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Lipid composition and oxidation status in brain of wild-caught size-class distributed *Parapenaeus longirostris* (Lucas, 1846)

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Abstract

The objectives of the study were to characterize the lipid composition, the oxidation status (represented by the levels of malondialdehyde (MDA)), vitamin E content and the fluorescence intensity of lipid-soluble fluorescent products (LSFP) in neural tissues from males and females of wild-caught size-class distributed of the pink shrimp *Parapenaeus longirostris* (Lucas, 1846), trawled in the south Atlantic coast of Spain. Moreover, the mechanisms that might produce the deposition of age-pigments in relation to the physiological age of this species in its natural environment were also investigated. Three different size classes were defined for each sex, resulting in lower sizes for males than for females. The proportion of polar lipids predominated over that of neutral lipids and no significant differences were observed either between different size-classes within sex or between different sex but belonging to the same size-class. The major polar lipid classes were phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) and no significant differences were observed between different groups. However, the cerebrosides fraction showed a significant upward trend from class I to III in both males and females. The fatty acid composition of total lipids, PC, PE and PS in the pink shrimp showed, in most cases, an increase in monounsaturated fatty acids (particularly 18:1(n-9) and 24:1(n-9)) and a decrease in polyunsaturated fatty acids (primarily 22:6(n-3)) with increasing size-age. In males, the concentration of MDA (nmol g^{-1} brain) and fluorescence intensity (% mg^{-1} TL) were positively correlated ($r = 0.77$; $P < 0.02$) but both were negatively correlated to carapace length in the different sizes ($r = -0.88$; $P < 0.002$ and $r = -0.81$; $P < 0.01$, respectively). The concentration of vitamin E (ng mg^{-1} brain) was positively correlated to carapace length ($r = 0.85$; $P < 0.005$) showing a parallel increase of brain membrane lipids and the most potent biological antioxidant vitamin. In females, the concentration of MDA (nmol g^{-1} brain), fluorescence intensity (% mg^{-1} TL) and vitamin E (ng mg^{-1} brain) were not correlated to carapace length of the different sizes (under study) and showed no significant differences between the three different size classes.The fluorescence analysis of brain LSFP was not a useful tool to separate the population into different size classes.

Keywords: Brain; Fatty acid; Lipid class; Lipid-soluble fluorescent products; Malondialdehyde; Parapenaeus longirostris; Physiological age; Vitamin E

1. Introduction

It has been postulated that the determination of the age of individuals in wild crustacean populations can be achieved by measuring in post-mitotic tissues, particularly in the brain, the accumulation of fluorescent age-pigments (lipofuscin) which are thought to be universal correlates of animal senescence [43]. In attempting to

Abbreviations: AA, arachidonic acid; 20:4(n-6); DMA, dimethyl acetal; EPA, eicosapentaenoic acid, 20:5(n-3); DHA, docosahexaenoic acid, 22:6(n-3); HUFA, highly unsaturated fatty acid (3 20:3); LSFP, lipid-soluble fluorescent products; MDA, malondialdehyde; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; TBARS, thiobarbituric acid reactive substances.

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quantify age pigments of crustaceans two major approaches have been developed; (1) measuring the lipofuscin concentration of histological preparations when excited with UV light in a microscope equipped with a fluorescence detector [2,45,46,53] and (2) measuring the fluorescence intensity of lipid/water soluble fluorescent products with a spectrofluorimeter [5,22,33,34,42,44]. The nature (structure and composition) of lipofuscin is best understood in the context of the processes involved in its formation. There are two contrasting theories; one is that lipofuscin accumulation is a storage phenomenon based on the inability of cells to degrade normal cellular constituents and the second is that lipofuscin is an end product of molecular damage to cell organelles by oxygen free radicals with lipid autooxidative reactions playing a crucial role [7,48]. However, not only lipid peroxidation products extracted with chloroform/methanol contribute to fluorescence and age pigments, since there is also polyene-related fluorescence derived from carotenoids, retinoids and dolichols, lipid glycosylation products and contaminating ascorbate-, protein- and nucleic acid oxidation products, most of which have no direct association with age pigment [54,55]. In any case, the carbonyl-related toxic reactions with the amino and thiol groups of biomolecules seem to represent a group of universal, essential and vital side reactions that lead to aging in biological systems [23–25]. Lipid oxidation includes, primarily, polyunsaturated fatty acids (PUFA) and secondly, cholesterol oxidation reactions which are believed (as above) to be responsible to a great extent for lipofuscinogenesis [24,36].

In order to further develop and apply the lipofuscin method in assessment of age in crustaceans it is useful to understand the mechanisms which affect the rate of production/deposition of lipofuscin/age pigments in the crustacean brain. One theory suggests that lipofuscin formation is the result of the balance between the rate of lipid peroxidation and the effectiveness of cellular protective mechanisms [23,56]. Lipids from marine organisms, including marine crustaceans, are rich in polyunsaturated fatty acids, primarily eicosapentaenoic acid (20:5(n-3); EPA) and docosahexaenoic acid (22:6(n-3); DHA) [39]. DHA and EPA are major components of neural and retinal tissues of vertebrates and marine invertebrates, including crustaceans [4,40,41]. In the present study, the aim was to characterize the lipid and fatty acid composition of neural tissues of a commercially important crustacean species, the pink shrimp *Parapenaeus longirostris*, which is of interest since there is limited data in the literature about neural lipids in crustaceans. Also investigated was the oxidation status of the same tissues by determining malondialdehyde (MDA) values which results from the breakdown of polyunsaturated fatty acid hydroperoxides [24], vitamin E levels and the presence and fluorescence intensity of lipid-soluble fluorescent products (LSFP), in relation to a size-class distribution of the natural population of this species. In this particular case we have tried to reproduce a number of previous studies which attempted to resolve age cohorts in unpurified lipid soluble fluorescent products [33,34,42,44]. A crucial objective of this study was to elucidate some of the mechanisms that might produce deposition of age-pigments in this species in its natural environment. The usefulness of the brain lipid-soluble fluorescence for detecting age-cohorts [5,34] in the prawn was also assessed.

