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Quantification of the Age-Pigment Lipofuscin in Brains of Known-Age, Pond-Reared Prawns *Penaeus japonicus* (Crustacea, Decapoda)

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ABSTRACT A quantitative study of the lipofuscin content was carried out by image analysis in brains of known-age, pond-reared *Penaeus japonicus* (Crustacea, Decapoda) with the aim of assessing the applicability of the lipofuscin technique as an estimator of the physiological age in penaeids. With this purpose, three distinct measurements of lipofuscin levels (% area fraction, granule density and mean granule size) were recorded in ten sections of the olfactory lobe cell mass (OLCM) per animal. The image analysis was based on the autofluorescence emitted by the pigment, which accentuates the contrast between the lipofuscin granules and the background tissue. The concentration of lipofuscin increased significantly with age and was independent of sex. The relationship between age and lipofuscin concentration (area fraction and granule density) was best described by a seasonalized von Bertalanffy function, since the accumulation rate of the pigment dramatically slowed down in fall-winter, probably as a result of reduced seasonal metabolism. The present results confirm the potential of the lipofuscin method in the estimation of physiological age in penaeids and suggest that the application of this methodology can be useful in studies of age structure in wild populations and in the assessment of natural resources. *J. Exp. Zool. 286:120–130, 2000.*

A precise knowledge of the age structure in crustacean populations is essential for the assessment of these resources and fisheries management. However, reliable age estimations in crustaceans run into difficulties due to the high variability in molt frequencies and growth rates, in addition to the impossibility of using permanent hard parts as age indicators. Thus, the establishment of growth parameters in crustaceans has been assessed traditionally by studies of either specimens grown in captivity, tagging and recapture experiments, or size-frequency data from wild populations (Pauly et al., '84). More recently, quantitative studies of lipofuscin content have been used in age estimations on the basis that a universal characteristic of ageing in animals is the deposition of lipofuscin in non-dividing cells. This is the reason why lipofuscin is currently believed to be a reliable marker of age (Sohal and Wolfe, '86; Brunk et al., '92a; Marzabadi et al., '92).

Lipofuscin is a lipopigment that is produced in secondary lysosomes as a result of the cellular metabolism (Dowson and Harris, '81). In spite of its highly variable composition, a universal property of lipofuscin is the emission of yellow to greenish autofluorescence when excited with ultraviolet and blue light (Sohal and Wolfe, '86; Brunk et al., '92a,b). This feature has the advantage that measurements of the autofluorescence emitted by histological samples of post-mitotic tissues can serve to quantify the amount of lipofuscin accumulated by the cells and consequently may be of application in the assessment of ageing or in diagnosis of certain pathologies in animals, including man (Dowson and Harris, '81; Dowson, '82; Marzabadi et al., '92). The analysis of extractable fluorescent age pigments by spectrofluorometry was preliminarily used to assess ageing in crustaceans by Ettershank ('83, '84), but a good deal of further studies revealed that the biochemical procedure is not useful as an accurate age index

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without improvement of the original spectrofluorometric technique (Nicol, '87; Sheehy and Ettershank, '88; Hill and Womersley, '91; Brunk et al., '92b; Marzabadi et al., '92; Sheehy, '96; Mourente and Díaz-Salvago, '98).

Promising results in ageing crustaceans were achieved by in situ quantification of lipofuscin granules on histological sections of nervous tissue using fluorescence microscopy (Sheehy, '89). Indeed, morphological lipofuscin has been found to occur in all cell masses of the brain and eyestalks of decapod crustaceans (Sheehy, '89, '90a; Belchier et al., '94; Sheehy and Wickins, '94; Sheehy et al., '96), being particularly conspicuous in the globuli cell masses associated with the olfactory lobe. Whereas a single cluster of these globuli cells is located posterior to the olfactory lobe in Dendrobranchiata, two (anterior and posterior) clusters are found in the Pleocyemata, the anterior one representing probably an apomorphic character of this group (Sandeman et al., '93; Sandeman and Scholtz, '95). Most of the research aimed at correlating lipofuscin accumulation with ageing in decapod crustaceans has been developed on reptantian species (Sheehy, '89, '90a,b,c, '92, '96; Tully, '93; Belchier et al., '94; Sheehy and Wickins, '94; Sheehy et al., '94, '95b, '96; de Kerros et al., '95; O'Donovan and Tully, '96; Wahle et al., '96). Only two studies (Sheehy, '90a; Sheehy et al., '95a) are available on the shorter-lived dendrobranchiates, even though investigations in this field would be valuable to determine age classes in this commercially important group of decapods. Although in his first attempt Sheehy ('90a) was not able to find significant levels of fluorescent lipofuscin granules in three penaeid species, a few years later he and co-workers showed that morphological quantification of lipofuscin does represent a useful tool for the assessment of age structure in dendrobranchiates (Sheehy et al., '95a).

The aim of the present study is twofold: first, to gain a deeper knowledge of the pattern of lipofuscin accumulation with age in crustacean brains, and second, to assess the application of the lipofuscin technique as an accurate estimator of physiological age in short-lived decapods.

