

Preliminary assessment for age estimation of wild population of mud crab (*Scylla olivacea*) in Pak Phanang Bay, Thailand, using histologically quantified lipofuscin as age marker

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Abstract: The age structure of wild mud crab (*Scylla olivacea*), one of the most important mangrove fisheries resource in the Southeast Asian region, was explored using autofluorescent age pigment, lipofuscin. Samples were collected from the mangrove swamp area in Pak Phanang Bay, Thailand. The carapace width-frequency distribution did not show any distinct modes of the sample population. Lipofuscin concentration in the olfactory lobe cell mass (OLCM) of the brain was measured using image analysis of fluorescent micrographs and its concentration showed positive correlation with carapace width. The lipofuscin concentration (% of area fraction) ranged from 0.09 to 0.28 with the formation of three modes. Strong correlation was found between lipofuscin concentration and mode numbers observed in the lipofuscin concentration histogram ($R^2=0.99$) and when modes were considered as distinct ages, the lipofuscin accumulation rate showed almost constant (0.07% of area fraction) in each year. Although, existence of wide size ranged population in a lipofuscin concentration mode, the analysis suggested that *S. olivacea* live in the mangrove ecosystem at best of 2⁺ year class.

Keywords: Mud crab, lipofuscin quantification, age estimation, mangrove ecosystem

1. Introduction

Mud crabs of the genus *Scylla*, commercially important and conspicuous crustacean found in intertidal and subtidal coastal habitats (KEENAN *et al.*, 1998), are traditionally exploited in a number of ways by artisanal fishermen (MACHINTOSH *et al.*, 1993). Mud crabs provide basic source of income for coastal fishing communities throughout the Indo-Pacific region, especially in Thailand (MOSER *et al.*,

2002). However, this important resource is presently vulnerable and proper management is becoming a key issue. Understanding the age structure of wild population of mud crabs is undoubtedly necessary for better stock management. 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, which is 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; VAY *et al.*, 2007) or using specimens cultured in captive condition (PLAUT and FISHELSON, 1991; HILL, 1992). Recently, quantitative studies of lipofuscin have encouraged researchers to determine age on the basis of chronological deposition of lipofuscin in neuron cell masses.

Lipofuscin is a lipopigment that is produced in secondary lysosomes as a result of cellular metabolism (DOWSON and HARRIS, 1981). Since

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the formation of lipofuscin is dependent upon metabolism, it should increase in concentration as long as the cell is alive (HARVEY *et al.*, 1999). 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 the determination of ages (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. Pioneering work in the analysis of extractable fluorescent age pigments using spectrofluorometry was done by ETTERS HANK (1983 and 1985), who used lipofuscin for aging crustaceans but was criticized on the spectrofluorometric technique in the following years (NICOL, 1987; HILL and WOMERSLEY, 1991; SHEEHY, 1996). Later, 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). SHEEHY (1990a) was the first to confirm the widespread occurrence of lipofuscin-like fluorescent material in the brain of crustaceans and also to find a broad correlation between the adult body size of the species and the occurrence of the fluorescence. In fact, morphological lipofuscin has been found to occur in (associated with) the neuron soma in all cell masses of the brain and eyestalks of decapod crustaceans (SHEEHY, 1989 and 1990a; SHEEHY and WICKINS, 1994, SHEEHY *et al.*, 1996), being particularly conspicuous in the globule cell masses associated with the olfactory lobe.

To date, the quantification of lipofuscin method were successfully applied in many studies of wild population of crustaceans (*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, 1990b and 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, 1990a), lipofuscin concentration has never been used as an age marker. However, the wide application of this technique on other crustaceans to estimate population age encouraged us to apply histological lipofuscin quantification methods for wild population of mud crabs. The present study was conducted to gain a deeper knowledge in the existence of lipofuscin in mud crabs (*Scylla olivacea*) and to use the lipofuscin quantification technique to assess the age of wild population in the tropical mangrove forest, Pak Phanang, Thailand.

2. Materials and methods

2.1 Study site

Pak Phanang Bay is located in Nakhon Si Thammarat province in the southeastern part of Thailand, covering an area of 126 km². The eastern side of the bay is largely occupied by mangrove forest (approximately 90 km²) and an extensive mudflat (1–3 km wide) emerges at low tide. The present study was conducted within the eastern mangroves that cover 6994 ha, 82% of the total Pak Phanang district mangroves (Fig. 1). THAMPANYA *et al.* (2002) mentioned that there are three distinct seasons; hot-dry season (February–May), rainy season (June–September) and the highest rainfall period of monsoon season (October–January) with water temperature ranges between 25 and 36°C. The average rainfall in this area 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.