2. Materials and methods

2.1. Shrimp collection and dissection of shrimp brain

Wild-caught deep water pink shrimp *Parapenaeus longirostris* (Lucas, 1846) were trawled between 150 and 400 m depth, in the Atlantic waters of SW Spain (Gulf of Cádiz) in April 1996, by the Spanish oceanographic vessel 'Cornide de Saavedra' during the scientific trawling campaign ARSA 04 96. The population was separated by size-frequency modes and males and females classified in three groups of similar orbital carapace length (Table 1). About 100 individuals for every size group were sampled onboard. Shrimp carapaces were dissected at the mouthend and the anterior portion containing the brain, which was extracted and immediately frozen in liquid nitrogen before transfer to the -80°C freezer in the land laboratory. Triplicate samples of whole brains cut by the circumoesophageal commissures were prepared for total lipid extraction ($n = 10$ to $n = 42$ for each sample depending on size and sex), thiobarbituric acid reactive substances (TBARS) quantification ($n = 4$ to $n = 20$) and vitamin E content ($n = 5$ to $n = 20$).

2.2. Lipid extraction

The brains were weighed and placed in ice-cold chloroform/methanol (2:1, v/v) plus 0.01% (w/v) butylated hydroxytoluene (BHT) as an antioxidant. Total lipid was extracted from the brains according to the method of Folch et al. [18]. After homogenization in approximately 10 vol. of ice-cold chloroform/methanol (2:1; v/v) plus BHT, the homogenates were filtered and 0.25 vol. of 0.88% KCl added and the solution mixed. After separation the chloroform layer was removed, refiltered and the solvent evaporated by flushing with nitrogen. The lipid was dried in a vacuum desiccator before weighing. The lipid extracts were redissolved in chloroform/methanol (2:1, v/v) plus BHT at a final concentration of 10 mg ml^{-1} and stored at -20°C between procedures.

Table 1
Biometric data, total lipid and vitamin E contents, TBARS value and relative lipofuscin levels in brain of size-class distributed males and females of the pink shrimp *Parapenaeus longirostris*

Sample	Males			Females		
	M1	M2	M3	F1	F2	F3
Age class	I	II	III	I	II	III
Carapace length (mm)	<17	20–23	>26	<17	25–27	>30
Wet weight per brain (mg)	3.0 ± 0.4^{a}	6.9 ± 0.5^{b}	11.8 ± 0.8^{c}	2.8 ± 0.2^{a}	7.5 ± 0.5^{b}	12.7 ± 0.9^{c}
Total lipid per brain (mg)	0.28 ± 0.03^{a}	0.75 ± 0.06^{b}	1.39 ± 0.11^{c}	0.27 ± 0.02^{a}	0.78 ± 0.07^{b}	1.43 ± 0.09^{c}
Total lipid (wet weight%)	9.3 ± 0.5	11.2 ± 1.1	11.8 ± 0.4	9.6 ± 0.5	10.4 ± 0.7	11.1 ± 0.5
TBARS (MDA μmol per brain)	105.7 ± 7.7^{a}	152.4 ± 24.7^{a}	234.0 ± 42.4^{b}	$77.0 \pm 14.3^{a*}$	$188.3 \pm 41.6^{b*}$	$278.8 \pm 52.2^{c*}$
LSFP (% fluorescence per brain)	2.1 ± 0.2^{a}	4.4 ± 0.6 ab	7.0 ± 0.6^{bc}	2.1 ± 0.2^{a}	$6.1 \pm 1.8^{b*}$	$9.7 \pm 0.7^{c*}$
Vitamin E (ng/brain)	207 ± 33^{a}	922 ± 43^{b}	1690 ± 184^{c}	222 ± 23^{a}	$568 \pm 27^{b*}$	$1049 \pm 125^{c*}$
TBARS (MDA nmol g^{-1} brain)	35.0 ± 2.5^{a}	22.1 ± 3.6^{b}	19.8 ± 3.6^{b}	27.4 ± 5.1	25.0 ± 5.5	21.9 ± 4.1
LSFP (% fluorescence mg^{-1} TL)	7.6 ± 0.9^{a}	5.9 ± 0.7^{ab}	5.1 ± 0.4^{b}	7.9 ± 0.8	7.9 ± 1.7	6.8 ± 0.5
Vitamin E (ng mg^{-1} brain)	68 ± 10^{a}	134 ± 6^{b}	140 ± 15^{b}	77 ± 8	$76 \pm 3*$	$83 \pm 9*$

TBARS, thiobarbituric acid reactive substances; MDA, malondialdehyde; LSFP, lipid soluble fluorescent products.
Results are means of pooled brain triplicates $\pm$ SD.
Values within the same sex bearing different superscript letter are significantly different ($P<0.05$) and values within the same age-class bearing * are also significantly different ($P<0.05$).

