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Characterization of antioxidant systems, oxidation status and lipids in brain of wild-caught size-class distributed *Aristeus antennatus* (Risso, 1816) Crustacea, Decapoda

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Abstract

The objectives of the study were to characterize the enzymic antioxidant system (free radical scavenging enzymes such as catalase, superoxide dismutase, glutathione peroxidases, glutathione transferase and glutathione reductase), dietary antioxidants (vitamin E), the oxidation status (malondialdehyde (MDA) levels and the fluorescence intensity of lipid-soluble fluorescent products (LSFP)) and lipid composition (lipid classes and polyunsaturated fatty acids (PUFA) as pro-oxidants) in neural tissues from males and females of wild-caught size-class distributed blue and red marine shrimp *Aristeus antennatus* (Risso, 1816), trawled off the south coast of Spain. Moreover, the mechanisms that may result in the deposition of age-pigments in relation to the physiological age of this species in its natural environment were investigated. Three different size classes were defined for males and four for females, and differences were observed for the different variables measured between sexes. The proportion of polar lipids (primarily phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine) predominated over that of neutral lipids, increasing significantly in males but decreasing in females. However, cerebrosides increased significantly from size–age class I to III in males but no significant differences were observed in females. The fatty acid composition showed increases in monounsaturated fatty acids (particularly 18:1 and 24:1 isomers) and dimethyl acetals, but decreases in PUFA (primarily 22:6(n-3)) with increasing size–age in both sexes. The concentration of MDA (nmol g^{-1} brain) did not present any marked trend with size–age in both sexes. In contrast, fluorescence intensity showed increasing trends in both sexes with increasing size–age, when expressed as % fluorescence $brain^{-1}$ ($\lambda_{ex/em}$ 350–445 nm and $\lambda_{ex/em}$ 400–455). However, when expressed as % fluorescence mg^{-1} brain total lipid, only males presented an upward trend with size–age ($\lambda_{ex/em}$ 400–455). The concentration of vitamin E (ng mg^{-1} brain) did not show significant differences between different size–age classes within the same sex and showed a molar ratio of one molecule of vitamin E per approximately 200 molecules of PUFA in brain membranes. The antioxidant enzyme activities showed clearer patterns with increasing size–age in males than in females, with catalase and glutathione transferase presenting downward trends and superoxide dismutase and total glutathione peroxidase showing upward trends. The fluorescence analysis of brain LSFP was not a useful tool to separate the population into different size–age classes, although the different patterns encountered between sexes for the variables measured points to males as better subjects for this type of study. © 1999 Elsevier Science Inc. All rights reserved.

Keywords: Brain; Fatty acids; Lipid classes; Lipid-soluble fluorescent products; Malondialdehyde; *Aristeus antennatus*; Catalase; Superoxide dismutase; Glutathione peroxidases; Glutathione transferase; Glutathione reductase; Vitamin E; Size–age

Abbreviations: AA, arachidonic acid, 20:4(n-6); CAT, catalase; DHA, docosahexaenoic acid, 22:6(n-3); DMA, dimethyl acetal; EPA, eicosapentaenoic acid, 20:5(n-3); GPX-Se, glutathione peroxidase Se dependent; GPX-total, total glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GST, glutathione-*S*-transferase; HUFA, highly unsaturated fatty acid ($\geq$ 20:3); LSFP, lipid-soluble fluorescent products; MDA, malondialdehyde; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; SOD, superoxide dismutase; TAG, triacylglycerol; TBARS, thiobarbituric acid reactive substances.

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1. Introduction

It has been hypothesized that the determination of the age of individuals in wild crustacean populations can be achieved by measuring in post mitotic tissues, particularly in brain, the accumulation of fluorescent age-pigments (lipofuscin) which are thought to be universal correlates of animal senescence [55]. In attempting to 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 [3,57,58,66] and (2) measuring the fluorescence intensity of lipid/water soluble fluorescent products with a spectrofluorometer [7,28,43,44,54,56]. 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 [9,61]. 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 [68,69]. 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 [29,32,33]. 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 [32,47].

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 antioxidant protective mechanisms [29,47]. Lipids from marine organisms, including marine crustaceans, are rich in highly unsaturated fatty acids (HUFA), primarily eicosapentaenoic acid (20:5(n-3); EPA) and docosahexaenoic acid (22:6(n-3); DHA) [42,43,51]. DHA and EPA are major components of neural and retinal tissues of vertebrates and marine invertebrates, including crustaceans [6,52,53]. In a previous study, we investigated the relation between the oxidation status and the intensity of fluorescence of lipid-soluble fluorescent products (LSFP) in brain of size-class distributed *Parapenaeus longirostris* to detect correlations between fluorescence derived from oxidative processes and size–age of the shrimp population [40]. In the present, parallel study with another deep sea marine shrimp species (*Aristeus antennatus*), we aimed to characterize the brain antioxidant enzyme system (represented by free radical scavenging enzymes such as catalase, superoxide dismutase, glutathione peroxidases, glutathione transferase and glutathione reductase) and vitamin E content (dietary antioxidant) in relation to the oxidation status (represented by the levels of malondialdehyde (MDA), derived from the breakdown of PUFA hydroperoxides [32], and the fluorescence intensity of LSFP, prooxidant levels (represented by brain lipids, particularly PUFA and HUFA), and the size–age of the population. We also aimed to reproduce a number of previous studies which attempted to resolve age cohorts in unpurified lipid soluble fluorescent products [43,44,54,56] by investigating the usefulness of the brain lipid-soluble fluorescence for detecting age-cohorts [7,44] in this species. Moreover, a crucial objective of this study was to further elucidate the mechanisms that may produce deposition of age-pigments in the brain of crustacean in its natural environment.