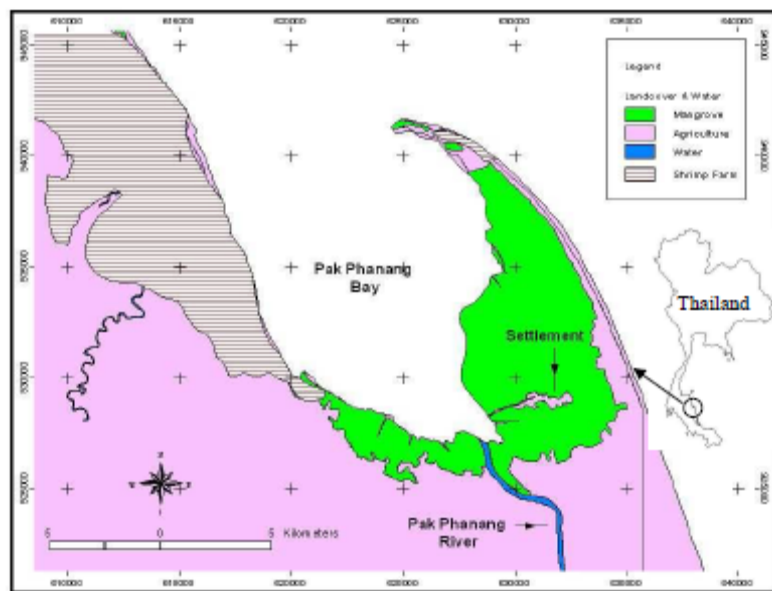


Fig. 1. Study area, Pak Phanang mangrove ecosystem and the sampling place (fishermen settlement) inside of the mangrove.

2.2 Samples

Crab trap is the main gear using for mud crab fishery in the Pak Phanang mangrove swamps. Crabs also captured by bare hand and as bi-catch of channel trap that used for shrimp fishing. Samples were collected randomly from the middlemen traders in the mangrove communities on October and November in 2006 and on April and May in 2007. 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. Crab species were identified from their color and external morphology as suggested by KEENAN *et al.* (1998). Three species (*Scylla olivacea*, *S. paramamosain*, and *S. serrata*) were identified with *S. olivacea* accounting for the highest composition of 46%. The present study on lipofuscin analysis focused on the May 2007 samples where 43% (21 out of 45) were identified as *S. olivacea*. 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.

2.3 Identification and quantification of lipofuscin

The brain samples were dehydrated in ascending ethanol concentrations from 70% to 100%, transferred to lemosol and embedded in paraffin. Serial vertical cross sections of the samples were cut at $5\ \mu\text{m}$. For confirmation of the position of the olfactory lobe (OL), some of the histological samples were stained with haematoxylin-eosin and then examined under

the optical microscope. Longitudinal serial sections were prepared from the left side of brain in dorsal view position. All sections were de-waxed through three 10-min xylene changes and mounted without staining. The observed different clusters of cell bodies were numbered according to SANDEMAN *et al.* (1992). The number 10 cell mass (corresponding to olfactory lobe cell mass; OLCM) was used for fluorescent concentration analysis in the present study as it was large and clearly visible.

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 $40\times$ 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. We used ImageJ software (National Institute of Health, USA) 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.

2.4 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 121 specimens, using class interval of 5 mm. A lipofuscin concentration-frequency distribution (LFD) was constructed from samples (21 specimens; May 2007) used for the pigment concentration analysis with 0.02% class interval. Potential cluster of samples were identified by plotting ICW against lipofuscin concentration. The observed clusters were further substantiated and the mean values of the peaks were estimated with the Hass elblad's (HASSELBLAD, 1966) methods as

supportive information.

3. Results

3.1 Carapace width-frequency

A total of 61 males and 60 females were sampled during the study period and the length frequency distribution is showed in figure 2. Numbers of immature (<94 mm ICW; KOOLKALYA *et al.*, 2006) and mature crabs were 84 (69%) and 37 (31%), respectively for all the samples while in May 2007 samples used in the lipofuscin study contained 12 (57%) and 9 (43%), respectively. The male-female distribution did not differ significantly (Kolmogorov-Smirnov test, $P > 0.05$) in any month.