2.3. Lipid class separation and quantification

Approximately 10 μg of total lipid were spotted on high-performance thin-layer chromatography (HPTLC) plates that had been pre-run in diethyl ether and activated at 110°C for 0.5 h. The plates were developed for 6 cm in methyl acetate/isopropanol/chloroform/methanol/0.25% aqueous KCl (25:25:25:10:9, by vol.) to separate polar lipid classes with neutral lipids running at the solvent front [52]. After drying, the plates were developed fully in hexane/diethyl ether/acetic acid (80:20:2, v/v/v) to separate the neutral lipids and cholesterol [12]. Lipids were stained by charring at 160°C for 20 min after spraying with 3% copper acetate/8% orthophosphoric acid and identified by comparison with pure, commercial standards [16]. Lipid classes were quantified by calibrated densitometry using a Shimadzu CS-9001PC dual wavelength flying spot scanner [35]. HPTLC ($10 \times 10 \times 0.15$ mm) glass plates, precoated with silica-gel 60 (without fluorescence indicator), were obtained from Merck (Darmstadt, Germany).

2.4. Total lipid fatty acid analysis

Fatty acid methyl esters from total lipids were prepared by acid-catalyzed transmethylation for 16 h at 50°C, using tricosanoic acid (23:0) as the internal standard [13]. After extraction and purification, the fatty acid methyl esters were determined quantitatively in a Hewlett-Packard 5890A Series II gas chromatograph equipped with a chemically bonded (PEG) Supelcowax-10 fused silica wall-coated capillary column (30 m $\times$ 0.32 mm i.d., Supelco, Bellefonte, USA), an on-column injection system and FID. Hydrogen was used as the carrier gas with an oven thermal gradient from initial 50 to 180°C at 35°C per min and then to a final temperature of 235 at 3°C per min. The final temperature was maintained for 10 min. Individual fatty acid methyl esters were identified by comparison with known standards and quantified using HP ChemStation software with instrument control and data acquisition module in a computer linked to the gas chromatograph.

2.5. Measurements of thiobarbituric acid reactive substances

The measurements of TBARS in triplicate samples of brains from different size-class males and females were carried out using a method adapted from that used by Burk et al. [8]. Up to 20–30 mg of brain tissue per sample was homogenized in 1.5 ml of 20% trichloroacetic acid (w/v) containing 0.05 ml of 1% BHT in methanol. To this was 2.95 ml of freshly prepared 50 mM thiobarbituric acid solution was added. The reagents were mixed in a stoppered test tube and heated at 100°C for 10 min. After cooling the tubes and removing protein precipitates by centrifugation at 2000 rpm, the supernatant was read in an spectrophotometer at 532 nm. The absorbance was recorded against a blank at the same wavelength. The concentration of TBA-malondialdehyde (MDA) expressed as nmol MDA g^{-1} of brain and nmol MDA/brain was calculated using the extinction coefficient 0.156 μM cm^{-1}.

2.6. Analysis of brains for tocopherol

Vitamin E concentrations (as tocopherol plus α-tocopheryl esters) were measured in brain using high-performance liquid chromatography (HPLC). Samples were weighed, homogenized and saponified as described by Bieri [6], but using a single-step hexane extraction [3]. HPLC analysis was performed using a 250×4.6 mm reverse phase Spherisorb ODS2 column (Sigma, St. Louis, MO) essentially as described by Carpenter [11]. The mobile phase was 98% methanol pumped at 2 ml min^{-1}, the effluent from the column was monitored at a UV wavelength of 293 nm and the quantitation achieved by comparison with (±)-α-tocopherol (Sigma, St. Louis, MO) as external standard (10 μg ml^{-1}).

2.7. Measurement of lipid-soluble fluorescent products

The lipid-soluble fluorescent products was measured basically according to Fletcher et al. [17], with the innovation of using the same total lipid extracts used for lipid analysis, since the extraction procedures are almost identical. For measurement of fluorescence, aliquots of 100 μl of total lipid extract at a concentration of 10 mg ml^{-1} in chloroform/methanol (2:1, v/v) plus BHT were diluted to 500 μl with chloroform/methanol (2:1, v/v) without BHT in 700 μl capacity quartz cuvettes. Fluorescence intensity was determined in a Perkin-Elmer LS-5 spectrofluorimeter at an exciter λ of 350 nm and analyzer λ of 445 nm [17,20]. The slit arrangement was 2.5 and 2.5 for excitation and emission, respectively and sensitivity was set at 10. Quinine sulphate at a concentration of 1 μg ml^{-1} in 1 M H_2SO_4 was used as standard for fluorescence intensity and the results were expressed in arbitrary units defined as the percent fluorescence of the standard corresponding to 1 mg of total lipid extracted. For quantitative comparisons and linearity determinations, fluorescence of the standard was recorded just before recording the sample fluorescence. Linearity of instrumental response over the range of fluorescence intensities encountered in brain extracts was confirmed by using serial dilutions of samples (0.3–3.5 μg μl^{-1} brain total lipid extracts). Data fitted to the equation $FI = 1.0 + 3.3^{*} C$, where FI is the fluorescence intensity and C the concentration of the total lipid extract (μg μl^{-1}), being 0.4 and 0.2 the SE of the y intercept and the slope, respectively ($r^2 = 0.95$; $P < 0.0001$). It was also confirmed that the inclusion of BHT as antioxidant in the solvents (0.01% w/v) did not interfere in the measurement of the fluorescence intensity of the samples.