2. Materials and methods

2.1. Shrimp collection and dissection of shrimp brain

Wild-caught deep water blue and red shrimp *A. antennatus* (Risso, 1816) were trawled at 700 m depth, in the Mediterranean waters of SE Spain in June 1997, by a commercial shrimp trawler. The population was separated by size-frequency modes, and males and females classified into three and four groups of similar orbital carapace length, respectively (Table 1). All sampled animals were hard shelled and in intermoult period. About 100 individuals for each size group were sampled onboard. Shrimp carapaces were dissected at the mouthend and the anterior portion containing the brain, which was then 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 ($n = 10–42$ for each sample depending on size and sex), thiobarbituric acid reactive substances (TBARS) quantification, vitamin E content and antioxidant enzymes assays ($n = 5–20$).

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

Table 1
Biometric data, total lipid, TBARS (MDA) values, relative LSFP levels, vitamin E contents and antioxidant enzyme activities in brain of size-class distributed males and females of wild-caught *A. antennatus*[a]

Sample	Males			Females			
	M1	M2	M3	F1	F2	F3	F4
Size/age class	I	II	III	I	II	III	IV
Carapace length (mm)	<20	23–26	30–32	<28	31–38	42–50	>55
Live mass/brain (mg)	9.6 ± 0.3^{b}	16.2 ± 0.5^{c}	20.8 ± 0.6^{d}	11.2 ± 0.2^{b}	20.2 ± 1.6^{c}	28.0 ± 0.8^{d}	53.1 ± 2.7^{e}
Total lipid/brain (mg)	0.9 ± 0.0^{b}	1.4 ± 0.0^{c}	2.6 ± 0.2^{d}	1.0 ± 0.0^{b}	2.1 ± 0.1^{bc}	3.3 ± 0.1^{c}	6.7 ± 0.9^{d}
Total lipid (live mass %)	9.2 ± 0.2^{b}	8.9 ± 0.2^{b}	12.3 ± 0.8^{c}	9.1 ± 0.4^{b}	10.4 ± 0.4^{bc}	11.8 ± 0.5^{bc}	12.6 ± 1.7^{c}
Oxidation status							
TBARS (MDA pmol brain^{-1})	784.4 ± 67.2^{b}	1589.8 ± 57.2^{c}	1255.0 ± 142.3^{d}	1190.4 ± 200.6^{b}	1533.5 ± 119.5^{b}	3454.6 ± 475.6^{c}	4759.8 ± 784.0^{d}
TBARS (MDA nmol g^{-1} brain)	81.3 ± 7.0^{b}	98.2 ± 3.5^{c}	60.3 ± 6.8^{d}	105.9 ± 17.9^{bc}	76.0 ± 5.9^{d}	123.4 ± 17.0^{b}	89.6 ± 14.8^{cd}
LSFP (% fluorescence brain^{-1})							
$\lambda_{ex/em} = 350-445$nm	2.6 ± 0.6^{b}	2.8 ± 0.6^{b}	9.6 ± 1.8^{c}	3.4 ± 0.2^{b}	4.7 ± 1.6^{b}	6.1 ± 0.7^{b}	20.1 ± 4.0^{c}
$\lambda_{ex/em} = 400-455$nm	12.9 ± 3.5^{b}	17.9 ± 2.8^{b}	86.5 ± 25.2^{c}	25.5 ± 10.0^{b}	36.7 ± 6.2^{b}	62.4 ± 15.5^{b}	126.0 ± 31.3^{c}
LSFP (% fluorescence mg^{-1} total lipid)							
$\lambda_{ex/em} = 350-445$nm	2.9 ± 0.7	2.0 ± 0.4	3.7 ± 0.7	3.3 ± 0.2	2.2 ± 0.8	1.9 ± 0.2	3.0 ± 0.6
$\lambda_{ex/em} = 400-455$nm	14.6 ± 4.0^{b}	12.4 ± 1.9^{b}	33.7 ± 9.8^{c}	24.8 ± 10.0	17.5 ± 3.0	18.9 ± 4.7	18.9 ± 4.7
Vitamin E (ng brain^{-1})	644.6 ± 23.1	1302.6 ± 187.1	1323.9 ± 393.9	820.0 ± 143.2^{b}	1500.6 ± 17.4^{bc}	1942.0 ± 279.4^{cd}	2972.1 ± 569.1^{d}
Vitamin E (ng mg^{-1} brain)	66.8 ± 2.4	80.4 ± 11.6	63.6 ± 18.9	73.0 ± 12.7	74.4 ± 0.9	69.4 ± 10.0	56.0 ± 10.7
Molar ratio vitamin E/PUFA	$1/236.1 \pm 4.7^{bc}$	$1/179.7 \pm 24.6^{b}$	$1/226.4 \pm 45.4^{bc}$	$1/217.4 \pm 23.8^{b}$	$1/226.1 \pm 11.2^{bc}$	$1/237.5 \pm 23.4^{bc}$	$1/299.4 \pm 36.6^{c}$
Antioxidant enzyme activities							
CAT (μmol min^{-1} mg^{-1} protein)	19.8 ± 2.3^{b}	11.6 ± 4.6^{bc}	6.0 ± 2.8^{c}	26.2 ± 3.2^{b}	67.7 ± 16.2^{c}	28.5 ± 4.5^{bc}	17.1 ± 8.4^{b}
SOD (SOD Units mg^{-1} protein)	5.4 ± 0.0^{b}	9.0 ± 0.1^{c}	13.4 ± 2.1^{d}	9.2 ± 0.6^{b}	8.6 ± 0.2^{b}	7.1 ± 0.6^{bc}	6.0 ± 0.2^{c}
GPX-Se (nmol min^{-1} mg^{-1} protein)	56.2 ± 1.3	49.2 ± 0.7	62.3 ± 17.6	101.0 ± 0.7^{b}	54.4 ± 2.9^{c}	65.7 ± 14.1^{c}	78.0 ± 8.7^{bc}
GPX-total (nmol min^{-1} mg^{-1} protein)	62.5 ± 1.3^{b}	66.8 ± 2.2^{bc}	74.4 ± 3.6^{c}	94.5 ± 1.5	72.6 ± 2.9	69.6 ± 8.6	81.2 ± 5.3
GST (nmol min^{-1} mg^{-1} protein)	222.1 ± 10.2^{b}	188.8 ± 2.5^{c}	163.9 ± 9.7^{c}	170.3 ± 2.2^{b}	268.4 ± 10.3^{c}	265.8 ± 13.9^{c}	318.7 ± 26.0^{c}
GR (nmol min^{-1} mg^{-1} protein)	99.8 ± 21.5	138.2 ± 56.6	78.1 ± 5.8	75.1 ± 4.6^{b}	140.2 ± 43.5^{bc}	166.3 ± 11.7^{c}	123.3 ± 25.0^{bc}