Though the immature crabs (<94 mm ICW) were noted in each sampling time, small crabs (<70 mm ICW) were noticed to be more abundant in October 2006. In the ICFD, one to three modes were observed but the numbers and position of modes were not consistent over the sam-

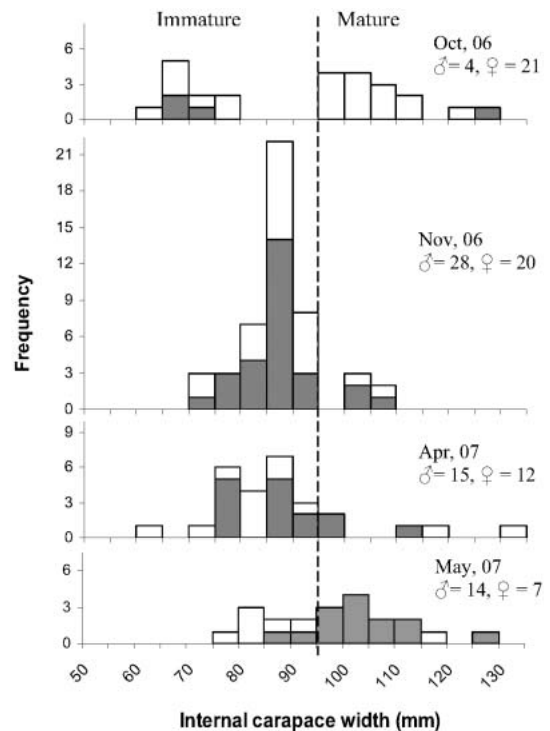


Fig. 2. Size-frequency distributions for samples of *Scylla olivacea* examined from Pak Phanang mangrove ecosystem during October 2006 to May 2007. The dash lines show division between immature and mature crabs.

pling months (Fig. 2). Some modes were not visually obvious.

3.2 Morphology of OLCM and lipofuscin

The olfactory lobes are clearly delineated as spheres lying on each side of the brain (Fig. 3A). The OLCM lies posterior to ventral of olfactory lobe in the brain of *S. olivacea*. These neuron groups are easily distinguishable from other neuronal aggregates because they consist of crescent-shaped, compact clusters of small-sized globule cells (Fig. 3B). Lipofuscin was identified by its bright yellow autofluorescence and by its round or irregular granular shape, usually $=2\ \mu\text{m}$ in diameter, which sometimes formed in aggregates of several granules (Fig. 4A & B). In the present study, although we found different clusters of cell bodies in the

crab brain, OLCM was selected for the lipofuscin study because of its relatively large size and clear indication of position.

3.3 Lipofuscin concentration

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 0.09 and 0.28% area fraction. Lipofuscin concentration progressively increased with the increase in ICW and the relation could be linearly regressed ($L = 0.0024\ \text{ICW} - 0.07$; $R^2 = 0.38$, $P < 0.05$) that showed three clusters in the sample population (Fig. 5). Adjacent lipofuscin groups did not overlap largely. Each cluster was numbered in

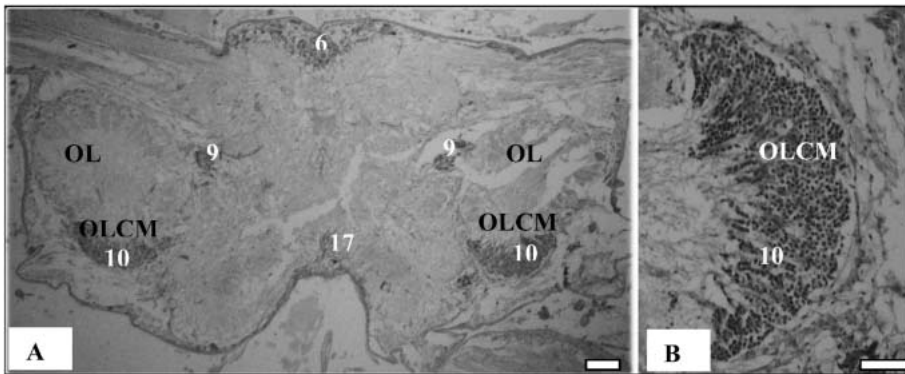


Fig. 3. A horizontal section through the brain of *Scylla olivacea*. A, The olfactory lobe (OL) lying in each side of the brain and the olfactory lobe cell mass (OLCM); scale bar = $200\ \mu\text{m}$. B, The close view of the OLCM; scale bar = $100\ \mu\text{m}$. The different clusters of cell bodies were numbered according to the SANDEMAN *et al.*, 1992.

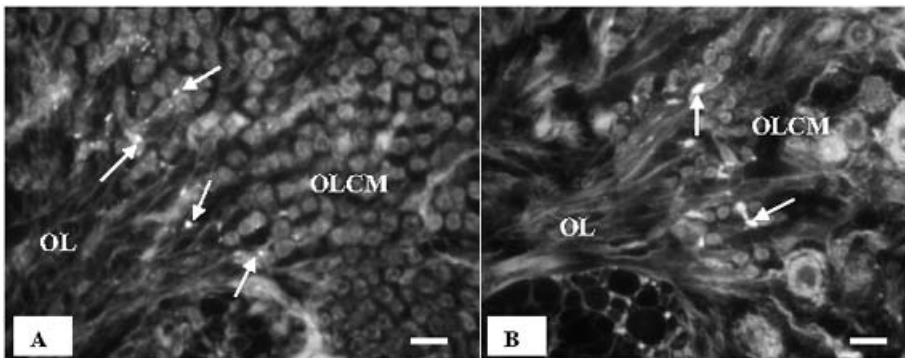


Fig. 4. The accumulated fluorescent lipofuscin granules (some arrowed) in the olfactory lobe cell mass of *Scylla olivacea*. A, Common granule type lipofuscin and B, aggregated lipofuscin granules. Scale bar = $10\ \mu\text{m}$.

