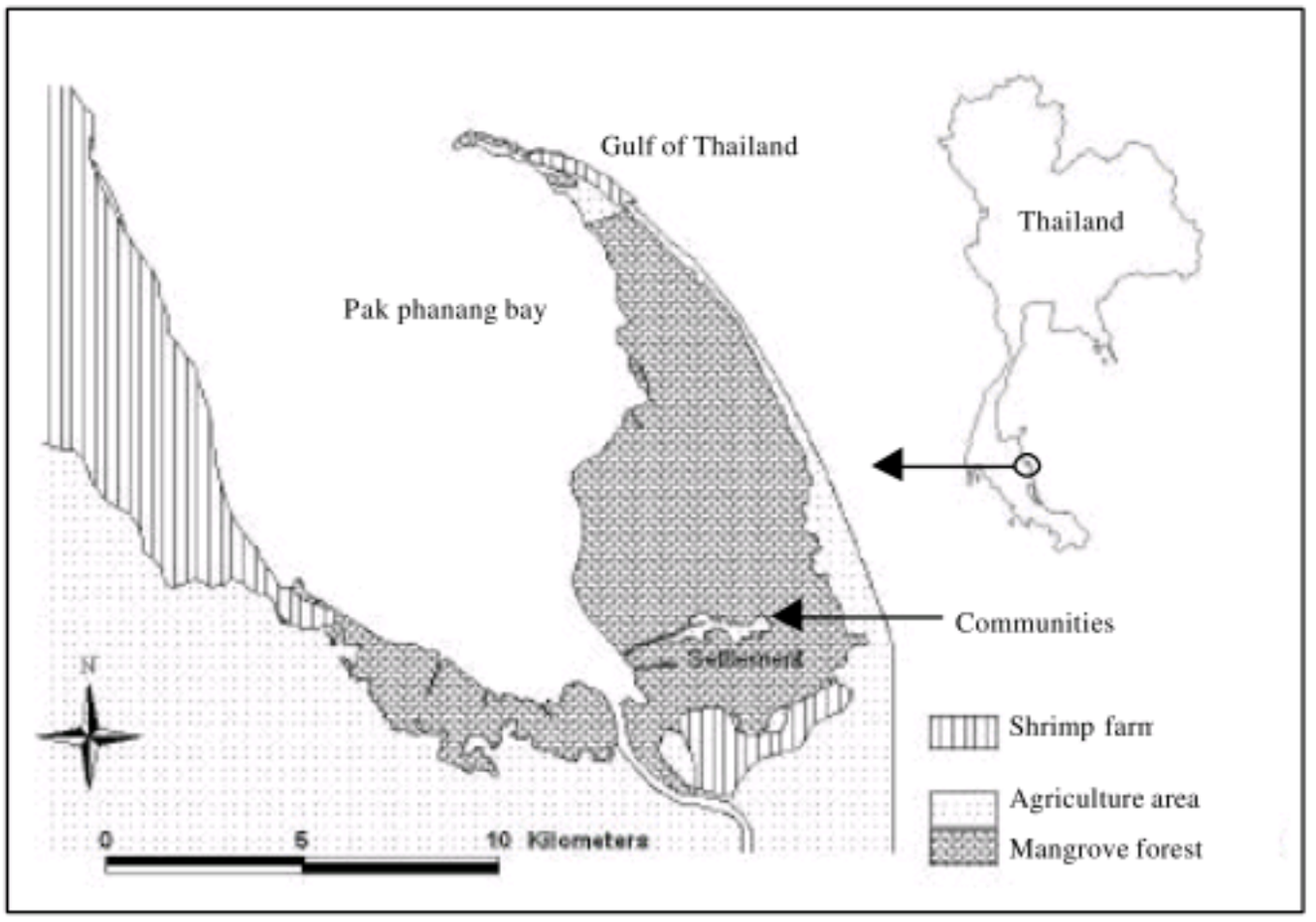

Fig. 1: Study area, Pak Phanang mangrove ecosystem and the sampling place (fishermen communities) inside of the mangrove