[a] Results are means of pooled brain triplicates ± S.D. Values bearing different superscript letters within the same row and sex are significantly different ($P < 0.05$). LSFP, lipid soluble fluorescent products; TBARS, thiobarbituric acid reactive substances; MDA, malondialdehyde; PUFA, polyunsaturated fatty acid; CAT, catalase; SOD, superoxide dismutase; GPX-Se, glutathione peroxidase Se-dependent; GPX-total, total glutathione peroxidase; GST, glutathione transferase; GR, glutathione reductase.

hydroxytoluene (BHT) as an antioxidant. Total lipid was extracted from the brains according to the method of Folch et al. [22]. 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 and stored at −20°C between procedures.

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. Lipid classes were separated in single-dimension double-system development. First, 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 [65]. 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 [15]. 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 [20]. Lipid classes were quantified by calibrated densitometry using a Shimadzu CS-9001PC dual wavelength flying spot scanner [45]. HPTLC (10 × 10 × 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 internal standard [16]. 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 × 0.32 mm i.d., Supelco, Bellefonte, USA), an on-column injection system and FID. Hydrogen was used as carrier gas with an oven thermal gradient from initial 50 to 180°C at 35°C min^{-1} and then to a final temperature of 235°C at 3°C min^{-1}. 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 (TBARS)

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. [11]. Between 20 and 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 added 2.95 ml of freshly prepared 50 mM thiobarbituric acid solution. 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 rev./min, 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} brain and nmol MDA $brain^{-1}$ was calculated using the extinction coefficient 0.156 $\mu M^{-1} cm^{-1}$.

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 [8], but using a single-step hexane extraction [5]. HPLC analysis was performed using a 250 × 4.6 mm reverse phase Spherisorb ODS2 column (Sigma, St Louis, MO) essentially as described by Carpenter [14]. The mobile phase was 98% methanol pumped at 2 ml/min, 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).

2.7. Measurement of lipid-soluble fluorescent products (LSFP)

The lipid-soluble fluorescent products were measured basically according to Fletcher et al. [21], 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 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 spectrofluorometer at an excitation wavelength of 350 nm and emission wavelength 445 nm

[21,25] and were also measured at an excitation wavelength of 400 nm and emission wavelength of 455 nm which are characteristic of lipid complexes formed by the cross-linking of aldehyde groups of malondialdehyde with the amino groups of PE and PS [30]. 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 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 (1.0–5.0 μg/μl brain total lipid extracts). Data fitted to the equations for (1) $\lambda_{ex/em}$ (350/445 nm): $FI = 0.06 + 1.18 \times C$ ($r^2 = 0.97$; $P < 0.0001$), and (2) $\lambda_{ex/em}$ (400/455 nm): $FI = 5.4 + 7.45 \times C$ ($r^2 = 0.91$; $P < 0.0001$), where FI is the fluorescence intensity and C the concentration of the total lipid extract (μg/μl). 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 [40].

2.8. Determination of catalase, SOD, GPX, GST and GR activities in brain homogenates

Samples of brain were homogenised in 9 vol. of 20 mM phosphate buffer pH 7.4, 1 mM EDTA and 0.1% Triton X-100 and the homogenates centrifuged at $600 \times g$, to remove debris, and the resultant supernatants used directly for enzyme assays by continuous spectrophotometric rate determination.

Catalase (CAT) (EC 1.11.1.6) activity was measured by following the reduction of hydrogen peroxide at 240 nm using the extinction coefficient 0.04 mM^{-1} cm^{-1} [2]. Immediately before assay, 50 ml of 67 mM potassium phosphate buffer pH 7.0 was mixed with 80 ml of 30% (v/v) hydrogen peroxide. The assay cuvette (quartz) contained 3.0 ml of above buffered hydrogen peroxide solution plus 50 μl of sample.

Total superoxide dismutase (SOD) (EC 1.15.1.1) activity was assayed by measuring the inhibition of the oxygen-dependent oxidation of adrenalin (epinephrine) to adenochrome by xanthine oxidase plus xanthine [46]. Plastic mini-cuvettes containing 0.5 ml of 100 mM potassium phosphate buffer pH 7.8/0.1 mM EDTA, 200 μl adrenaline, 200 μl xanthine and 50 μl distilled water (uninhibited control) or 50 μl sample were prepared and the reaction initiated by the addition of 10 μl xanthine oxidase. The reaction was followed at 480 nm and 1 U of superoxide dismutase activity is described as the amount of the enzyme which inhibits the rate of adenochrome production by 50%.