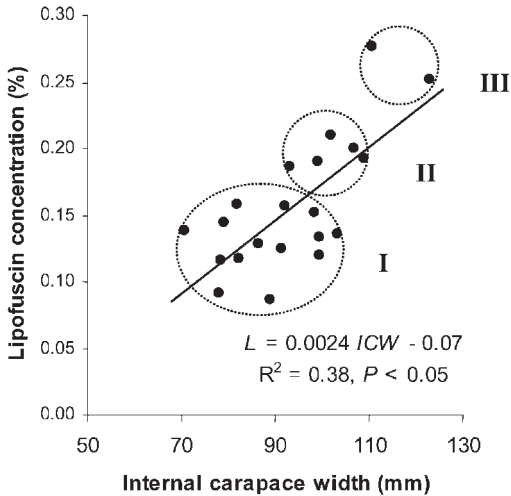


Fig. 5. Scatter plot of lipofuscin concentration against internal carapace width of *Scylla olivacea* collected from Pak Phanang mangrove ecosystem, Thailand, during May 2007. The linear relationship of lipofuscin concentration with growth and possible clusters (dotted circle) of population regarding lipofuscin accumulation.

ascending order as Mode M (M = I, II, III). The mean values of the three peaks I, II, and III in the lipofuscin concentration distribution were 0.14 ± 0.02 , 0.21 ± 0.01 , 0.28 ± 0.01 respectively (Fig. 6). The relationship between lipofuscin concentration L and mode numbers M is shown in figure 7 and the linear regression equation defining the relationship is $L = 0.07 M + 0.07$ ($R^2 = 0.99, P < 0.05$). Despite a positive correlation between ICW and lipofuscin concentration (correlation coefficient $r = 0.72; P < 0.05$), there was a considerable dispersion of ICW within each lipofuscin groups. Several of the lipofuscin groups were noticed in each size class (Fig. 8).

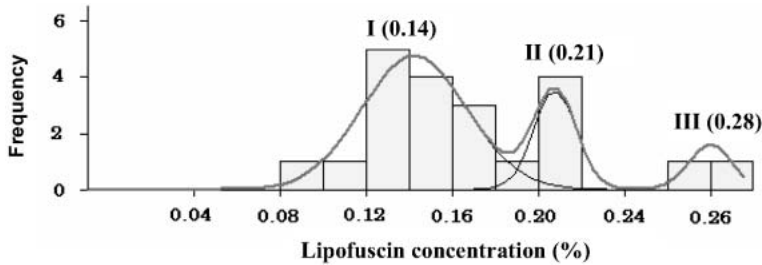


Fig. 6. Frequency distribution of the lipofuscin concentration of *Scylla olivacea* collected from Pak Phanang mangrove ecosystem, Thailand, in May 2007. The potential mean values in parenthesis of each peak estimated by Hasselblad's method.

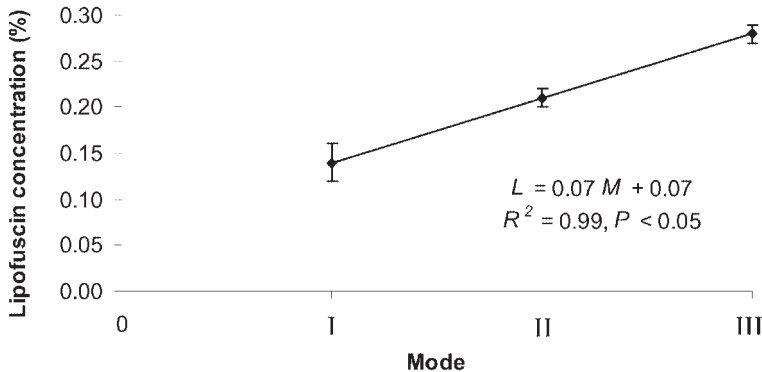


Fig. 7. Relationship between lipofuscin concentration and number of modes of lipofuscin concentrations of *Scylla olivacea* collected from Pak Phanang Bay, Thailand. Vertical bars show 95% confidence limits.