MATERIALS AND METHODS

Animals

A single brood of *Penaeus japonicus* Bate, 1888, hatched on February 15, 1996, was reared in a commercial aquaculture facility located in Ayamonte (Huelva, Spain). Juveniles were placed in a 30,000 m^2 earthen pond at a density of 20 individuals/m^2 and fed a high quality pelleted diet (52% protein). The daily average water temperature varied between about 15°C in winter and 26°C in summer, and the daily water renewal ranged from 5% to 25%; surface aerators were used to maintain the oxygen levels above 75% of the saturation concentration. At intervals of about one week, samples of around 100 prawns were taken and the body weight recorded for monitoring the growth of the animals in the pond. Random subsamples of some of the periodical samplings, consisting of at least ten males and ten females, were removed every month for histological studies, starting at the age of 4.6 months (July 10, 1996), since an earlier study (Sheehy et al., '95) found it difficult to detect and quantify lipofuscin in younger animals. On December 15, 1996 (age of 11.2 months), the market-sized individuals were selectively fished for commercial sale and the remainder of the original stock, consisting of the smallest individuals (see Table 2 for comparison of mean weights between the samples of December and January), was moved to an indoor tank, from which samples were drawn every 2 months up to the age of 15.2 months (May 1997). The last sampling point included only three males due to high mortality towards the end of the experiment.

Light and fluorescence microscopy and lipofuscin quantification

After anesthetizing the animals by chilling, the brains were removed and fixed for 24 hr in 10% formaldehyde in filtered seawater, and subsequently dehydrated through an ethanol series and embedded in paraffin wax. Serial frontal sections of brains were cut at 6 μm. To facilitate localization of the OLCM, some of the histological samples were stained with haematoxylin-eosin. All sections containing OLCM tissue were de-waxed through three 10-min xylene changes, mounted without staining and examined under a Leica DMLB epifluorescence microscope equipped with a mercury 50-watt lamp and an I3 (450–490 nm) excitation filter.

For determination of the relative lipofuscin content in brains, images of ten centralmost sections of the OLCM per animal at a magnification of 40× were captured and digitized. A cooled CCD (charge-coupled device) integrating camera sampled the images at defined intervals and integrated a set number of samplings thereby enhancing the clarity of the resulting images.

After discrimination of the proper grey-scale levels, edited binary images of the lipofuscin granules were quantified using the Leica Quantimet Q500 MC image processing and analysis system. The largest rectangular frame inscribed into the OLCM profile was selected for image analysis. Three measurements of lipofuscin contents were recorded for each image: the lipofuscin area fraction (percentage of the cell mass occupied by pigment granules), density (number of lipofuscin granules per 100 μm^2), and mean size (mean diameter of lipofuscin granules). The means of these three measurements in the ten brain sections analysed were taken as the representative values of each individual in statistical treatments.

Electron microscopy

For transmission electron microscopy, brains of 10-month-old prawns were dissected out and cut longitudinally into two halves. The half brains were fixed for 2–4 hr with 2.5% glutaraldehyde in sodium cacodylate buffer 0.1 M at pH 7.2, supplemented with 3% sucrose. Subsequently, the samples were washed for 30 min in cacodylate buffer and postfixed for 1.5 hr at 4°C in osmium-reduced ferrocyanide (Bozzola and Russell, '92). After several short rinses in cacodylate buffer followed by a 30-min change of 70% acetone, the brain tissue was stained in block with 1% uranyl acetate in 70% acetone for 1.5 hr. Then, it was dehydrated in an acetone series and embedded in epoxy resin (Spurr, '69). Semithin (1-μm thick) sections stained with toluidine blue were used as controls to localize the globuli cells prior to the ultrastructural study. Ultrathin sections were doubly stained with uranyl acetate and lead citrate and examined in a Jeol 1200 EX transmission electron microscope.

Analysis of data and statistical treatment

To assess differences between sexes in the content of lipofuscin at each sampled age, the data on lipofuscin area fraction, granule density and mean granule size were compared between males and females by Student's *t*-tests. Homocedasticity was previously confirmed by *F*-tested comparison of variances. Two-factor analysis of variance (ANOVA) was carried out to assess the effect of sex and age on lipofuscin levels. Prior to the analyses, the data were arcsin transformed to eliminate heterocedasticity (Zar, '84). The exact location of significant differences among ages was determined by Tukey's multiple range test.

Linear and non-linear regression models were fitted to the relationships between age and lipofuscin measurements and to the relationship between age and weight by means of the least-squares method through Prism v. 2.01 and FiSAT. The goodness of fit was assessed by the coefficient of determination, r^2, and the visual appearance of the plotted data. The relationship between age and lipofuscin area fraction was best modelled by von Bertalanffy seasonal gowth function, according to the equation:

$$L_t = L_\infty \left\{1 - e^{[-K(t-t_0)-(CK/2\pi)(sin2\pi(t-t_s)-sin2\pi(t_0-t_s))]}\right\},$$

where L_t is the lipofuscin concentration (body length in the original formula) at age t, L_∞ is the mean lipofuscin concentration of infinitely old prawns (originally, the asymptotic length), K is a curvature parameter which determines how fast the animal approaches its L_∞, t_0 refers to the age in which $L = 0$, t_s is the so-called summer point, i.e., the time of the year when the growth rate is highest, and C is a parameter expressing the intensity of the seasonal growth oscillation (Pauly et al., '84; Sparre and Venema, '92). The program FiSAT estimates the "winter point" ($t_w = t_s + 0.5$) rather than t_s, that is, the time when the growth rate becomes lowest (Gayanilo et al., '96).