Glutathione peroxidase (GPX) (EC 1.11.1.9) was assayed by following the rate of NADPH oxidation at 340 nm by the coupled reaction with glutathione reductase [4]. Plastic mini-cuvettes containing 0.75 ml of 60 mM potassium phosphate buffer pH 7.4/1 mM EDTA/2 mM sodium azide, 50 μl reduced glutathione, 100 μl NADPH and 5 μl glutathione reductase were prepared. The basal reaction was initiated by the addition of either 50 μl hydrogen peroxide solution or 50 μl cumene hydroperoxide (as substrates for selenium-dependent and total GPX activities). The non-enzymic rate without sample added was measured for later subtraction. Sample (50 μl) was added and the assay continued by measuring absorbance at 340 nm with specific activities determined using the extinction coefficient of 6.22 mM^{-1} cm^{-1}.

Glutathione transferase (GST) activity was determined by following the formation of glutathione-CDNB adduct at 340 nm. Standard plastic cuvettes containing 2.5 ml of 120 mM potassium phosphate buffer pH 6.5, 100 μl GSH and 100 μl CDNB were prepared and the reaction initiated by the addition of 50 μl sample. Specific activities were determined using an extinction coefficient of 9.6 mM^{-1} cm^{-1} [24].

Glutathione reductase (GR) (EC 1.6.4.2) activity was assayed as described by Racker [49] by measuring the oxidation of NADPH at 340 nm using the extinction coefficient 6.22 mM^{-1} cm^{-1}. Plastic mini-cuvettes containing 0.6 ml of 0.2 M potassium phosphate buffer pH 7.0/2 mM EDTA, 200 μl oxidised glutathione and 100 μl NADPH were prepared and the reaction initiated by the addition of 100 μl of sample.

Protein content in the homogenates was determined by the Folin-phenol reagent method, according to Lowry et al. [37] following digestion in NaOH/SDS.

2.9. 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 and to establish the relation between the different variables measured. Results are presented as means ± S.D. ($n = 3$) and parametric and non parametric statistics were used to analyze differences between mean values corresponding to different size-class individuals of the same sex by one-way ANOVA or Kruskal–Wallis tests followed (where appropriate) by a multiple comparison test (Tukey) [71]. Data were checked for homogeneity of the variances by the Bartlett test and, where necessary, the data were arcsine transformed before further statistical analysis.

3. Results

Biometric data, total lipid, TBARS values, relative LSFP levels, vitamin E contents and antioxidant enzyme activities in brain of size-class distributed males and females *A. antennatus* are shown in Table 1. Size-class distribution was defined according to the size-frequency modes separated statistically in the whole sample of the population. Brain live mass was 1.8- and 2.2-fold greater for males of class II and III, respectively, compared to those of class I. On the other hand, brain live mass of class II, III and IV females were, respectively, 1.8-, 2.5- and 4.7-fold larger than those of class I. Total lipid content on a brain live mass percentage basis was significantly higher in brain of class III males and of class IV females, with values in all classes at approximately 10%. Brains of class II and III males were 1.5- and 2.9-fold richer in total lipids than brains of the class I male. Similarly, brains of class II, III and IV females showed 2.1-, 3.3- and 6.7-fold more total lipids than brains of class I. In males, total MDA content per brain was significantly different between classes I, II and III, increasing by 2.0-fold from class I to class II but decreasing by 1.3-fold from class II to class III. When the results were expressed as nmol MDA g^{-1} brain, the MDA content significantly increased (by 17.2%) from class I to class II and decreased significantly (by 38.6%) from class II to class III. In females, total MDA content was identical in classes I and II but 2.2–2.9 and 3.1–4.0 times significantly higher in class III and class IV brains, respectively, in comparison to that of classes I and II. In contrast, when MDA content in female brains was expressed as nmol g^{-1} brain the lowest value was shown by class II brains and the highest by class III brains (Table 1).

The intensity of fluorescence of LSFP from male brain when expressed per whole brain showed, in both pairs of excitation–emission wavelength measured, identical values in classes I and II but significantly higher in class III. A similar result was observed for females, which presented identical intensity of fluorescence in classes I, II and III but significantly higher in class IV. However, when the intensity of fluorescence was expressed as % fluorescence per mg of lipid soluble extract, the male brain presented no significant differences between classes for the pair $\lambda_{ex/em}$ 350–445 nm but showed that the intensity of fluorescence was significantly higher in class III when measured at $\lambda_{ex/em}$ 400–455 nm. On the other hand, female brain did not show a significant difference between the classes when measured with both pairs of wavelengths.

Vitamin E content, when expressed per whole brain (ng $brain^{-1}$), showed increasing, but not statistically significant, trends between the different classes in both males and females. When data were presented as ng mg^{-1} brain, no significant differences between different classes were observed within male or female brains. The molar ratio of vitamin E (antioxidant) to PUFA (prooxidant) in male and female *A. antennatus* is one molecule of vitamin E per approximately 200 molecules of PUFA in brain membranes (Table 1).