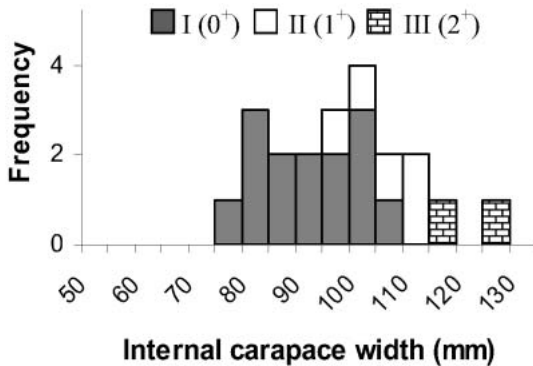


Fig. 8. The internal carapace width-frequency distribution of *Scylla olivacea* samples during May 2007. The modes developed by the lipofuscin analysis are applied to the ICW histogram. Presumable age groups are also shown in parentheses.

4. Discussion

No clear continuous mode groups can be differentiated from the carapace width-frequency analysis to estimate the wild population age (Fig. 2). There is no continuity of modes and hence age determination of *S. olivacea* is difficult. This discontinuity may be caused by the different growth rates of individuals in a cohort from fast growing to slow growing individuals (MOSER *et al.*, 2002). 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). The size range among all male and female *S. olivacea* recorded in the study was larger (70–123 mm ICW), particularly, when compared with the result by MOSER *et al.* (2005) from the Ranong province, Thailand. In the present study, samples were taken from commercial middlemen, hence the smaller crabs less than the commercial size (50 mm, 4–5 months aged; MOSER *et al.*, 2005) were not included in the present analysis.

The position of olfactory and the neuron cell

masses of *S. olivacea* were similar to those reported in *S. serrata* (SANDEMAN *et al.*, 1992). The morphology of the OLCM and globuli cells of *S. olivacea* is also similar with other brachyuran (SANDEMAN *et al.*, 1992 and 1993). Lipofuscin granules in *S. olivacea* were not greater in numbers or light intensity compared with other species (*Homarus gammarus*, SHEEHY *et al.*, 1996; *Marsupenaeus japonicus*, VILA *et al.*, 2000; *Oratosquilla oratoria*, KODAMA *et al.*, 2005). However, dense aggregations of lipofuscin granules (Fig. 4B) were often found similar to the species of *Birgus latro* (SHEEHY, 1990a).

Although microscope-based quantification of lipofuscin is rather time-consuming and labor intensive (SHEEHY, 1990b and 1996; VILA *et al.*, 2000; BLUHM and BREY, 2001), this method can give very precise measurements (SHEEHY, 2002) than other biochemical extractable lipofuscin quantification (ETTERSHANK, 1985) for crustacean age determination. SHEEHY (1996) reported that there was no relationship between intensity of autofluorescence of extracted lipofuscin and in situ lipofuscin concentration based on microscope observation of same tissue. He concluded that the extraction of lipofuscin was not stable and recommended to avoid quantification by chemical extraction. Moreover, SHEEHY *et al.* (1998) and KODAMA *et al.* (2006) verified the ageing technique of wild crustacean population using lipofuscin quantification by microscopic method. This encouraged us to apply the same procedure for wild mud crab population for the first time.

Lipofuscin accumulates in postmitotic nerve cells where cellular metabolic activity is high (SHEEHY, 1989). There were some specific nerve cell mass in different parts of the crustacean species that have been used for the purpose of histological lipofuscin quantification such as OLCM (SHEEHY, 1989; SHEEHY and WICKINS, 1994; VILA *et al.*, 2000; BLUHM and BREY, 2001), protocerebral bridge cell mass (KODAMA *et al.*, 2005) and eyestalk ganglia (SHEEHY *et al.*, 1996). In decapod species, the lipofuscin concentration is especially dense in the OLCM (SHEEHY, 1989; SHEEHY and WICKINS, 1994; VILA *et al.*, 2000; BLUHM and BREY, 2001). In the present study, we also noticed the high

concentration of lipofuscin granules in the OLCM of the brain of *S. olivacea*. We, therefore, used the OLCM for the lipofuscin quantification and hence to confirm its usefulness in age determination of *S. olivacea*.

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) and *Oratosquilla oratoria* (KODAMA *et al.*, 2005). Thus, the combination of data from both sexes was used for analysis in this study.

We observed a linear relationship between size and lipofuscin concentration in the samples in May 2007 (Fig. 5). From this, we can conclude that lipofuscin concentration increases with growth of *S. olivacea* as in other species (SHEEHY, 1990b; SHEEHY *et al.*, 1998; KODAMA *et al.*, 2005). When we compared 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. 5). In the regression analysis between order of peaks and mode numbers (Fig. 7), a higher regression coefficient was observed indicating that the peaks have the same interval with the lipofuscin accumulation period. Those results strongly support that the order of peaks indicating the order of age as shown in previous studies in wild population of other crustaceans (SHEEHY *et al.*, 1998; BLUHM and BREY, 2001; KODAMA *et al.* 2005) and show the applicability of microscopic quantification of lipofuscin as a tool for cohort analysis and age determination.