RESULTS

Morphology

The olfactory lobe cell masses (OLCMs) lie posterior and ventral to each olfactory lobe in the brain of *Penaeus japonicus*. These groups of neurones are easily distinguishable from other neuronal aggregates because they consist of crescent-shaped, compact clusters of small-sized globuli cells that stain darker with conventional dyes (haematoxylin-eosin and toluidine blue stainings). Their cytoplasm appears as a thin band around the spherical nucleus (Fig. 1a, b). While localization of lipofuscin was not feasible in the youngest animals sampled (aged 4.6 months), autofluorescent lipofuscin granules were already observed with clarity in 5.5-month-old male and female prawns (Fig. 2a), and their frequency increased in older animals (Fig. 2b). Under the electron microscope, the lipofuscin granules are spheroidal organelles measuring ~1 μm in diameter that show ultrastructural features of secondary lysosomes. They exhibit a heterogeneous matrix in which some membrane remnants are embedded in a finely granular content (Fig. 1c).

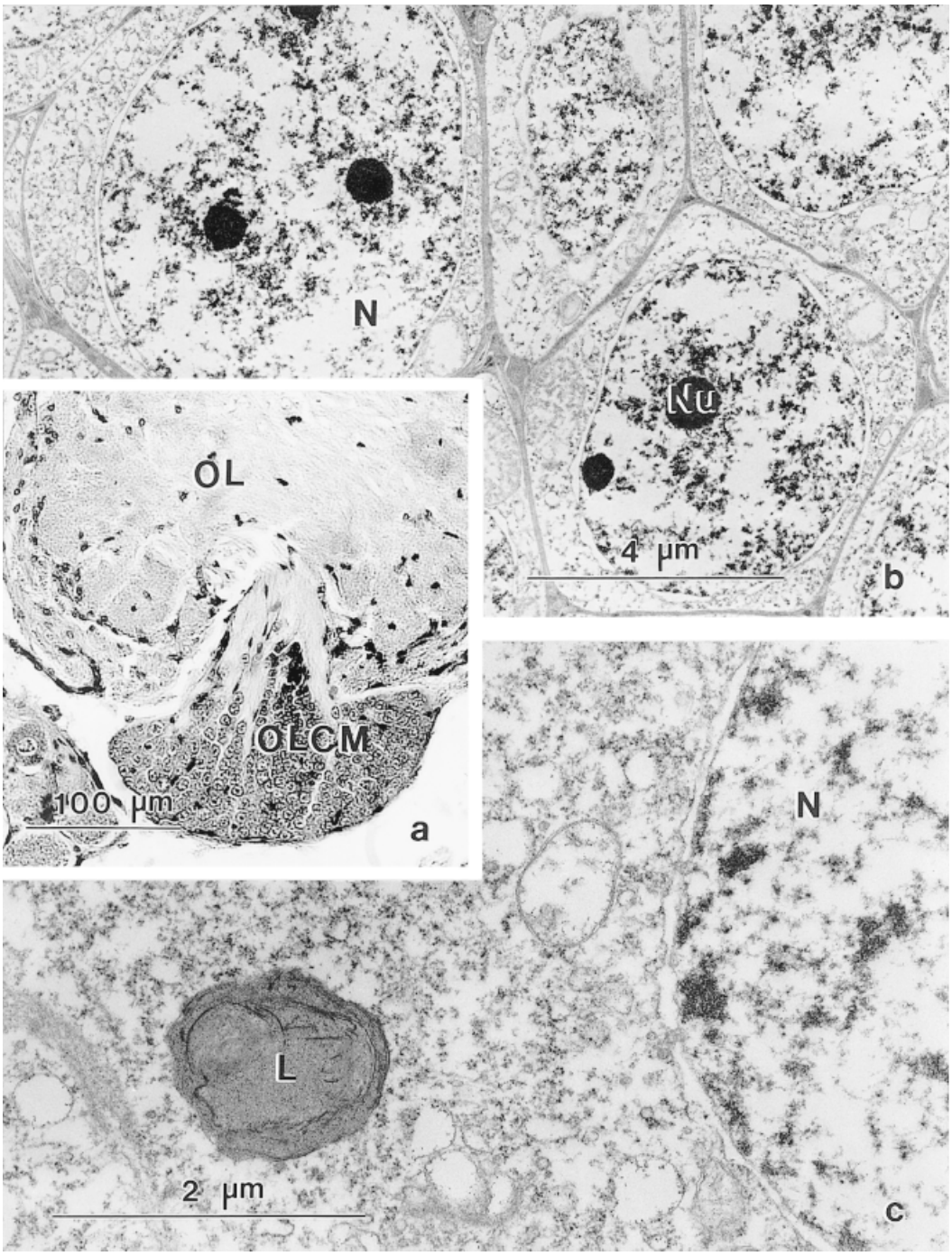

Fig. 1. Light (a) and transmission electron (b) micrographs of *Peneaus japonicus* OLCM globuli cells. (**a**) Paraffin cross section of the OLCM of a 5.5-month-old female stained with haematoxylin-eosin. (**b**) OLCM globuli cells of a 10-month-old female. (**c**) Portion of globuli cell of a 10-month-old male showing a pigment granule (secondary lysosome). L, lipofuscin granule; N, nucleus; Nu, nucleolus; OL, olfactory lobe; OLCM, olfactory lobe cell mass.