2.8. Statistical analysis

Linear regression analysis was used to check the linearity of the response of the fluorescence intensity depending on the concentration of the total lipid extracted from the sample, whereas linear correlation analysis was used to establish the relation between the different variables measured. Results are presented as means ± SD ($n = 3$) and data were checked for homogeneity of the variances by the Bartlett test and where necessary, the data were arc-sin transformed before further statistical analysis. Differences between mean values corresponding to different size-class individual of the same sex were analyzed by one-way ANOVA followed (where appropiate) by a multiple comparison test (Tukey). Differences between mean values within the same size-class and different sex were analyzed by a two-sample comparison t-test [57].

3. Results

Biometric data, total lipid, MDA, vitamin E contents and lipid soluble fluorescent products levels from male and female *P. longirostris* are shown in Table 1. Size-class distribution was defined according to commercial criteria for this species and caparace lengths for males and females shown were separated by size-frequency modes in the sampled population. Brain wet weight was 2.3 times and 3.9 times greater for males of class II and III, respectively, compared to those of class I. On the other hand, brain wet weight of class II and III females were, respectively, 2.7- and 4.5-fold larger than those of class I. However, total lipid content on a wet weigth basis was not significantly different in brains of all classes at approximately 10%. Brains of class II and III males were 2.7 and 5-fold richer in total lipids than brains of the class I male. Similarly, brains of class II and III females showed 2.9 and 5.3-fold more total lipids than brains of the class I. In males, total MDA content per brain was not significantly different between classes I and II but increased significantly by 2.2-fold from class II to class III. When the results were expressed as U^{-1} of brain weight, nmol MDA g^{-1} brain, the MDA content significantly decreased (by 36.8%) from class I to class II but the decrease observed from class II to class III was not statistically significant. In females, total MDA content was 2.4 and 5.3 significantly higher in class II and class III brains, respectively, in comparison to that of class I. In contrast, when MDA content in the females brain is expressed as nmol g^{-1} brain no significant differences were observed between the classes.

The intensity of fluorescence of LSFP from male and female brain when expressed per whole brain showed in both cases significant upward trends. However, when

Table 2
Lipid class compositions (percentage of total lipid) of brain lipid from wild-caught (size-class distributed) males and females of the pink shripm *Parapenaeus longirostris*

Sample	Males			Females		
	M1	M2	M3	F1	F2	F3
Total polar lipid	65.3 ± 1.5	66.1 ± 0.9	65.9 ± 1.9	66.7 ± 0.9	66.8 ± 1.1	67.8 ± 0.9
Phosphatidylcholine	18.9 ± 0.4	18.8 ± 1.0	16.9 ± 1.0	18.6 ± 0.3	18.2 ± 0.5	18.4 ± 0.1
Phosphatidylethanolamine	13.7 ± 0.3	13.6 ± 0.1	12.8 ± 0.7	14.7 ± 0.2	13.7 ± 0.4	13.9 ± 0.3
Phosphatidylserine	10.7 ± 0.4	10.9 ± 0.2	10.5 ± 0.3	10.8 ± 0.2	10.9 ± 0.2	10.6 ± 0.1
Phosphatidic acid/cardiolipin	8.9 ± 0.8	8.5 ± 0.3	9.4 ± 0.3	9.1 ± 0.9	9.4 ± 0.4	8.5 ± 0.6
Cerebrosides	6.3 ± 0.0[a]	7.6 ± 0.2 b	8.7 ± 0.1[c]	6.6 ± 0.2[a]	7.4 ± 0.3[b]	8.8 ± 0.2[c]
Phosphatidylinositol	1.9 ± 0.2	1.5 ± 0.1	1.5 ± 0.2	2.0 ± 0.2	1.6 ± 0.1	1.4 ± 0.1
Sphingomyelin	2.1 ± 0.0	2.3 ± 0.1	2.1 ± 0.2	1.9 ± 0.1	2.1 ± 0.2	2.2 ± 0.1
Lyso-phosphatidylcholine	0.2 ± 0.0[a]	0.3 ± 0.1 ab	0.4 ± 0.1[b]	0.1 ± 0.0[a]	0.2 ± 0.0[ab]	0.2 ± 0.0[b]
Others	2.4 ± 0.2[a]	2.6 ± 0.1 a	3.5 ± 0.2[b]	2.7 ± 0.2	3.1 ± 0.7	3.7 ± 0.1
Total neutral lipid	34.6 ± 1.5	33.7 ± 0.9	34.1 ± 1.9	33.3 ± 0.9	33.2 ± 1.1	32.1 ± 0.9
Cholesterol	29.8 ± 1.6	29.2 ± 0.2	29.4 ± 1.8	29.3 ± 0.6	29.4 ± 1.2	30.2 ± 1.1
Free fatty acid	3.2 ± 0.2[a]	2.1 ± 0.2[b]	2.9 ± 0.1[a]	2.9 ± 0.3[a]	2.5 ± 0.3[ab]	1.0 ± 0.7[b]
Triacylglycerol	1.7 ± 0.4	2.4 ± 0.9	1.8 ± 0.3	1.1 ± 0.2	1.2 ± 0.1	0.9 ± 0.1

Results are means of triplicate samples of pooled brain ± SD.
Values within the same sex bearing different superscript letters are significantly different ($P<0.05$).

the intensity of fluorescence was expressed as fluorescence per mg of lipid soluble extract, the male brain presented a downward trend with a significant difference between class I and class III observed (decreased by 32.9%). On the other hand, the female brain did not show a significant difference between the classes. Our colleagues in this project have confirmed this by fluorescense microscopy and are currently studying and quantifying morphological lipofuscin in brain of *P. Longirostris*.