The activity of CAT (μmol min^{-1} mg^{-1} protein) in brain of males showed a significant downward trend whereas no particular trend was observed for the activity of this enzyme in brain of females, with the significantly highest value in class II. SOD activity (SOD units mg^{-1} protein) showed a significant upward trend in male brain from class I to class III, whereas in female brain, SOD decreased significantly from class I to class IV. Se-dependent GPX activity (nmol min^{-1} mg^{-1} protein) showed no significant differences between classes in male brain and no particular trend was observed in female brain for the activity of this enzyme between size–age classes. Total GPX activity (nmol min^{-1} mg^{-1} protein) presented an upward trend from class I to class III in males whereas in females no significant differences were observed in activities between classes. GST activity (nmol min^{-1} mg^{-1} protein) in brain presented a significant downward trend from class I to class III in males whereas in females it showed a significant upward trend from class I to class IV. GR activity (nmol min^{-1} mg^{-1} protein) in brain of males showed no significant differences between the different classes whereas in females GR increased from class I to class III and then decreased in class IV (Table 1).

Lipid class composition of total lipids from brain of size–class distributed males and females of *A. antennatus* are presented in Table 2. The proportion of total polar lipids predominated over that of total neutral lipids, and, whereas polar lipids in male brain showed a significant upward trend (concomitant significant downward trend for neutral lipids) from class I to class III, no significant trend was observed in females for the different size/age classes. The major polar lipid classes were phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidic acid/cardiolipin (PA/CL) and no significant trends were observed between different groups. However, the percentage of cerebrosides showed a significant upward trend from class I to III in males but no significant differences were observed between different classes in females. In males, the proportion of cerebrosides increased by 4.9 and 22.8% from class I to II and from class II to III, respectively. In total neutral lipids, free cholesterol (C) was the major fraction comprising about 75% but showing no significant trends between different groups in males or females. However, it is noteworthy that triacylglycerol fraction (TAG) decreased from class I to class III in lipids of male brain, whereas in female brain, it increased from class I to class IV (Table 2).

Table 2
Lipid class composition (total lipid percentage) of brain lipid from wild-caught males and females of *A. antennatus* size-class distributed[a]

Sample	Males			Females			
	M1	M2	M3	F1	F2	F3	F4
Sphingomyelin	1.3 ± 0.1	1.6 ± 0.0	1.8 ± 0.3	1.9 ± 0.6	1.5 ± 0.2	1.3 ± 0.1	1.6 ± 0.1
Phosphatidylcholine	22.6 ± 1.0	24.7 ± 0.4	23.3 ± 1.5	25.1 ± 1.0[b]	21.9 ± 1.2[bc]	23.3 ± 0.4[bc]	20.4 ± 1.6[c]
Phosphatidylserine	9.6 ± 0.5[b]	10.7 ± 0.2[bc]	11.1 ± 0.2[c]	11.0 ± 0.5	9.7 ± 0.7	9.7 ± 0.4	10.8 ± 0.5
Phosphatidylinositol	1.7 ± 0.1[b]	1.5 ± 0.2[bc]	1.2 ± 0.0[c]	1.7 ± 0.1[b]	1.0 ± 0.1[c]	0.9 ± 0.2[c]	0.8 ± 0.2[c]
Phosphatidic acid/cardiolipin	10.2 ± 0.6	8.7 ± 0.2	9.6 ± 0.8	10.4 ± 0.7	10.2 ± 0.6	9.3 ± 0.7	9.6 ± 0.4
Phosphatidylethanolamine	13.9 ± 0.2	14.5 ± 0.8	13.5 ± 0.3	13.4 ± 0.4	13.7 ± 0.5	13.5 ± 0.1	13.8 ± 0.5
Lyso-phosphatidylcholine	0.4 ± 0.2	0.3 ± 0.1	0.4 ± 0.0	0.5 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	0.2 ± 0.0
Cerebrosides	7.7 ± 0.0[b]	8.1 ± 0.2[c]	10.5 ± 0.1[d]	8.4 ± 0.2	8.4 ± 0.3	8.4 ± 0.5	9.4 ± 0.5
Pigments	2.0 ± 0.2[b]	2.1 ± 0.2[b]	4.1 ± 0.5[c]	2.7 ± 0.1[b]	2.3 ± 0.3[bc]	1.9 ± 0.2[c]	3.0 ± 0.3[b]
Total polar lipids	69.3 ± 1.0[b]	72.3 ± 0.4[c]	75.5 ± 0.4[d]	75.1 ± 0.7[b]	69.0 ± 3.4[c]	68.6 ± 0.8[c]	69.5 ± 1.5[bc]
Cholesterol	22.7 ± 0.5[b]	22.2 ± 0.4[b]	20.8 ± 0.4[c]	21.2 ± 0.8[b]	25.6 ± 2.8[bc]	26.8 ± 0.3[c]	24.2 ± 0.1[bc]
Free fatty acid	3.4 ± 0.4[b]	2.1 ± 0.2[c]	2.4 ± 0.1[c]	2.7 ± 0.2[b]	2.7 ± 0.4[b]	1.9 ± 0.2[c]	2.0 ± 0.2[bc]
Triacylglycerol	4.5 ± 0.8[b]	3.4 ± 0.8[b]	1.3 ± 0.1[c]	0.9 ± 0.0[b]	2.7 ± 0.9[bc]	2.8 ± 0.8[bc]	4.4 ± 1.3[c]
Total neutral lipids	30.6 ± 1.1[b]	27.7 ± 0.4[c]	24.5 ± 0.4[d]	24.9 ± 0.7[b]	31.0 ± 3.4[c]	31.4 ± 0.8[c]	30.5 ± 1.5[bc]

[a] Results are means of pooled brain triplicates ± S.D. Values bearing different superscript letters within the same row and sex are significantly different ($P<0.05$).