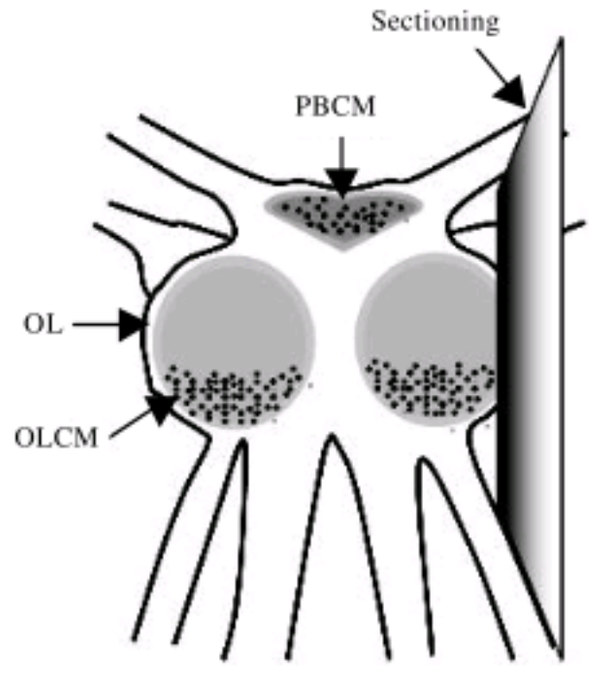

Fig. 2: Schematic drawing of dorsal view of the brain of mud crab and the position of Olfactory Lobe Cell Mass (OLCM) and Protocerebral Lobe Cell Mass (PBCM). The OLCM (dotes) in the Olfactory Lobe (OL) was dissected longitudinally for the lipofuscin analysis in crab brain

Quantification of Lipofuscin

The brain samples were dehydrated in ascending ethanol concentrations from 70 to 100%, transferred to lemosol and embedded in paraffin. Longitudinal serial sections of the samples were cut at 5 μm (Fig. 2). All sections were de-waxed through three 10 min xylene changes and mounted without staining. The Olfactory Lobe Cell Mass (OLCM) was noticed large and easily visible and used to quantify lipofuscin in the present study.

Fluorescent microscope (Olympus-BX51, Japan) was used to detect autofluorescence of lipofuscin. The histological sections of OLCM at the left side of the brain were excited at a 488 nm excitation wavelength and images were taken with 40x lenses. A total of 10 central most OLCM digital images were taken from each brain with a resolution of 512×512 pixels. The images were edited and quantified lipofuscin concentration using Photoshop CS2 image processing software. The outline of the OLCM in the image was traced manually to select the area of analysis and then maximizing the contrast of lipofuscin by using gray-scale thresholding binary image. The ImageJ software (National Institute of Health, USA) was used to measure the area fraction (%) of lipofuscin granule in earlier outlined OLCM area. The geometric average area fraction was calculated from the 5-10 sections of an individual and then used for statistical treatments.

Modal Analysis

The Kolmogorov-Smirnov test (Sokal and Rohlf, 1995) was conducted to identify any difference in frequency distributions between the sexes. An internal carapace width-frequency distribution (ICFD) was established from the size-data of 129 specimens, using class interval of 5 mm. A lipofuscin concentration-frequency distribution (LFD) was constructed from samples in May (19 individuals) used for the pigment concentration analysis with 0.02% class interval. Potential age groups were identified by fitting normal components to modes in the LFD histogram using the modal progress analysis routine of FiSAT II (FAO-ICLARM stock analysis tools, FAO, Rome, Italy). Within this program, Bhattacharya (1967) method was applied to obtain initial values for mode means, which were refined using NORMSEP (after Hasselblad, 1966) to obtain the mode distribution parameters (mean, standard deviation and number of individuals) for each normal distribution. To reveal the age composition within each size class, potential age groups based on the lipofuscin analysis were applied to the ICW histogram of the sample used for the lipofuscin analysis.

RESULTS

Carapace Width-Frequency

The male-female distribution did not differ significantly (Kolmogorov-Smirnov test, $p>0.05$) in any month (Fig. 3). The immature and mature crabs (>110 mm ICW; Overton and Macintosh, 2002) were noted in each sampling time. In the ICFD, one to three modes were observed but the numbers and position of modes were not consistent over the sampling months (Fig. 3). Some modes were not visually obvious.