Lipofuscin quantification

The concentration of lipofuscin, as expressed in terms of area fraction and granule density, clearly increased with age in OLCM of male and female *Penaeus japonicus* brains (Table 1). Application of the two-sample Student's *t*-test at each age revealed that the amount of lipofuscin accumulated in the globuli cells of the olfactory lobe is not de-

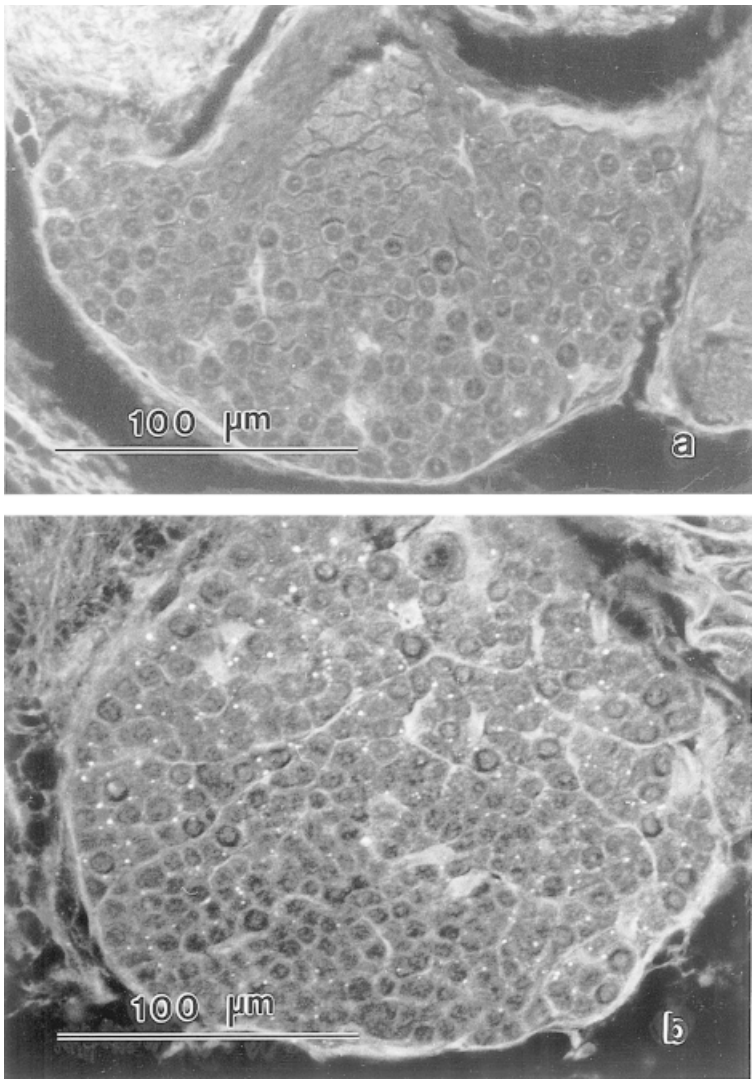

Fig. 2. Fluorescence micrographs of (**a**) 5.5- and (**b**) 15.2-month-old female *Penaeus japonicus*. Note the significantly greater number of autofluorescent lipofuscin granules in the older prawn.

pendent on the sex of the prawns ($\alpha = 0.05$ for area fraction and density at all ages, except for area fraction at the age of 9 months, where $\alpha = 0.01$) (see Table 1). A two-factor ANOVA again showed no sexual differences in the lipofuscin concentration (as both, area fraction and density) ($P > 0.5$), but did reveal a marked dependence on age ($P < 0.0001$). In view of the statistical results, the data from males and females were pooled into one single set of data for further analyses. The mean size of lipofuscin granules was similar in both sexes ($P > 0.25$), but proved different at various ages ($P < 0.0001$). However, such differences did not reflect a progressive increasing trend, but rather an irregular pattern that is difficult to explain in terms of aging.

Table 2 summarizes the pooled data of lipofuscin contents from males and females at the eight sampled ages. It is inferred that the lipofuscin levels (as expressed in both area fraction and granule density, but not mean granule size) increased over 11-fold throughout the 290 days of duration of the experiment. The relationship between age and either area fraction or granule density (Fig. 3) fitted relatively well to a linear model ($r^2 = 0.69$ and $r^2 = 0.63$, respectively; $P < 0.0001$). Somewhat closer was the linear relationship between age and body weight ($r^2 = 0.76$, $P = 0.005$) (Fig. 3). In contrast, the granule size was found not to be correlated with age ($r^2 = 0.097$, $P < 0.0001$) (Fig. 3), hence this index appears inadequate as age indicator and will not be taken into consideration fur-

TABLE 1. Lipofuscin levels in male and female Penaeus japonicus *of eight different ages*[1]