Vitamin E content, when expressed per whole brain (ng vit E per brain), showed significant upward trends between the three different classes in both males and females. However, when data were presented as ng vit E mg^{-1} brain, males increased significantly by 2-fold from class I to class II with no significant difference between classes II and III. In contrast, female brains did not show significant differences regarding vitamin E content between different classes when expressed per unit of brain weight. It is noteworthy that the brains from the class II and III males showed 1.8- and 1.7-times more vitamin E per unit of brain weight than the brains of females from the same classes, respectively.

Lipid class composition of total lipids from brain of size-class distributed males and females of *P. longirotris* are presented in Table 2. The proportion of total polar lipids predominated over that of total neutral lipids and no significant differences were observed either between different size-classes within sex or between different sex but belonging to the same size-class. The major polar lipid classes were phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) and no significant differences were observed between different groups. However, the cerebrosides fraction showed a significant upward trend from class I to III in both males and females. In males, the proportion of cerebrosides increased by 17.1 and 12.6% from class I to II and from class II to III, respectively, whereas in females, the increases were by 10.8 and 15.9%, respectively. In total neutral lipids, free cholesterol was the major fraction comprising about 87% but showing no significant differences between different groups.

In total lipid, the proportion of total saturated fatty acids (primarily 16:0 and 18:0) were significantly lower in brain lipid from males of size-class III but no significant differences were shown between different size-classes in females (Table 3). In contrast, total monoenes (primarily 18:1(n-9) and 24:1) showed a significant upward trend in both males and females. In particular, 24:1(n-9) significantly increased by 31.4 and 26.1% from class I to II and from class II to III in males and by 26.5 and 20.9% in females, respectively. Total dimethyl acetals (DMA) (derived from alkenyl-linked lipids) showed no significant differences between different groups. The percentages of total polyunsaturated fatty acids (predominantly 20:5(n-3) and 22:6(n-3)) showed significantly higher values in brain lipid of size class I, independently of the sex (Table 3).

4. Discussion

In males of *P. longirostris*, the concentration of MDA (nmol g^{-1} brain) and fluorescence intensity (%

Table 3
Total lipid fatty acid composition (weight percentage) from brain of wild-caught (size-class distributed) males and females of the pink shrimp *Parapenaeus longirostris*