Lipofuscin Concentration

Lipofuscin was identified by its bright yellow autofluorescence and by its round or irregular granular shape, usually = 2 μm in diameter, which sometimes formed in aggregates of several granules (Fig. 4). There was no significant difference between the sexes in the lipofuscin concentration frequency distribution (Kolmogorov-Smirnov test, $p>0.05$), hence sexes were not treated separately in further analysis. Lipofuscin concentrations varied between 0.06 and 0.26% area fraction. Lipofuscin concentration progressively increased with increasing ICW (Fig. 5) and the relation could be linearly regressed ($L = 0.003\ ICW - 0.13$; $R^2 = 0.74$, $p<0.05$) that showed three clusters in the sample population (Fig. 5). Adjacent lipofuscin groups did not overlap largely. Each cluster was numbered in ascending order as Mode M (M = I, II, III).

The formation of clusters in lipofuscin concentrations were justified by the Hasselblad`s method. This modal showed similar three regularly-spaced and visually-obvious normal distributions in the lipofuscin-concentration frequency (Fig. 6). The highest proportion (47%) of individuals was belonging

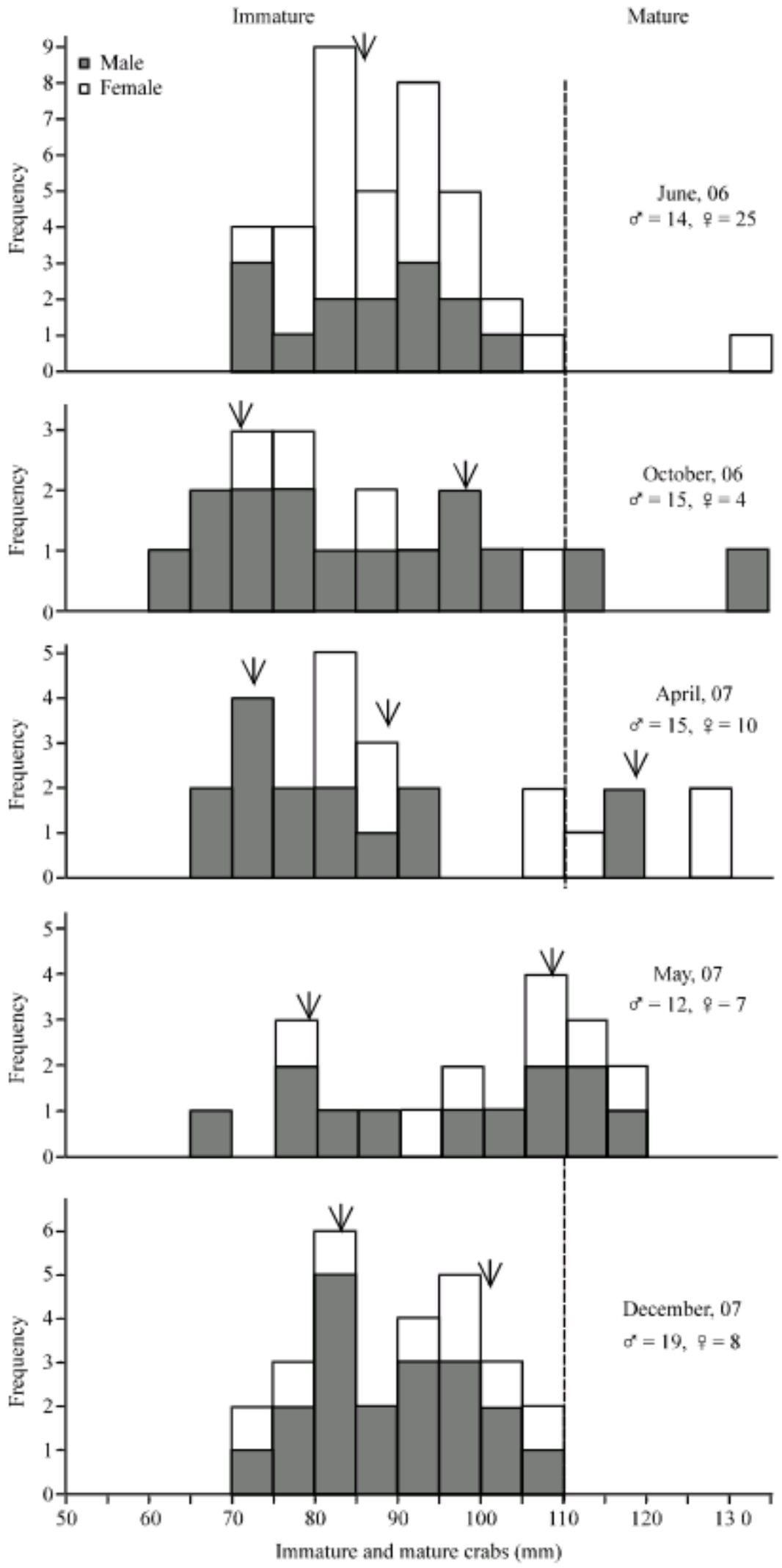

Fig. 3: Size-frequency distributions for samples of *Scylla paramamosain* examined from Pak Phanang mangrove ecosystem during June 2006 to December 2007. Arrows indicate modes separated by Hasselblad`s method. The dash lines show division between immature and mature crabs, respectively

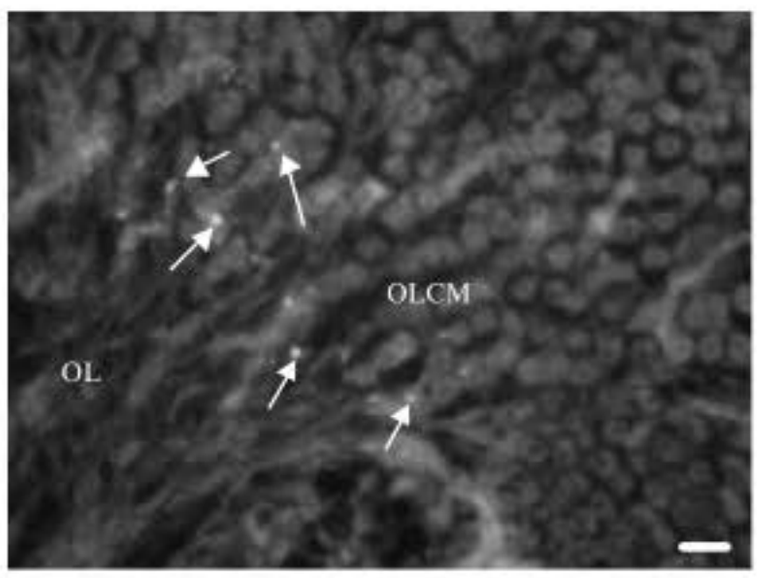

Fig. 4: The accumulated fluorescent lipofuscin granules (some arrowed) in the olfactory lobe cell mass of *Scylla olivacea*. Scale bar = 10 µm