In total lipid, the proportion of total saturated fatty acids (primarily 16:0 and 18:0) decreased in male brain lipids from class I to class III and also in females from class I to class IV (Table 3). In contrast, total monoenes (primarily 18:1(n-9)) showed a significant increasing trend in both males and females. Total dimethyl acetals (DMA) (derived from alkenyl-linked lipids) increased significantly in both males and females throughout the class groups. The percentages of total PUFA (predominantly 20:5(n-3) and 22:6(n-3)) were significantly higher in brain lipid of size class I, and decreased with age class in both sexes. It is noteworthy that the proportion of 20:5(n-3) remained more constant before decreasing in the highest age class, whereas the proportions of 22:6(n-3) decreased significantly by 13.8% from class I to class II and by 40% from class II to class III in males, and by 11.2% from class I to class II, by 12.7% from class II to class III and by 38.7% from class III to class IV in females. Arachidonic acid (20:4(n-6)) remained constant throughout all classes in males and increased significantly at class IV in females (Table 3).

4. Discussion

Brains of males and females of *A. antennatus* were larger than those of male and female *P. longirostris*, trawled between 150 and 400 m depth in the Atlantic waters of SW Spain (Gulf of Cádiz), but presented the same total lipid content [40]. In males of *A. antennatus*, the concentration of MDA (nmol g^{-1} brain) and fluorescence intensities (% fluorescence mg^{-1} total lipid soluble extract) at $\lambda_{ex/em}$ (350–445 nm) and $\lambda_{ex/em}$ (400–455 nm) were positively correlated ($r^2=0.98$; $P=0.09$ and $r^2=0.85$; $P=0.25$, respectively). However, MDA content (nmol g^{-1} brain) was not correlated with carapace length and decreased with size/age, whereas fluorescence intensities (%/mg TL) at $\lambda_{ex/em}$ (350–445 nm) and $\lambda_{ex/em}$ (400–455 nm) were both positively correlated with carapace length ($r^2=0.44$; $P=0.05$ and $r^2=0.62$; $P=0.01$, respectively), which does not indicate that the brain growth rate is higher than the brain oxidation rate as shown in a previous study for *P. longirostris* [40] but show that LSFP increase with size/age in males of this species. In contrast, in females of *A. antennatus*, the concentration of MDA (nmol g^{-1} brain) and fluorescence intensities (% fluorescence mg^{-1} total lipid soluble extract) for both pairs of wavelengths analyzed were not correlated with carapace length in the present study and showed no significant differences between the four different size classes. In particular, the intensity of fluorescence (% fluorescence mg^{-1} total lipid soluble extract) analysed for $\lambda_{ex/em}$ (400–455 nm), which is characteristic of lipid complex formed by the cross-linking of aldehyde groups of malondialdehyde with the amino groups of PE and PS [30] was higher than that analysed for $\lambda_{ex/em}$ (350-445 nm).

The MDA levels (nmol g^{-1} brain) in brains of male and female *A. antennatus* were higher than those found in brains of male and female *P. longirostris*, whereas the values for the intensity of fluorescence (% fluorescence mg^{-1} total lipid soluble extract) measured at $\lambda_{ex/em}$ (350–445 nm) were similar [40]. As in *P. longirostris*, the statistical analysis of fluorescence intensity from

brain LSFP in the present study showed no significant differences between the different size classes that could be assigned as belonging to different age classes. As a result, 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 [54]. A likely explanation is perhaps the non-specificity of the assay procedure [54,69] rather than a problem with LSFP/lipofuscin as an age determinant. In insects, several studies have demonstrated that the accumulation

Table 3
Total lipid fatty acid composition (mass percentage) from brain of wild-caught males and females of *A. antennatus* size-class distributed[a]