Fatty acid	Males			Females		
	M1	M2	M3	F1	F2	F3
14:0	0.4 ± 0.1	2.5 ± 1.5	0.4 ± 0.2	0.4 ± 0.1	1.7 ± 1.5	1.0 ± 0.9
15:0	2.2 ± 0.2	1.8 ± 0.2	1.9 ± 0.1	2.1 ± 0.3	2.2 ± 0.1	2.2 ± 0.1
16:0DMA	0.6 ± 0.0^{a}	0.6 ± 0.0^{a}	0.8 ± 0.0^{b}	0.6 ± 0.0	0.7 ± 0.0	0.8 ± 0.1
16:0	9.4 ± 0.1^{ab}	10.1 ± 0.6^{a}	8.7 ± 0.2^{b}	9.8 ± 0.2*	10.0 ± 0.5	9.2 ± 0.7
16:1(n-7)	3.6 ± 0.1^{a}	3.3 ± 0.1^{b}	3.4 ± 0.0^{ab}	3.7 ± 0.1	3.9 ± 0.2*	4.1 ± 0.0*
16:2	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.2 ± 0.1	0.2 ± 0.0
17:0	0.5 ± 0.1^{a}	0.1 ± 0.0^{b}	0.4 ± 0.0^{a}	0.6 ± 0.1^{a}	0.4 ± 0.1^{b}*	0.3 ± 0.0^{b}*
16:3	0.8 ± 0.1^{a}	0.5 ± 0.0^{b}	0.9 ± 0.1^{a}	0.8 ± 0.0	1.3 ± 0.8	0.9 ± 0.3
18:0DMA	1.7 ± 0.2	1.5 ± 0.2	1.3 ± 0.0	1.6 ± 0.1	1.5 ± 0.1	1.3 ± 0.1
18:1DMA	1.7 ± 0.1	1.9 ± 0.1	2.0 ± 0.1	1.8 ± 0.0^{a}	2.0 ± 0.0^{b}	2.0 ± 0.0^{b}
18:0	7.2 ± 0.2^{a}	6.4 ± 0.1^{b}	5.9 ± 0.1^{c}	7.6 ± 0.1^{a}*	6.6 ± 0.2^{b}	6.2 ± 0.2^{b}
18:1(n-9)	20.5 ± 0.2^{a}	23.9 ± 1.4^{b}	26.0 ± 0.7^{b}	21.2 ± 0.3^{a}	24.3 ± 0.4^{b}	25.2 ± 0.1^{b}
18:1(n-7)	2.5 ± 0.0^{a}	1.8 ± 0.2^{b}	1.9 ± 0.0^{b}	2.7 ± 0.2^{a}	2.0 ± 0.2^{b}	2.0 ± 0.1^{b}
18:2(n-6)	1.1 ± 0.1^{ab}	2.1 ± 0.6^{a}	0.9 ± 0.0^{b}	1.1 ± 0.0	1.7 ± 1.1	0.8 ± 0.1
18:3(n-3)	0.2 ± 0.0^{a}	0.2 ± 0.0^{ab}	0.2 ± 0.0^{b}	0.2 ± 0.0^{a}	0.2 ± 0.0^{ab}	0.1 ± 0.0^{b}
18:4(n-3)	0.3 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
20:0	0.7 ± 0.0^{a}	0.6 ± 0.0^{b}	0.5 ± 0.0^{b}	0.7 ± 0.0^{a}	0.6 ± 0.0^{b}	0.5 ± 0.0^{b}
20:1(n-9)	1.0 ± 0.0	0.8 ± 0.1	0.9 ± 0.1	1.1 ± 0.0*	1.0 ± 0.1	1.0 ± 0.2
20:1(n-7)	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0*	0.1 ± 0.0	0.1 ± 0.0
20:2(n-6)	0.4 ± 0.1	0.6 ± 0.1	0.4 ± 0.0	0.4 ± 0.1	0.5 ± 0.2	0.3 ± 0.1
20:4(n-6)	3.3 ± 0.1^{a}	2.8 ± 0.2^{b}	3.3 ± 0.1^{a}	3.5 ± 0.0^{a}	2.6 ± 0.2^{b}	2.9 ± 0.2^{b}
20:3(n-3)	0.2 ± 0.0^{ab}	0.2 ± 0.0^{a}	0.7 ± 0.0^{b}	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
20:4(n-3)	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0^{a}*	0.2 ± 0.0^{ab}	0.2 ± 0.0^{b}*
20:5(n-3)	8.3 ± 0.2^{a}	6.8 ± 0.6^{b}	7.6 ± 0.3^{ab}	8.9 ± 0.1*	7.4 ± 0.8	7.5 ± 0.7
22:0	1.2 ± 0.0^{a}	0.7 ± 0.1^{b}	0.8 ± 0.1^{b}	0.8 ± 0.1*	0.8 ± 0.1	0.8 ± 0.1
22:1	3.1 ± 0.0	2.9 ± 0.2	3.1 ± 0.1	2.3 ± 0.1	2.9 ± 0.2	2.1 ± 0.1
22:5(n-6)	0.4 ± 0.0^{a}	0.2 ± 0.0^{b}	0.3 ± 0.0^{ab}	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
22:4(n-3)	0.3 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.0
22:5(n-3)	0.7 ± 0.0^{a}	0.4 ± 0.0^{b}	0.3 ± 0.0^{c}	0.7 ± 0.0^{a}	0.4 ± 0.0^{b}	0.3 ± 0.0^{c}
22:6(n-3)	7.1 ± 0.2^{a}	5.4 ± 0.5^{b}	5.6 ± 0.1^{b}	7.3 ± 0.1^{a}	6.0 ± 0.6^{ab}	5.4 ± 0.6^{b}
24:0	1.3 ± 0.1	1.4 ± 0.0	1.4 ± 0.0	1.2 ± 0.0	1.2 ± 0.1*	1.3 ± 0.0
24:1(n-9)	3.5 ± 0.1^{a}	5.1 ± 0.5^{b}	6.9 ± 0.2^{c}	3.6 ± 0.0^{a}	4.9 ± 0.2^{b}	6.2 ± 0.1^{c}*
24:1(n-7)	4.3 ± 0.0^{a}	4.7 ± 0.4^{b}	5.9 ± 0.2^{b}	4.6 ± 0.0^{a}*	4.6 ± 0.2^{a}	5.8 ± 0.1^{b}
Total saturated	23.0 ± 0.2^{ab}	23.7 ± 2.1^{a}	20.1 ± 0.2^{b}	23.3 ± 0.1	23.6 ± 1.9	21.6 ± 1.7
Total monoenes	38.6 ± 0.3^{a}	42.9 ± 2.8^{a}	48.4 ± 0.9^{b}	40.3 ± 0.5^{a}*	43.7 ± 1.4^{b}	47.5 ± 0.2^{c}
Total polyenes	26.4 ± 0.3^{a}	22.3 ± 0.5^{b}	22.7 ± 0.3^{b}	26.7 ± 0.1^{a}	22.7 ± 0.3^{b}	21.3 ± 1.7^{b}
Total DMA	4.1 ± 0.2	4.0 ± 0.1	4.2 ± 0.1	4.0 ± 0.1	4.2 ± 0.3	4.1 ± 0.1
Unknown	7.9 ± 0.7	7.1 ± 1.5	4.6 ± 1.0	5.7 ± 0.3	5.8 ± 0.9	5.4 ± 1.0
Total (n-9)	25.4 ± 0.4^{a}	30.4 ± 1.9^{b}	34.4 ± 0.8^{c}	26.3 ± 0.4^{a}	30.7 ± 0.8^{b}	32.8 ± 0.2
Total (n-7)	10.5 ± 0.1^{ab}	10.0 ± 0.7^{a}	11.4 ± 0.2^{b}	11.1 ± 0.1^{ab}	10.5 ± 0.5^{a}	12.1 ± 0.2^{b}*
Total (n-6)	7.6 ± 0.0	7.8 ± 0.5	7.2 ± 0.1	7.9 ± 0.1*	7.1 ± 1.1	6.4 ± 0.2*
Total (n-3)	18.8 ± 0.4^{a}	14.5 ± 1.0^{b}	15.5 ± 0.2^{b}	18.8 ± 0.1^{a}	15.5 ± 1.3^{ab}	14.9 ± 1.6^{b}
HUFA (n-6)	5.8 ± 0.1^{a}	4.8 ± 0.3^{b}	5.6 ± 0.1^{a}	6.0 ± 0.1^{a}	4.7 ± 0.2^{b}	5.0 ± 0.2^{b}
HUFA (n-3)	17.5 ± 0.3^{a}	13.7 ± 0.9^{b}	14.5 ± 0.2^{b}	17.6 ± 0.1^{a}	14.6 ± 1.1^{ab}	13.9 ± 1.3^{b}