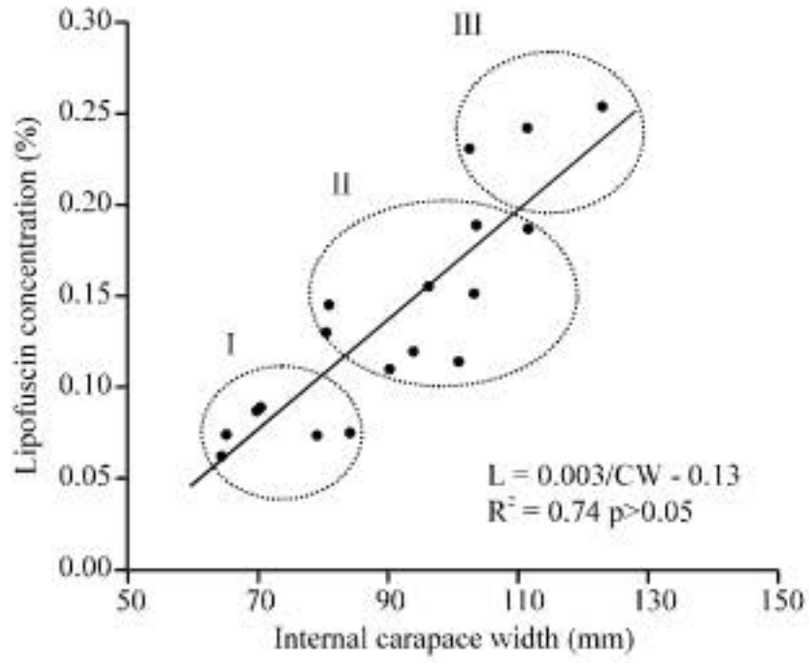

Fig. 5: Scatter plot of lipofuscin concentration against internal carapace width of *Scylla paramamosain* collected from Pak Phanang mangrove ecosystem, Thailand, during May 2007. The short horizontal lines show the mean value of each mode and vertical lines representing ±2SD (95% confidence limit)

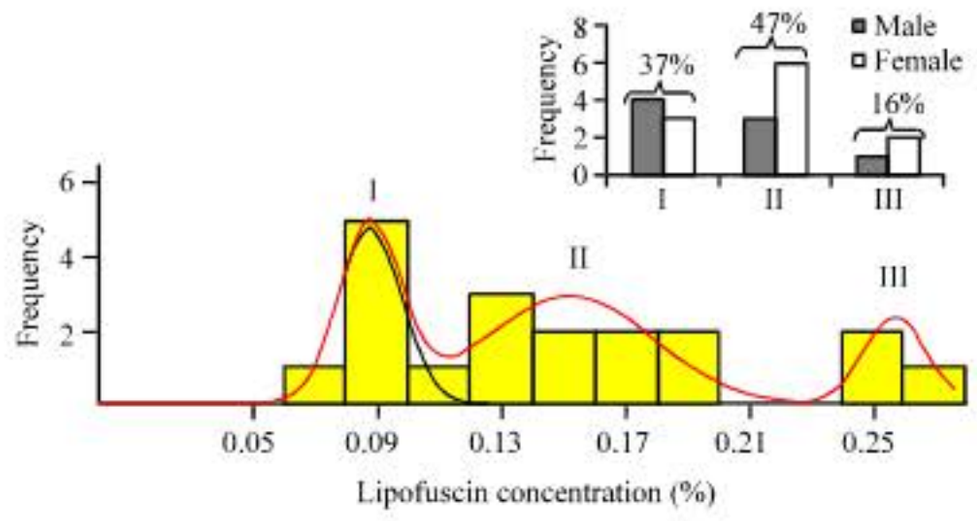

Fig. 6: Frequency distribution of the lipofuscin concentration of *Scylla paramamosain* collected from Pak Phanang mangrove ecosystem, Thailand, in May 2007. The potential age classes are shown as numbers (I, II, III) that separated by Hasselblad's method. The populations occupied by the modal groups are also shown (inset)

Table 1: The population demography of *Scylla paramamosain* collected in May 2007 at the Pak Phanang mangrove swamps, Thailand

LF mode	LF index	Individuals	Size class	Range (mm ICW)
I	0.08±0.01	7	75±10	65-91
II	0.16±0.02	9	94±12	80-110
III	0.25±0.01	3	117±10	103-123

Mean±SD. Lipofuscin (LF) modes, LF index, population size and size class developed by the Hasselblad's model analysis