Date of sampling	Age (days/months)	Lipofuscin area fraction (%)		Granule density (no. of granules/100 μm^2)		Granule mean size (μm)	
		Males	Females	Males	Females	Males	Females
Jul 29, 1996	165/5.5	0.09 ± 0.04 (n = 10)	0.07 ± 0.03 (n = 9)	0.06 ± 0.03 (n = 10)	0.04 ± 0.02 (n = 9)	1.94 ± 0.22 (n = 10)	1.75 ± 0.25 (n = 9)
Sep 10, 1996	208/6.9	0.24 ± 0.08 (n = 10)	0.32 ± 0.09 (n = 10)	0.15 ± 0.07 (n = 10)	0.19 ± 0.07 (n = 10)	1.84 ± 0.18 (n = 10)	1.85 ± 0.12 (n = 10)
Oct 9, 1996	237/7.9	0.34 ± 0.09 (n = 9)	0.30 ± 0.06 (n = 10)	0.18 ± 0.07 (n = 9)	0.16 ± 0.05 (n = 10)	2.06 ± 0.16 (n = 9)	2.08 ± 0.15 (n = 10)
Nov 12, 1996	271/9.0	0.33 ± 0.06* (n = 11)	0.42 ± 0.06* (n = 10)	0.22 ± 0.05 (n = 11)	0.26 ± 0.05 (n = 10)	1.78 ± 0.12 (n = 11)	1.84 ± 0.08 (n = 10)
Dec 11, 1996	300/10.0	0.33 ± 0.05 (n = 10)	0.35 ± 0.11 (n = 10)	0.19 ± 0.04 (n = 10)	0.21 ± 0.08 (n = 10)	1.93 ± 0.13 (n = 10)	1.94 ± 0.16 (n = 10)
Jan 15, 1997	335/11.2	0.47 ± 0.08 (n = 10)	0.41 ± 0.11 (n = 11)	0.32 ± 0.07 (n = 10)	0.26 ± 0.08 (n = 11)	1.70 ± 0.05 (n = 10)	1.88 ± 0.12 (n = 11)
Mar 18, 1997	397/13.2	0.58 ± 0.08 (n = 10)	0.55 ± 0.14 (n = 9)	0.40 ± 0.06 (n = 10)	0.40 ± 0.17 (n = 9)	1.71 ± 0.04 (n = 10)	1.84 ± 0.08 (n = 9)
May 15, 1997	455/15.2	0.80 ± 0.12 (n = 3)	0.94 ± 0.12 (n = 10)	0.53 ± 0.12 (n = 3)	0.68 ± 0.11 (n = 10)	1.66 ± 0.07 (n = 3)	1.64 ± 0.05 (n = 10)

[1]The ages are given in days and months along with the corresponding date of sampling. The values of lipofuscin measurements are expressed as means ± SD; n is the number of individuals examined. At all ages the lipofuscin levels were independent of sex (Student's *t*-tests, α = 0.05; except in values bearing *, where α = 0.01). Data of 4.6-month-old prawns are not included as no significant specific fluorescence was observed.

ther on. A high positive correlation occurred between weight and lipofuscin area fraction ($r = 0.83$, $P < 0.05$) (Fig. 3).

A deeper analysis of Table 2 and plots of age versus lipofuscin measurements shows that the lipofuscin concentration increased from July to October at an accumulation rate of 3.33×10^{-3}% area/day (1.7×10^{-3} granules/100 μm^2/day), then dramatically dropped by ~90% between October and December (accumulation rate: 3.17×10^{-4}% area/day, 4.76×10^{-4} granules/100 μm^2/day), but recovered in December and showed an increase through May (accumulation rate: 3.67×10^{-3}% area/day, 2.84×10^{-3} granules/100 μm^2/day). This relationship was best described by a seasonalized von Bertalanffy growth equation fitted through computer-optimized (FiSAT) parameters (Fig. 4). The area fraction data fitted this non-linear model more closely ($r^2 = 0.76$) than did granule density ($r^2 = 0.69$) (Table 3). When the relevant routine of FiSAT was initiated with the default range parameter constraints, the upper limit of 1.83 was selected as the best estimate for L_∞ in terms of lipofuscin % area fraction. The estimated values for the other parameters were $K = 0.96$ year^{-1}, $t_0 = 0.42$ years, $C = 0.92$, and $t_s = 0.27$ years. The estimate of $L_\infty = 1.83$% area fraction means that an infinitely old prawn would be expected to accumulate an amount of lipofuscin under 2% of the OLCM volume. The closeness of the C estimate to 1 indicates that the pattern of lipofuscin accumulation in the OLCM is markedly seasonalized in this prawn species. The values of t_0 and t_s correspond to days 153 and 99, respectively, hence t_w

TABLE 2. Pooled lipofuscin data of males and females[1]

Date of sampling	Age (days/months)	Body weight (g)	n (Σn = 153)	Lipofuscin area fraction (% of area)	Granule density (no. of granules/100 μm^2)	Granule mean size (μm)
Jul 29, 1996	165/5.5	12.5 ± 1.93	19	0.08 ± 0.04^{a}	0.05 ± 0.02^{a}	1.85 ± 0.24^{b}
Sep 10, 1996	208/6.9	18.2 ± 3.44	20	0.28 ± 0.09^{b}	0.17 ± 0.17^{b}	1.85 ± 0.15^{b}
Oct 9, 1996	237/7.9	20.4 ± 4.84	20	0.32 ± 0.08^{b}	0.17 ± 0.06^{b}	2.06 ± 0.17^{c}
Nov 12, 1996	271/9.0	25.1 ± 5.54	21	0.37 ± 0.07^{b,c}	0.24 ± 0.05^{b,c}	1.81 ± 0.10^{a,b}
Dec 11, 1996	300/10.0	27.6 ± 5.99	20	0.34 ± 0.08^{b,c}	0.20 ± 0.06^{b}	1.94 ± 0.15^{b,c}
Jan 15, 1997	335/11.2	24.7 ± 5.25	21	0.44 ± 0.10^{c}	0.29 ± 0.08^{c,d}	1.79 ± 0.11^{a,b}
Mar 18, 1997	397/13.2	25.8 ± 6.19	19	0.57 ± 0.11^{d}	0.40 ± 0.12^{d}	1.77 ± 0.08^{a,b}
May 15, 1997	455/15.2	29.6 ± 4.12	13	0.91 ± 0.12^{e}	0.64 ± 0.12^{e}	1.64 ± 0.06^{a}