Data are means $\pm$ SD ($n = 3$).
SD = 0.0 implies and SD < 0.05.
Values within the same sex bearing different superscript letter are significantly different ($P < 0.05$) and values for males and females within the same age-class bearing * are also significantly different ($P < 0.05$).
nd, not detected; tr, trace; DMA, dimethyl acetals; HUFA, highly unsaturated fatty acids.

mg^{-1} TL) were positively correlated ($r = 0.77$; $P < 0.02$) but both were negatively correlated to carapace length in the different sizes under study ($r = -0.88$; $P < 0.002$ and $r = -0.81$; $P < 0.01$, respectively). This indicates that the brain growth rate is higher than the brain oxidation rate although in absolute terms (pmol MDA per brain and fluorescence intensity% per brain) they all increase, denoting the formation of LSFP by the increase of lipid oxidation products. The concentration of vitamin E (ng mg^{-1} brain) was positively

correlated to carapace length ($r = 0.85$; $P < 0.005$) showing a parallel increase of brain membrane lipids and the most potent biological antioxidant vitamin of exogenous origin, in order to protect neural tissues from oxidative stress.

In females of *P. Longirostris*, the concentration of MDA (nmol g^{-1} brain) and fluorescence intensity (% mg^{-1} TL) were not correlated to carapace length of the different sizes under study and showed no significant differences between the three different size classes. In consequence, when expressed in absolute terms (pmol MDA per brain and fluorescence intensity% per brain), both variables presented significant upward trends and the values corresponding to stages II and III were significantly higher than those observed in males. This could be due to the higher size of the females, since it is well known that in penaeids the sexes show differential growth with higher growth and metabolic rates in females [14]. However, the concentration of vitamin E per unit of brain weight (ng mg^{-1} brain) was not correlated to carapace length. Vitamin E plays a very significant role in reproduction of Penaeids, since omission of this vitamin from the diet of *Penaeus japonicus* broodstock yielded a lower gonado-somatic index compared to the control diet, indicating a requirement of this vitamin for gonadal development [1]. Improved reproductive performance of *Penaeus indicus* was also observed with supplementation of dietary α-tocopherol [10]. It is believed that shrimp may possess hydrolytic and transport mechanisms for vitamin E similar to those found in fish and other vertebrates [9,27]. The hepatopancreas seems to be the major storage organ for α-tocopherol (this function is performed by the liver in vertebrates), since vitamin E may be unevenly distributed among shrimp tissues but more concentrated in hepatopancreas [21]. The muscle, accounting for over 50% of body mass also contained a large amount of vitamin E, which can be transported from both hepatopancreas and muscle to ovary during vitellogenesis, via the haemolymph, by low and high density lipoproteins as shown for fish [27]. In the present study, the reason for the low concentration of brain α-tocopherol in class II and III females (only 56.7 and 59.3% of the concentration of vitamin E in the brain of males from the same age-size classes, respectively) might be due to a transference of vitamin E from the brain to the ovary in females, since ovarian maturation and spawning occur in this species at these stages [37]. However, the lower vitamin E concentration in the brain of the females did not imply an increase in oxidation, since no significant differences were found in the values for MDA and LSFP in brain between males and females at stages II and III. Taking into account that the total lipids from the female brain were not more unsaturated than those of the males, this fact suggests that the low values for vitamin E in females are sufficient to maintain the integrity of the neural and brain tissues or that other components of the brain antioxidant system (such as superoxide dismutase, glutathione peroxidase or catalase activities or ascorbic acid) combine to prevent an increase in brain oxidation. It is generally assumed that to be readily bioavailable, α-tocopherol exists as a component of membranes. This is an area not explored in crustaceans and very limited data exist regarding bioavailability, biokinetic, storage and mobilization of vitamin E during maturation.

In a *P. longirostris* population, juveniles of about 6 months (males of 19 mm and females of 21 mm carapace length), 1-year-old individuals (23 mm class males and 27 mm class females) and 1.5 (27 mm males and 32 mm females) and 2.5- (33 mm males and 38 mm females) years-old, respectively, were established in south Portugal waters by size frequency distribution [37]. In consequence, in the present study, the statistical analysis of fluorescence intensity from brain LSFP showed no significant differences between the three different size classes that could belong to different age classes. In contrast, in this case, the fluorescence analysis of brain LSFP is not a useful tool to separate the population into different age classes as proposed by Sheehy [42]. The most likely explanation is perhaps the non-specificity of the assay procedure [42,55] rather than a problem with lipofuscin as an age determinant. However, in insects, several studies have demonstrated that the accumulation of LSFP is a linear function of time, metabolic rate, lipid peroxidation potential and aging [15,29,30,48,49].