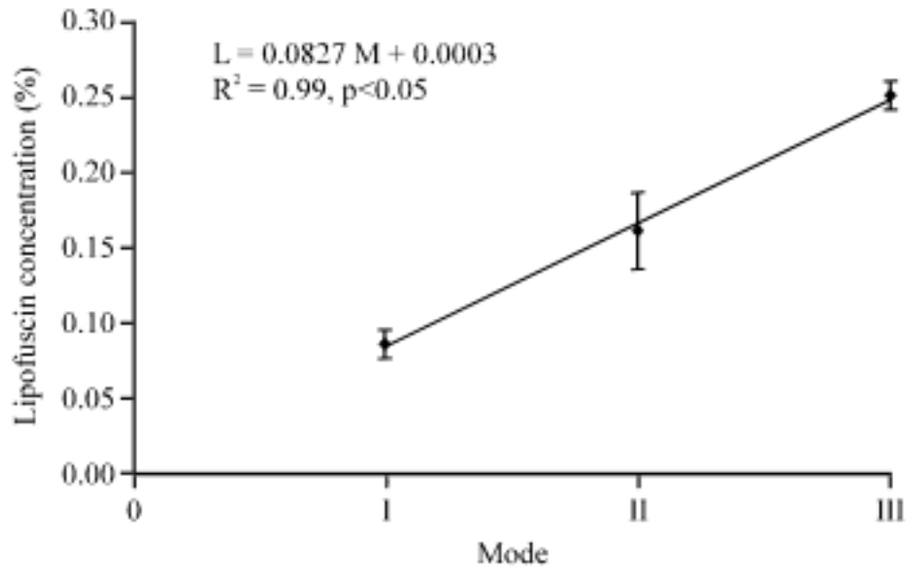

Fig. 7: Relationship between lipofuscin concentration and modes of three groups formed by the modal analysis of frequency distribution of lipofuscin concentrations of *Scylla paramamosain* collected from Pak Phanang Bay, Thailand. Vertical bars show 95% confidence limits

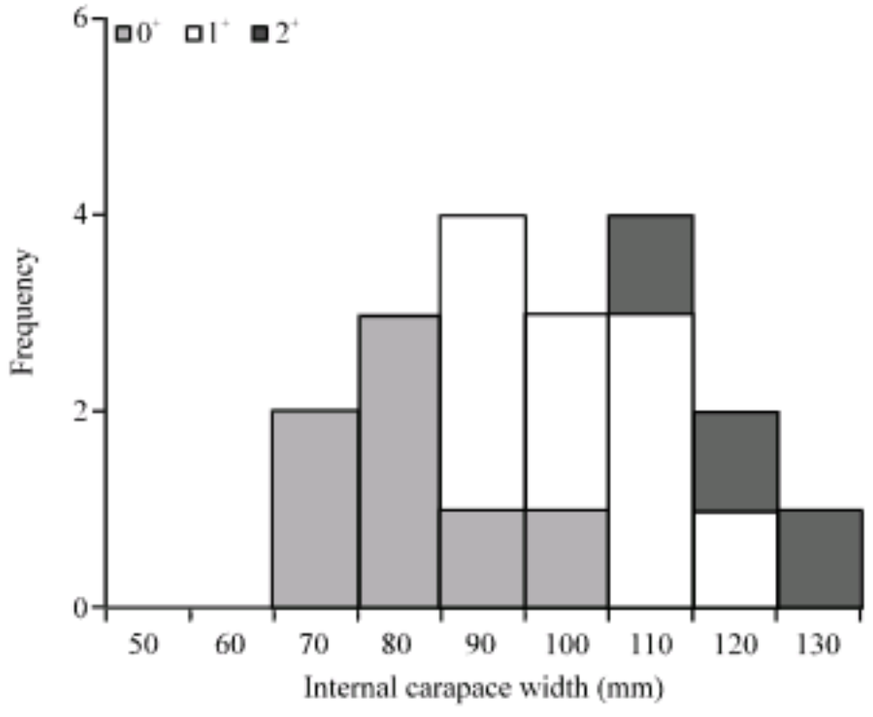

Fig. 8: The internal carapace width-frequency distribution of *Scylla paramamosain* samples during May 2007. The modes developed by the lipofuscin analysis are applied to the ICW histogram

to mode II. The mean values of the three peaks I, II and III in the lipofuscin concentration distribution were 0.08±0.01, 0.16±0.01, 0.25±0.01, respectively (Table 1). The mean size of the lipofuscin modes I, II and III were 75, 94 and 117 mm ICW, respectively (Table 1).