Fatty acid	Males			Females			
	M1	M2	M3	F1	F2	F3	F4
14:0	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1
15:0	2.5 ± 0.4^{b}	2.1 ± 0.2^{b}	3.7 ± 0.4^{c}	2.8 ± 0.3	2.6 ± 0.7	2.2 ± 0.1	2.3 ± 0.1
16:0	7.6 ± 0.3^{b}	7.2 ± 0.1^{b}	6.1 ± 0.1^{c}	7.3 ± 0.0^{b}	6.8 ± 0.2^{c}	6.7 ± 0.3^{c}	6.1 ± 0.2^{d}
17:0	0.4 ± 0.0^{b}	0.3 ± 0.0^{c}	0.2 ± 0.0^{d}	0.4 ± 0.0^{b}	0.3 ± 0.0^{b}	0.2 ± 0.1^{c}	0.2 ± 0.0^{c}
18:0	5.4 ± 0.2^{b}	4.9 ± 0.1^{bc}	4.2 ± 0.7^{c}	5.5 ± 0.1^{b}	5.1 ± 0.2^{c}	4.9 ± 0.1^{c}	3.9 ± 0.1^{d}
20:0	0.5 ± 0.0^{b}	0.5 ± 0.0^{bc}	0.4 ± 0.0^{c}	0.5 ± 0.0^{b}	0.5 ± 0.0^{bc}	0.5 ± 0.0^{c}	0.5 ± 0.0^{b}
24:0	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.2 ± 0.2	nd
Total saturated	17.3 ± 0.3^{b}	15.9 ± 0.2^{bc}	15.3 ± 0.9^{c}	17.1 ± 0.3^{b}	15.9 ± 0.9^{bc}	15.0 ± 0.5^{c}	13.3 ± 0.5^{d}
16:1(n-7)	3.2 ± 0.1^{b}	3.5 ± 0.2^{b}	3.9 ± 0.2^{c}	3.4 ± 0.2^{b}	3.6 ± 0.1^{b}	4.1 ± 0.1^{c}	4.1 ± 0.1^{c}
18:1	26.6 ± 0.6^{b}	29.0 ± 0.1^{c}	30.1 ± 0.9^{c}	27.2 ± 1.0^{b}	29.9 ± 0.6^{c}	31.8 ± 0.2^{c}	32.6 ± 1.2^{c}
20:1(n-9)	2.4 ± 0.1^{b}	2.4 ± 0.0^{b}	1.7 ± 0.1^{c}	2.1 ± 0.0	2.1 ± 0.1	2.0 ± 0.0	2.1 ± 0.2
22:1(n-11)	2.2 ± 0.1	2.0 ± 0.1	2.0 ± 0.2	2.3 ± 0.2^{b}	2.0 ± 0.1^{bc}	1.8 ± 0.0^{cd}	1.6 ± 0.1^{d}
22:1(n-9)	1.5 ± 0.2	1.3 ± 0.2	1.4 ± 0.2	1.2 ± 0.1^{b}	1.4 ± 0.0^{bc}	1.4 ± 0.1^{c}	1.7 ± 0.1^{d}
24:1(n-9)	3.8 ± 0.2	4.1 ± 0.1	4.5 ± 0.5	3.9 ± 0.3^{b}	4.4 ± 0.1^{bc}	5.0 ± 0.3^{cd}	5.2 ± 0.1^{d}
24:1(n-7)	3.5 ± 0.1^{b}	4.0 ± 0.1^{bc}	4.5 ± 0.5^{c}	3.8 ± 0.1^{b}	4.2 ± 0.1^{bc}	4.5 ± 0.1^{c}	4.7 ± 0.5^{c}
Total monoenes	43.1 ± 1.0^{b}	46.3 ± 0.3^{bc}	48.1 ± 1.7^{c}	44.0 ± 1.4^{b}	47.6 ± 0.7^{bc}	50.7 ± 0.6^{cd}	51.9 ± 1.8^{d}
16:2(n-6)	1.5 ± 0.0^{b}	1.5 ± 0.1^{b}	2.1 ± 0.3^{c}	1.7 ± 0.0^{b}	1.5 ± 0.1^{bc}	1.4 ± 0.0^{bc}	1.3 ± 0.2^{c}
16:3(n-3)	0.9 ± 0.1	1.0 ± 0.0	1.0 ± 0.1	1.0 ± 0.0^{b}	0.9 ± 0.1^{b}	0.7 ± 0.1^{c}	1.0 ± 0.0^{b}
16:4(n-3)	0.8 ± 0.1^{bc}	0.6 ± 0.0^{b}	0.9 ± 0.1^{c}	0.9 ± 0.1^{b}	0.7 ± 0.1^{bc}	0.5 ± 0.0^{c}	0.6 ± 0.0^{c}
18:2(n-6)	1.2 ± 0.1	1.0 ± 0.1	1.1 ± 0.2	1.3 ± 0.1^{b}	1.1 ± 0.2^{bc}	1.0 ± 0.0^{c}	0.7 ± 0.1^{d}
20:2(n-6)	0.7 ± 0.0^{b}	0.6 ± 0.0^{c}	0.5 ± 0.1^{d}	0.7 ± 0.1^{b}	0.5 ± 0.1^{c}	0.4 ± 0.0^{d}	0.4 ± 0.0^{d}
20:3(n-6)	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	nd	nd
20:4(n-6)	4.4 ± 0.1	4.8 ± 0.1	4.9 ± 1.1	4.7 ± 0.5^{bc}	4.3 ± 0.3^{b}	4.1 ± 0.1^{b}	5.3 ± 0.1^{c}
20:3(n-3)	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	tr
20:4(n-3)	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	0.2 ± 0.0^{c}	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	0.6 ± 0.1^{c}	0.2 ± 0.0^{d}
20:5(n-3)	8.7 ± 0.3^{b}	8.5 ± 0.1^{b}	6.2 ± 0.4^{c}	8.3 ± 0.2^{b}	8.1 ± 0.4^{b}	8.1 ± 0.1^{b}	6.9 ± 0.1^{c}
22:2(n-6)	2.2 ± 0.2	2.0 ± 0.1	2.3 ± 0.2	2.2 ± 0.0^{b}	2.3 ± 0.0^{b}	2.4 ± 0.2^{b}	2.8 ± 0.2^{c}
21:5(n-3)	0.4 ± 0.3	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.1 ± 0.1	nd	tr
22:4(n-6)	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.1^{bc}	0.2 ± 0.0^{d}	0.3 ± 0.0^{cd}	0.4 ± 0.0^{b}
22:3(n-3)	0.3 ± 0.0^{b}	0.4 ± 0.0^{bc}	0.5 ± 0.1^{c}	0.4 ± 0.0^{bc}	0.4 ± 0.0^{bc}	0.4 ± 0.0^{b}	0.5 ± 0.0^{c}
22:5(n-6)	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	0.2 ± 0.0^{c}	0.3 ± 0.0^{b}	0.2 ± 0.0^{c}	0.2 ± 0.0^{c}	0.2 ± 0.0^{d}
22:5(n-3)	0.7 ± 0.0^{b}	0.6 ± 0.0^{bc}	0.5 ± 0.1^{c}	0.7 ± 0.0^{b}	0.6 ± 0.0^{c}	0.7 ± 0.0^{bc}	0.6 ± 0.0^{d}
22:6(n-3)	8.7 ± 0.5^{b}	7.5 ± 0.2^{c}	4.5 ± 0.4^{d}	8.0 ± 0.2^{b}	7.1 ± 0.4^{c}	6.2 ± 0.2^{d}	3.8 ± 0.2^{e}
Total polyenes	31.0 ± 1.0^{b}	29.3 ± 0.6^{b}	24.9 ± 1.4^{c}	30.4 ± 1.1^{b}	28.1 ± 0.5^{c}	26.6 ± 0.1^{cd}	24.5 ± 0.5^{d}
16:0DMA	1.1 ± 0.0^{b}	1.3 ± 0.0^{b}	1.8 ± 0.2^{c}	1.1 ± 0.0^{b}	1.2 ± 0.1^{bc}	1.3 ± 0.1^{c}	2.0 ± 0.4^{c}
16:1DMA	0.2 ± 0.0^{bc}	0.1 ± 0.0^{b}	0.3 ± 0.1^{c}	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1
18:0DMA	2.4 ± 0.0	2.7 ± 0.0	2.8 ± 0.3	2.5 ± 0.1^{b}	3.0 ± 0.2^{c}	3.2 ± 0.1^{cd}	3.6 ± 0.0^{d}
18:1(n-9)DMA	0.3 ± 0.0^{b}	0.4 ± 0.0^{b}	0.5 ± 0.0^{c}	0.3 ± 0.0^{b}	0.4 ± 0.0^{b}	0.4 ± 0.1^{b}	0.5 ± 0.0^{c}
18:1(n-7)DMA	0.4 ± 0.0^{b}	0.5 ± 0.0^{b}	0.7 ± 0.1^{c}	0.4 ± 0.0^{b}	0.4 ± 0.0^{b}	0.4 ± 0.1^{b}	0.7 ± 0.0^{c}
Total DMA	4.3 ± 0.1^{b}	5.0 ± 0.1^{c}	6.1 ± 0.4^{d}	4.4 ± 0.2^{b}	5.0 ± 0.3^{bc}	5.4 ± 0.2^{c}	7.0 ± 0.5^{d}
Unknown	3.4 ± 0.6	2.7 ± 0.4	4.6 ± 2.3	3.2 ± 0.7	2.7 ± 0.4	1.8 ± 0.1	2.7 ± 1.3
Total (n-9)	34.0 ± 0.9^{b}	36.5 ± 0.4^{c}	37.3 ± 1.4^{c}	34.2 ± 1.4^{b}	37.6 ± 0.6^{c}	40.1 ± 0.6^{d}	41.4 ± 1.1^{d}
Total (n-7)	6.8 ± 0.2^{b}	7.5 ± 0.1^{c}	8.4 ± 0.4^{d}	7.2 ± 0.2^{b}	7.8 ± 0.1^{b}	8.6 ± 0.1^{c}	8.7 ± 0.6^{c}
Total (n-6)	10.7 ± 0.2	10.7 ± 0.4	11.5 ± 0.9	11.2 ± 0.8^{b}	10.3 ± 0.5^{bc}	9.7 ± 0.2^{c}	11.1 ± 0.3^{b}
Total (n-3)	20.3 ± 0.9^{b}	18.7 ± 0.4^{b}	13.4 ± 0.8^{c}	19.2 ± 0.4^{b}	17.8 ± 0.6^{c}	16.9 ± 0.3^{c}	13.5 ± 0.4^{d}
HUFA(n-6)	7.3 ± 0.2	7.5 ± 0.2	7.8 ± 1.4	7.6 ± 0.6^{b}	7.1 ± 0.4^{b}	6.9 ± 0.2^{b}	8.7 ± 0.1^{c}
HUFA(n-3)	19.4 ± 1.0^{b}	17.7 ± 0.4^{b}	12.4 ± 0.9^{c}	18.2 ± 0.4^{b}	16.9 ± 0.6^{c}	16.1 ± 0.2^{c}	12.0 ± 0.3^{d}