[1]Lipofuscin measurements are expressed as means ± SD. Values of each lipofuscin measurement bearing different superscript letters are significantly different ($P < 0.05$).

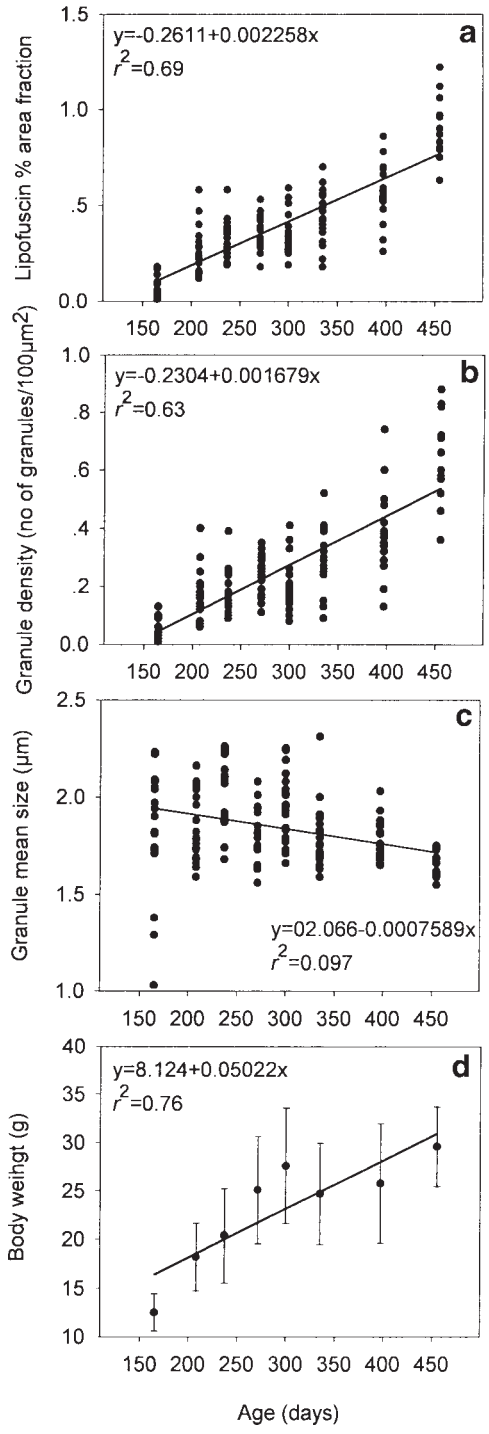

Fig. 3. Linear relationships between chronological age and (**a**) lipofuscin area fraction, (**b**) lipofuscin granule density, (**c**) lipofuscin granule mean size, and (**d**) body weight. The equations describing each relationship and the associated coefficients of determination are shown at the top (bottom in c) of the graphs. Vertical lines in (d) represent the SD.

= 281 days, that is, the theoretical lowest rate of lipofuscin accumulation occurred on the November 22, 1996. From the modelled equation, the 95% of the L_∞ value would be reached at the age of about 3.5 years.

DISCUSSION

Morphology

The morphology of the OLCM and globuli cells of *Penaeus japonicus* are similar to those described in others decapods, including the dendrobranchiate *Penaeus monodon* (Sandeman et al., '93; Sandeman and Scholtz, '95). As the animals grow older, the globuli cells accumulate increasing amounts of pigment granules (lipofuscin) which can be readily detected under fluorescence microscopy. Electron micrographs of the lipofuscin granules show the typical morphology of secondary lysosomes, resembling those of *Homarus gammarus* (Sheehy et al., '96). A pioneer study on dendrobranchiates (Sheehy, '90a) failed to unequivocally characterize autofluorescent lipofuscin in penaeid brains, as only one specimen of *P. esculentus*, out of several specimens of three *Penaeus* species surveyed, showed an appreciable amount of lipofuscin. A further study on one of these species (*P. monodon*), however, broadened the scope of the lipofuscin technique in the assessment of population dynamics in dendrobranchiates (Sheehy et al., '95a). In contrast to *P. monodon*, where the first appearance of technically resolvable autofluorescent lipofuscin granules is thought to occur not much before the age of eight months, in *P. japonicus* the pigment granules are already detectable at the age of 5.5 months, though at low concentrations. As lipofuscin was not conspicuously observed in 4.6-month-old individuals, it is deduced from the present observations that the lowest resolvable age limit in *P. japonicus* is about 5 months.