The relationship between lipofuscin concentration L and mode of each lipofuscin group M is shown in Fig. 7 and the linear regression equation defining the relationship is L = 0.0827 M+0.0003 (R^2 = 0.99, $p<0.05$), corresponding to an annual lipofuscin accumulation rate of 0.08% area fraction. Despite a positive correlation between ICW and lipofuscin concentration (correlation coefficient

r = 0.85; p<0.05), there was a considerable dispersion of ICW within each lipofuscin groups. Several of the lipofuscin groups (Fig. 5) were noticed in each size class (Fig. 8).

DISCUSSION

The discontinuities in mode distributions noticed in internal carapace width distributions (Fig. 3) and hence age determination of *S. paramamosain* is subjected to difficult. It might be due to different growth rates of individuals. Though there is no data regarding the growth pattern of *S. paramamosain* but different growth rate mentioned in other *Scylla* species. For example, Moser *et al*. (2002) mentioned different growth rates of individuals in a cohort from fast growing to slow growing individuals in case of *S. olivacea*. Thus, a cohort starting in a small size class will have some individuals reaching the largest size class very quickly, while the majority still remains in the medium or lower size classes. Moreover, in general, the increment of carapace width of crustaceans varies between individuals under-going the same molt (Hartnoll and Abele, 1982) and the interval between successive molts becomes longer as age increases, particularly after sexual maturity (Abe, 1982; Kodama *et al*., 2005). In the present study, samples were taken from commercial middlemen; hence the smaller crabs less than the sampled size (65 mm ICW) were not included in the present analysis.

In the present study, we could not find any difference in accumulation of lipofuscin between sexes. In other studies, differences were not found between male and female in the aspect of lipofuscin accumulation with growing age in other crustaceans like *Marsupenaeus japonicus* (Vila *et al*., 2000), *Homarus gammarus* (Sheehy *et al*., 1996; Uglem *et al*., 2005); *Cherax quadricarinatus* (Sheehy, 1992); *Oratosquilla oratoria* (Kodama *et al*., 2005) and *Scylla olivacea* (Islam *et al*., 2007). Thus, the combinations of data from both sexes were used for analysis in this study.

A linear relationship observed between size and lipofuscin concentration in the samples in May 2007 (Fig. 5) which indicate that lipofuscin concentration increases with growth of *S. paramamosain.* The similar phenomena also noticed in *S. olivacea* (Islam *et al*., 2007) as well as in other species (Sheehy, 1990a; Sheehy *et al*., 1998; Kodama *et al*., 2005). In the distribution in size and lipofuscin concentration of the samples, obvious breaks existed in lipofuscin concentration, which were not found in the size distribution (Fig. 3). In the regression analysis between order of peaks and modes of lipofuscin concentration (Fig. 7), a higher regression coefficient was observed indicating that the peaks has the same interval with the lipofuscin accumulation period. In addition, the lipofuscin accumulation period as observed in wild population of other crustaceans (Sheehy *et al*., 1998; Bluhm and Brey, 2001; Kodama *et al*., 2005; Islam *et al*., 2007) also show the applicability of microscopic quantification of lipofuscin as a tool for cohort analysis and age determination.

Though lack of life-pattern data on *S. paramamosain,* it is noticed that other *Scylla* species such as *S. olivacea* takes 3-4 weeks of larval development (Moser *et al*., 2005) and do not enter into the mangroves until the Instar 1 stage (Moser and Macintosh, 2001). In the Instar 1 stage, crabs settle in the mangrove ecosystem for at least 1 month old. They takes 3-4 months to reach the smallest size (50 mm ICW) to be caught by commercial fishermen and another 4-5 months to reach sexual maturity (>90 mm ICW; Moser *et al*., 2005). Therefore, the 1st lipofuscin mode in *S. paramamosain* (75 mm ICW; Table 1) and/or the youngest age group caught in May 2007 were undoubtedly less than 1-year age group. Also, 70-80 mm ICW classes are composed mainly of the 1st lipofuscin mode (Fig. 8), suggesting that the 1st lipofuscin mode corresponds to the 0^+ year age group.