There is very little information in the literature about the lipid content and the lipid composition of the brain in crustaceans [41] and only a few studies in fish [25,31,51]. In the present study, the brain of the pink shrimp *P. longirostris* showed a higher proportion of total neutral lipids, due to a higher proportion of free cholesterol, than in the brain of fishes like the rainbow trout, cod or Atlantic herring [31,51]. In contrast, despite the lower proportion of total polar lipids, the levels of PS, phosphatidic acid/cardiolipin and cerebrosides were higher than in the brains of the fish species mentioned above [31,51]. It is perhaps noteworthy, that there were significant differences observed between the levels of cerebrosides in the different size classes for both males and females. The significant increase in cerebrosides may be indicative of myelination processes [26,41]. The proportion of cerebrosides in brain total lipids were correlated to carapace length in males ($r = 0.99$; $P < 0.001$) and females ($r = 0.91$; $P < 0.001$). The significant increase during neural maturation of cerebrosides (glycosphingolipids) may be source of glycation reactions between the reducing sugar of the cerebroside and amino compounds which may give rise to age dependent pigment like flourophores not oxygen-dependent, analogues of secondary lipid peroxidation products [55].

An interesting characteristic of the fatty acid composition in pink shrimp brain lipids is the generally lower level of unsaturation in comparison to total lipid fatty acid acids from fish brain. The proportion of DHA in pink shrimp brain lipids ranged from 20–25% of the proportion of this fatty acid in the brain of fishes such as rainbow trout, cod or Atlantic herring but, in contrast, the proportions of EPA and arachidonic acid (AA; 20:4(n-6)) were higher in the shrimp [31,51].

The proportion of total monoenes was also higher than in fish brain and in the present study, the levels of 24:1(n-9) showed a significant upward trend in brain total lipids from stage I to stage III. This fatty acid was particularly abundant in PC (data not shown) as observed in fish [26,31,32,51]. The presence of significant levels of 24:1 fatty acids in brain phosphoglycerides, although common in cerebrosides, it has not been reported in mammals (the review of Sastry [41] shows that most of the omissions of 24:1 from mammal brain are the result of old or inadequate gas-liquid chromatography) and the role(s) that molecular species of phosphoglycerides containing 24:1 may have in fish and crustacean neural tissues remains to be elucidated. The proportion of 24:1(n-9) in total lipids from brain of male and female *P. longirostris* is positively correlated to the concentration of MDA per brain ($r = 0.90$; $P < 0.0001$ and $r = 0.92$; $P < 0.0001$, respectively) and to the fluorescence intensity per brain ($r = 0.99$; $P < 0.0001$ and $r = 0.96$; $P < 0.0001$, respectively).

It is noteworthy that the fatty acid composition of total lipids in the brain of pink shrimp showed an increase in monounsaturated fatty acids and a decrease in polyunsaturated fatty acids with increasing size, a fact that has also been observed in fish [31] and mammals [28]. There are several hypothesis to explain the disappearance of PUFA and the increase of monoenes in the brain during aging; (i) decreases in Δ6 and Δ5 desaturase activities and increase of Δ9 desaturase activity, (ii) deficiency in PUFA uptake, (iii) a change in the cellular makeup of the brain during aging, possibly involving a reduction in the number of dendrites (which are rich in PUFA) and (iiii) enhanced lipid peroxidation process during aging [28,47].

The presence of significant proportions of DMA was the result of synthesis and acumulation of PE-plasmalogens which are implicated in myelin membrane composition [41]. This fact and the increase of cerebrosides observed problably reflects maturation processes of the neural system. Plasmalogens are formed by oxidation of the corresponding alkyacylglycerophospholipid (the enzymes responsible for its synthesis are located in the peroxisomes) by a microsomal enzyme requiring NADPH and molecular oxygen [38,41,50]. On the other hand, cerebrosides contain a high proportion of hydroxy fatty acids which are synthesized by hydroxylation of acyl-CoA in the presence of molecular oxygen, NADH and iron [19,41].This situation could be prone to generate free radicals and oxidative stress capable of inducing lipid peroxidation and generate aldehydes (TBARS, MDA) which are precursors of fluorescent peroxidation products and age pigment-like fluorophores [54]. The proportion of cerebrosides in total lipids from brain of male and female *P. longirostris* is positively correlated to the concentration of MDA per brain ($r = 0.86$; $P = 0.0003$ and $r = 0.92$; $P < 0.0001$, respectively) and to the fluorescence intensity per brain ($r = 0.98$; $P < 0.0001$ and $r = 0.93$; $P < 0.0001$, respectively).

In conclusion, the brain lipids of the pink shrimp have a less unsaturated pattern than brain lipids of marine fish. Highly unsaturated fatty acids such as DHA and EPA are less abundant than in fish, whereas there are higher levels of monounsaturated fatty acids. In consequence, there is perhaps less risk from peroxidative attack than in marine fish brain and so a high concentration of antioxidants, such as vitamin E, may not be required in pink shrimp brain compared to other organisms with a higher degree of unsaturation in their brain lipids. In any case, further studies are required in crustaceans on brain lipids, oxidation status in neural tissues and the relation between LSFP, lipofuscin and age in fast growing species of decapods from temperate waters.

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