Lipofuscin quantification

Sexual differences were not found in animals of the same age in any of the three types of measurements of lipofuscin levels, hence further studies on lipofuscin in this species does not seem to require separate samples of either sex. The same is seemingly true for the reptantians *Cherax cuspidatus* (Sheehy, '90b), *C. quadricarinatus* (Sheehy, '90c; Sheehy et al., '94, '95b) and *Homarus gammarus* (Sheehy et al., '96).

The factor of age markedly affected the quantity of lipofuscin deposits in the OLCM. An increasing trend was observed in % area fraction

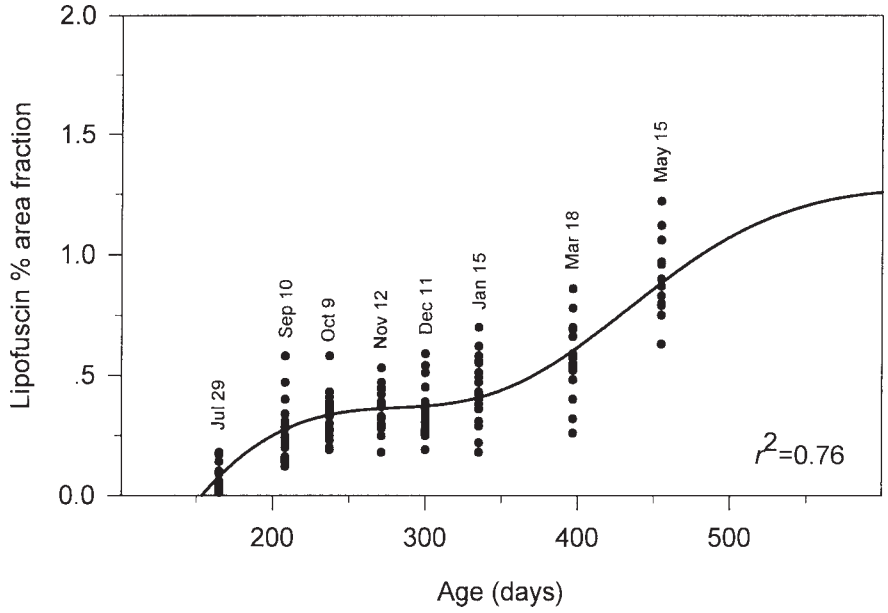

Fig. 4. Non-linear relationship between chronological age and lipofuscin area fraction as described by a seasonalized von Bertalanffy growth function (see text). For each age, the date of sampling is shown. The coefficient of determination is indicated at the bottom of the graph.

and granule density, though the granule mean size did not appear to follow such a trend. In fact, the average granule size measured by image analysis proved smaller in older prawns. This largely inexplicable situation may reflect an artifactual overestimation of the granule size as a result of forcing the threshold levels of the grey-scale to enhance fluorescent granules in poor samples of young specimens. In contrast, the fluorescence intensity of lipofuscin granules in old individuals was high enough to be discriminated with accuracy by the automatic grey-scale determination. Actually, lipofuscin granules in *P. japonicus* did not undergo an overall significant enlargement with age, though in older individuals some large granules were found. Therefore, the increasing concentration of pigment is largely accounted for by an increasing number of granules rather than by increasing volume of the granules as demonstrated to occur in other species (Tully, '93; Sheehy et al., '96; Wahle et al., '96).

The highest lipofuscin area fraction (= volume fraction) measured in *Penaeus japonicus* (1.22%) corresponded to a female aged 15.2 months (455 days); the mean value for this age was 0.91%, sd = 0.12%. These figures are noticeably greater (by about three-fold) than the lipofuscin levels of cultured *P. monodon* of approximately the same age (15.3 months) (Sheehy et al., '95a), but low as compared to similarly aged freshwater crayfishes, *Cherax cuspidatus* and *C. quadricarinatus* (Sheehy, '89, '90b,c, '92; Sheehy et al., '94). Thus, for instance, the average lipofuscin volume fractions measured in 300-day-old and 420-day-old crayfish were about 1% and 2%, respectively, against about 0.3% and 0.9% of 300-day-old and 455-day-old *P.*

TABLE 3. Estimation of the parameters of seasonalized von Bertalanffy functions modelled by the values of lipofuscin measurements[1]

	Estimated growth parameters					
Lipofuscin measurements	L_∞	K (year^{-1})	t_0 (years)	C	t_w (years)	r^2
Lipofuscin area fraction (%)	1.83	0.96	0.42	0.92	0.77	0.76
Granule density (no. of granules/ 100 μm^2)	1.32	0.91	0.42	0.87	0.74	0.69

[1]L_∞ is the asymptotic lipofuscin measurement, K is a curvature parameter, t_0 is the theoretical age at which the lipofuscin concentration is 0, C is a coefficient of seasonal oscillation, and t_w (t_s + 0.5) is the winter point (age at which the lipofuscin accumulation rate is lowest). The equation is better described by the area fraction values, with a significantly higher coefficient of determination (r^2).

japonicus, respectively. Interestingly, the values of lipofuscin area fraction observed in *P. japonicus* are comparable to those recorded in similarly aged decapod species of much longer life span: 13-month-old *Homarus americanus* OLCMs contained a lipofuscin area fraction of about 0.5% (Wahle et al., '96) (0.57% was measured in 13-month-old *P. japonicus*), whereas 10-month-old *H. gammarus* accumulated 0.4–0.5% lipofuscin area fration (Tully, '93; O'Donovan and Tully, '96) (0.34% was recorded in 10-month-old *P. japonicus*).