Despite the continuous year round recruitment of *S. olivacea* (MOSER *et al.*, 2002 and 2005) in Thailand, there were some periodic peaks (MOSER and MACHINTOSH, 2001). They noticed periodic portunid larvae recruitment during dry to wet (October-November) and wet to dry (March to April) seasons in Klong Ngao mangrove, Ranong province. The present study area showed different seasonal pattern, dry season in February-May, rainy season

starting in June, and heavy monsoon rains begin in October and prolonged until January (THAMPANYA *et al.*, 2002). Fishermen stated that mud crab recruitment period in Pak Phanang mangrove is from September to February (heavy rain monsoon). However, age group recruit in the mangrove system in September are not caught until February in the subsequent year due to lowest commercial crab size limit for *S. olivacea* (50 mm ICW; MOSER *et al.*, 2005).

On the other hand, periodic spawning period was also noticed for *S. olivacea*. This is around June and August-October in Ranong province (TONGDEE, 2001) and June-November in the Andaman sea, Thailand (KOOLKALYA *et al.*, 2006). Moreover, higher number of female crabs was noticed in commercial catch in May to July (MOSER *et al.*, 2005) while higher number of mature females can be observed in July-November (KOOLKALYA *et al.*, 2006) following the migration to offshore region for spawning. In the present study, female number decreased in May, 2007 that may be attributed to the migration of females to offshore for spawning in May. This is also supported by fishermen's observation of the crab's life cycle in the Pak Phanang mangroves. Crab larvae those are spawned around June reaching a 1-year age by next June.

Scylla 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 MACHINTOSH, 2001). In the Instar 1 stage, crabs settle in the mangrove ecosystem for at least 1 month old. *Scylla olivacea* 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 and/or the youngest age group caught in May 2007 were 9 to 10-months-old (0⁺ year). Also, 70-90 mm ICW classes are composed 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. 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). 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. When we consider the modes of lipofuscin as an age classes, the regression equation (Fig. 7) indicate that lipofuscin accumulation in OLCM of *S. olivacea* was at an almost constant annual accumulation rate of $7.0 \times 10^{-3}\%$ 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 and 2006). The present study also showed 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, 1980).

When we accepted the hypothesis that the cluster in figure 5B and/or the peaks in figure 6 as cohort of the age class 0^+ , 1^+ and 2^+ , respectively, there are considerable overlaps in the size between different age groups (Fig. 8). A possible explanation for the wide range of sizes can be partially explained by the long spawning period of the species in tropical area. However, the overlap of the size among size classes cannot be explained only by the long spawning season. Another explanation for the wide size class in same age group is the wide variation of growth rates in the same age group as reported by MOSER *et al.* (2002).

Conclusively, the present study showed the possible application of the lipofuscin microscopic observation for age determination of *S. olivacea*. 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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References

- ABE, K. (1982): The frequency of molting and growth of the horse crab. *Nippon Suisan Gakkaishi*, **48**, 157-163.
- BLUHM, B.A., T. BREY, and M. KLAGES (2001): The autofluorescent pigment lipofuscin: key to age, growth and productivity of the Antarctic amphipod *Waldechia obesa* (Chevreux, 1905). *J. Exp. Mar. Biol. Ecol.*, **258**, 215-119.
- BLUHM, B.A. and T. BREY (2001): Age determination in the Antarctic shrimp *Notocrangon antarcticus* (Crustacea: Decapoda), using the autofluorescent pigment lipofuscin. *Mar. Biol.*, **138**, 247-257.
- BOROMTHANARATH, S., S. COBB and V. LEE (1991): Coastal Management in Pak Phanang: A historical perspective of the resources and Issues. Hat Yai, Thailand: Coastal Resources Institute, Prince of Songkla University, p. 16-21.
- BRUNK, U.T., C.B. JONES and R.S. SOHAL (1992): A novel hypothesis of lipofuscinogenesis and cellular aging based on interactions between oxidative stress and autophagocytosis. *Mutation Res.*, **275**, 395-403.
- DOWSON, J.H. and S.J. HARRIS (1981): Quantitative studies of the autofluorescence derived from neuronal lipofuscin. *J. Microsc.*, **123** (Pt 3), 249-58.
- DOWSON, J.H. (1982): The evaluation of autofluorescence emission spectra derived from neuronal lipopigment. *J. Microsc.*, **128** (PT 3), 261-70.
- ETTERSHPANK, G. (1983): Age structure and cyclical annual size change in the Antarctic krill *Euphausia superba*. *Polar Biol.*, **2**: 189-193.
- ETTERSHPANK, G. (1985): Population age structure in