It was difficult to infer the 2nd and 3rd modes of lipofuscin as distinct age groups from our results. Due to the year-round existence of smaller and larger individuals in all sampling periods, they are continuous breeders. However, they showed some periodic peaks (Islam, 2008) and higher breeding was noticed during dry season (April-May). According to such periodic peak breeding, *S. paramamosain* would be recruited highly in very following month (Fig. 9). So, the individuals those

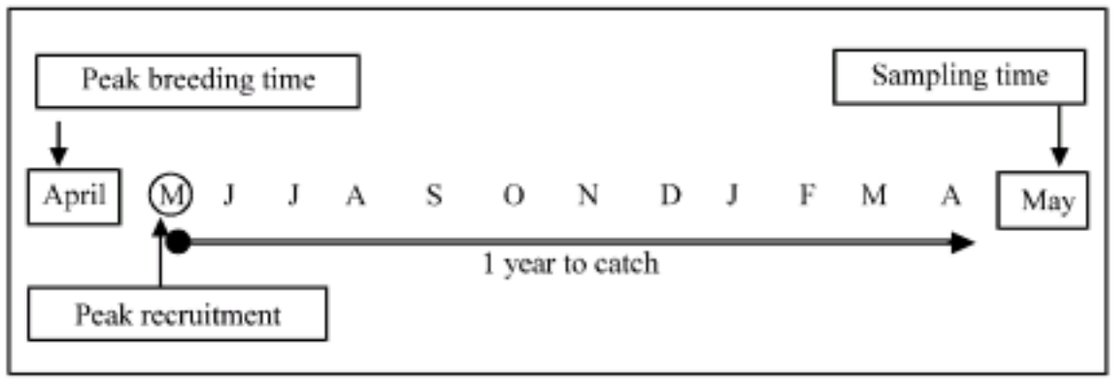

Fig. 9: The schematic diagram of the breeding and recruitment pattern of *Scylla paramamosain* in Pak Phanang mangrove swamps, Thailand, including the indication of presumable age-structure at the time of sampling, May 2007

recruited to the fishery in May they will be reached 1-year age by the next May. Thus, in the sample of May, higher individuals would be belonging to 2nd mode/cluster as they getting 1-year aged. In the lipofuscin concentration analysis, the same individuals patterns noticed at the time of May sample (Fig. 6). This indicates that each cluster of lipofuscin concentration exactly represented distinct age groups.

In other crustaceans, lipofuscin accumulates in nerve cell masses at an almost constant accumulation rate in rearing experiments (Sheehy *et al*., 1996) as well as from wild populations (Bluhm and Brey, 2001; Kodama *et al*., 2005; Islam *et al*., 2007). Sheehy *et al*. (1998) proved that annual accumulation rate of lipofuscin in western rock lobster *Panulirus cygnus* was constant in both wild and laboratory-reared specimens. It is considered that the modes of lipofuscin as an age classes, the regression equation (Fig. 7) indicate that lipofuscin accumulation in OLCM of *S. paramamosain* were at an almost constant annual accumulation rate of 8.0×10^{-2} % volume fraction that could be afforded that each of the groups corresponded to a distinct age class.

Moreover, regularly spaced modes in lipofuscin concentration histogram in wild population of other crustacean species have been observed in other studies, in which relationship between lipofuscin modes and age was established (Sheehy *et al*., 1998; Bluhm and Brey, 2001; Kodama *et al*., 2005, 2006; Islam *et al*., 2007). The present study also showed regularly spaced modes in lipofuscin concentration histogram with strong linear relationship between modes. Therefore, it would be presumable to regard groups I, II and III as a distinct age class of 0^+, 1^+ and 2^+, respectively that also supports the average 3 year life expectancy of mud crabs (Heasman *et al*., 1985).

CONCLUSION

Conclusively, the present study showed the possible application of the lipofuscin microscopic observation for age determination of *S. paramamosain*. The weakness of the present study for the validation of this method is small sample size and lack of seasonal movement of lipofuscin cohorts. For future validation purposes, a year-round observation of the lipofuscin cohorts and/or examination of lipofuscin concentrations of specimens with known ages are recommended.

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