In several crustacean species, lipofuscin has been shown to accumulate linearly with age (Sheehy, '90c; Tully, '93; O'Donovan and Tully, '96; Sheehy et al., '96) or even as a positive power function in young specimens (Wahle et al., '96). A linear relationship between age and lipofuscin concentration implies that the accumulation rate of the pigment is constant throughout the life span of the species. This type of model is ideal for prediction of age in wild populations, but unfortunately the linear trend often turns into a negative exponential or sigmoidal model when a wider range of ages is studied, since the lipofuscin accumulation rate decelerates with advancing age (Sheehy, '92; Sheehy et al., '94). Such non-linear models reflect the actual metabolic activity of the animals, as the production of lipofuscin in post-mitotic tissues depends on the metabolic rate at a given time and at given external conditions (Sohal and Wolfe, '86). For this reason, environmental factors affecting the metabolic rate, especially the ambient temperature, significantly affect lipofuscin accumulation (Sheehy, '90b,c; Sheehy et al., '94, '95b; Wahle et al., '96). Therefore, the lipofuscin levels measured in a crustacean brain give an approximate idea of the physiological age rather than simply the mere passage of time or chronological age (Sohal and Wolfe, '86; Sheehy, '90b,c; Tully, '93; Sheehy et al., '95b; Wahle et al., '96).

Our data on lipofuscin concentration in *Penaeus japonicus* (area fraction and granule density) can be fitted to a linear relationship with age, but the residuals from the regression would indicate that this is not an appropriate model. In fact, the examination of the plotted data reveals that the accumulation rate of the pigment is high in spring and summer, but dramatically slows down in autumn and winter, most probably as a result of reduced seasonal metabolism. Thus, the relationships between lipofuscin levels and age are best described by a seasonalized von Bertalanffy growth function, a model which was earlier shown to fit to age-pigment data in pond-reared *Cherax quadricarinatus* (Sheehy et al., '94). Both the linear and non-linear functions are best modelled when the data on lipofuscin concentration are expressed in % area fraction compared with the values of granule density. Therefore, this measurement is believed to be the most accurate indicator of age in *P. japonicus*. The shape of the curve of lipofuscin area fraction in relation to age is in full agreement with the observed growth curves of *Penaeus kerathurus* caught off the Gulf of Cádiz (Rodríguez, '77, '87), which can be fitted to a seasonalized von Bertalanffy growth equation, since the growth rate drops in winter (Pauly, '81; Rodríguez, '87). Curiously, our cultured *P. japonicus* continued to grow in autumn and winter, but, as judged from the data on lipofuscin measurements, their metabolic activity did appear to have slowed down. A plausible explanation for the sustained growth rate during winter is that an abundant supply of food, despite a low biological activity, results in biomass production. This situation differs from the wild, where the availability of resources is more limited and the food intake is likely to be mainly spent in the maintenance of the basal metabolism.

Seasonal growth has been observed in numerous penaeid species from temperate and subtropical waters (Pauly et al., '84; Rodríguez, '87; Dall et al., '90). Pauly et al. ('84) drew growth parameters from the length-frequency data provided by several authors by fitting the published values to von Bertalanffy growth curves with seasonal oscillations. In all cases, the goodness of fit was noticeably lower than that obtained in the present study with lipofuscin quantification. The estimates of the equation modelled to the values of lipofuscin area fraction obtained for *Penaeus japonicus* make good biological sense. Thus, the high value estimated for C (0.92) denotes an intense oscillation of the metabolic activity, which is related to the marked annual temperature fluctuations, and is in agreement with the $C = 0.9$ estimated for *P. kerathurus* from the same area (Pauly et al., '84). The estimated winter point ($t_w = 281$ days of age), however, falls at the end of November, somewhat later than the t_w calculated for wild *P. kerathurus* (Pauly et al., '84).

Although real data on longevity in penaeids are not abundant, the life span of coastal penaeids is believed to last between one and two years in the tropics, but is probably longer in temperate areas (Dall et al., '90). Rodríguez ('87) calculated an average longevity of around 20 months for wild *P.*

kerathurus, and similarly the life span of *P. japonicus* is considered not to extend much beyond two years. Therefore, the collection of data covers only 65% of the overall expected longevity, and for this reason the estimated L_∞ = 1.83% lipofuscin area fraction, though biologically reasonable, has to be taken with caution. To get an approximate idea of the meaning of this value, it could be assumed that 5% of the older specimens in the population reach 95% of L_∞ (Taylor, '58); then, the longevity of *P. japonicus* in the reported culture conditions would be calculated as the age at which L = 1.7385, which corresponds to about 3.5 years. Thus, specimens of *P. japonicus* weighing more than 100 g are sometimes found that certainly exceed the age of 3 years (J.F. Le Bitoux, J.M. Rodríguez-Higueras and T. Scovacricchi, pers. comm.).

In conclusion, the present observations confirm the potential of the lipofuscin method in the estimation of physiological age in penaeids. Likewise, they suggest that the application of this technique can become a useful complement to traditional investigations of natural resources based on length-frequency data of the stocks.

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