- males and juveniles of the Antarctic krill, *Euphausia superba*. Polar Biol., 4, 199–201.
- HARTNOLL, R.G. and L.G. ABELE (eds.) (1982): The Biology of Crustacea, Vol. 2. Embryology, Morphology and Genetics. Academic Press, New York, p. 111–196.
- HARVEY, H.R., D.H. SECOR and S-J. JU (1999): Biochemical measures of age in the Blue Crab, *Callinectes sapidus*. NOAA Chesapeake Bay Stock Assessment Committee workshop report 1998, p. 41–50.
- HASSELBLAD, V. (1966): Estimation of parameters for a mixture of normal distributions. Technometrics, 8, 431–444.
- HEASMAN, M.P. (1980): The biology of *Scylla serrata*. -Ph.D. thesis, Zoology Department, University of Queensland, Australia, p. 1–506.
- HILL, K.T. and C. WOMERSLEY (1991): Critical aspects of fluorescent age-pigment methodologies: Modification for accurate analysis and age assessments in aquatic organisms. Mar. Biol., 109, 1–11.
- HILL, C. (1992): Interactions between year classes in the benthic amphipod *Monoporeia affinis*- effects of juvenile survival and growth. Oecologia, 91, 157–162.
- KEENAN, C.P., P.F.J. DAVIE and D.L. MANN (1998): A revision of the genus *Scylla* De Haan, 1833 (Crustacea: Decapoda: Branchyura: Portunidae). The Raffles Bull. Zool., 46, 217–245.
- KODAMA, K., T. YAMAKAWA, T. SHIMIZU and I. AOKI (2005): Age estimation of the wild population of Japanese mantis shrimp *Oratosquilla oratoria* (Crustacea: Stomatopoda) in Tokyo Bay, Japan, using lipofuscin as an age marker. Fish. Sci., 71, 141–150.
- KODAMA, K., H. SHIRAIISHI, M. MORITA and T. HORIGUCHI (2006): Verification of lipofuscin-based crustacean ageing: seasonality of lipofuscin accumulation in the stomatopod *Oratosquilla oratoria* in relation to water temperature. Mar. Biol., 150, 131–140.
- KOOLKALYA, S., T. THAPANAND and S. TUNKIJJANUJLJ (2006): Aspects in spawning biology and migration of the mud crab *Scylla olivacea* in the Andaman Sea, Thailand. Fish. Manag. Ecol., 13, 391–397.
- MACHINTOSH, D.J., C. THONGKUM, K. SWAMY, C. CHEEWASEDTHAM and N. PAPHAVISIT (1993): Brood stock management and the potential to improve the exploitation of mangrove crabs, *Scylla serrata* (Forsk.) through pond fattening in Ranong, Thailand. Aqua. Fish. Manag., 24, 261–269.
- MARZABADI, M.R., D. YIN, and U.T. BRUNK (1992): Lipofuscinogenesis in a model system of cultured cardiac myocytes. in Free radicals and aging, EMERIT, I. and B. CHANCE (eds.). Birkhäuser Verlag, Basel, Switzerland, p. 78–88.
- MEDINA, A., Y. VILA, C. MEGINA, I. SOBRINO and F. RAMOS (2000): A histological study of the age-pigment, lipofuscin, in dendrobranchiate shrimp brains. J. Crust. Biol., 20 (3), 423–430.
- MOSER, S. and D.J. MACHINTOSH (2001): Diurnal and lunar patterns of larval recruitment of Brachyura into a mangrove estuary system in Ranong Province, Thailand. Mar. Biol., 138, 827–841.
- MOSER, S., D.J. MACHINTOSH, S. PRIPANAPONG and N. TONGDEE (2002): Estimated growth of the mud crab *Scylla olivacea* in the Ranong mangrove ecosystem, Thailand, based on a tagging and recapture study. Mar. Fresh. Res., 53, 1083–1089.
- MOSER, S., D.J. MACHINTOSH, S. LAOPRASERT and N. TONGDEE (2005): Population ecology of the mud crab *Scylla olivacea*: a study in the Ranong mangrove ecosystem, Thailand, with emphasis on juvenile recruitment and mortality. Fish. Res., 71, 27–41.
- NICOL, S. (1987): Some limitations in the use of the lipofuscin ageing technique. Mar. Biol., 93, 609–614.
- NICOL, S., M. STOLP and G.W. HOSIE (1991): Accumulation of fluorescent age pigments in a laboratory population of Antractric krill *Euphausia superba* Dana. J. Exp. Mar. Biol. Ecol., 146, 113–161.
- PLAUT, I., L. FISHELSON (1991): Population structure and growth in captivity of the spiny lobster *Panulirus penicillatus* from Dahab, Gulf of Aqaba, Red Sea. Mar. Biol., 111, 467–472.
- SANDEMAN, D., R. SANDEMAN, C. DERBY and M. SCHMIDT (1992): Morphology of the Brain of Crayfish, Crabs, and Spiny Lobsters: A Common Nomenclature for homologous structures. Biol. Bull., 183, 304–326.
- SANDEMAN, D., G. SCHOLTZ and R.E. SANDEMAN (1993): Brain evolution in decapod Crustacea. J. Exp. Zool., 265, 112–133.
- SHEEHY, M.R.J. (1989): Crustacean brain lipofuscin: an examination of the morphological pigment in the fresh-water crayfish *Cherax cuspidatus* (Parastacidae). J. Crust. Biol., 9, 387–391.
- SHEEHY, M.R.J. (1990a): Widespread occurrence of fluorescent morphological lipofuscin in the crustacean brain. J. Crust. Biol., 10 (4), 613–622.
- SHEEHY, M.R.J. (1990b): Potential of morphological lipofuscin age-pigment as an index of crustacean age. Mar. Biol., 107, 439–442.
- SHEEHY, M.R.J. (1992): Lipofuscin age-pigment accumulation in the brains of aging field- and laboratory-reared crayfish *Cherax quadricarinatus* (von Martens) (Decapod: Parastacidae). J. Exp. Mar. Biol. Ecol., 161, 79–89.
- SHEEHY, M.R.J. and J.F. WICKINS (1994): Lipofuscin

- age pigment in the brain of the European lobster (*Homarus gammarus*) (L). *Microsc. Anal.*, **40**, 23–25.
- SHEEHY, M.R.J., J.G. GREENWOOD and D.R. FIELDER (1994): More accurate chronological age determination of crustaceans from field situations using the physiological age marker, lipofuscin. *Mar. Biol.*, **121**, 237–245.
- SHEEHY, M.R.J. (1996): Quantitative comparison of in situ lipofuscin concentration with soluble autofluorescence intensity in the crustacean brain. *Exp. Gerontol.*, **31**, 421–432.
- SHEEHY, M.R.J., P.M.J. SHELTON, J.F. WICKINS, M. BELCHIER and E. GATEN (1996): Ageing the European lobster, *Homarus gammarus*, by the lipofuscin in its eyestalk ganglia. *Mar. Ecol. Prog. Ser.*, **143**, 99–111.
- SHEEHY, M.R.J., N. CAPUTI, C. CHUBB and M. BELCHIER (1998): Use of lipofuscin for resolving cohorts of western rock lobster (*Panulirus cygnus*). *Can. J. Fish. Aquat. Sci.*, **55**, 925–936.
- SHEEHY, M.R.J. (2002): Role of environmental temperature in aging and longevity: insights from neurolipofuscin. *Arch. Gerontol. Geriatr.*, **34**, 287–310.
- SOHAL, R.S. and L.S. WOLFE (1986): Lipofuscin: characteristics and significance. In: SWAAB, D.F., E. FLIERS, M. MIRMIRAN, W.A. Van GOOL and F. V. HAAREN (eds). *Progress in Brain Research*, **70**, 171–183.
- SOKAL, R.R. and F.J. ROHLF (1995): *Biometry*. W. H. Freeman and Co., San Francisco.
- THAMPANYA, U., J.E. VERMAAT and C.M. DUARTE (2002): Colonization success of common Thai mangrove species as a function of shelter from water movement. *Mar. Ecol. Prog. Ser.*, **237**, 111–120.
- TONGDEE, N. (2001): Size Distribution, Sex ratio and Size at maturity of mud crab (*Scylla spp.*) in Ranong province, Thailand. *Asia. Fish. Sci.*, **14**, 113–120.
- UGLEM, I., M. BELCHIER and T. SVASAND (2005): Age determination of European lobsters (*Homarus gammarus* L.) by histological quantification of lipofuscin. *J. Crust. Biol.*, **25** (1), 95–99.
- VAY, L.Le., V.N. UT and M. WALTON (2007): Population ecology of the mud crab *Scylla paramamosain* (Estampador) in an estuarine mangrove system; a mark-recapture study. *Mar. Biol.*, **151**, 1127–1135.
- VILA, Y., A. MEDINA, C. MEGINA, F. RAMOS and I. SOBRINO (2000): Quantification of the age-pigment lipofuscin in brains of known-age, pond-reared prawns *Marsupenaeus japonicus* (Crustacea, Decapoda). *J. Exp. Zool.*, **286**, 120–130.

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