[a] Data are means ± S.D. ($n = 3$). S.D. = 0.0 implies a S.D. < 0.05; nd, not detected; tr, trace < 0.05. Values within the same sex bearing different superscript letters are significantly different ($P < 0.05$). DMA, dimethyl acetal; HUFA, highly unsaturated fatty acids.

of LSFP is a linear function of time, metabolic rate, lipid peroxidation potential and aging [19,38,39,61,62]. The increase in the level of oxidative stress with aging is thought to arise from an imbalance between pro-oxidants and antioxidants, more probably due to an increase in the rates of oxidant generation than to a decline in the level of antioxidant defences. Tissue protection against cytotoxicity originated by lipid peroxidation is due, in part, to mitochondrial oxidation of MDA [59], a capacity which is substantially lost with age [31]. This could provide an explanation for the accumulation of MDA and LSFP lipofuscin-like materials, according to the fact that the level of oxidative stress increases during aging, causing oxidative damage and increasing mitochondrial oxidant generation [62].

In males and females, the concentration of vitamin E (ng mg^{-1} brain) was not correlated with carapace length, as it was shown for *P. longirostris* [40]. Although vitamin E plays a very significant role in reproduction of Penaeids [1,13,17] and it is believed that shrimp may possess hydrolytic and transport mechanisms for vitamin E similar to those found in fish and other vertebrates [12,27,35], no evidence was found of lower vitamin E concentration in the brain of females at reproductive stages in *A. antennatus*. The values for vitamin E in males and females of *A. antennatus* were similar to those found in *P. longirostris* and are thought to be sufficient to help to maintain the integrity of the neural and brain tissues [40], although limited data exist regarding bioavailability, biokinetics, storage and mobilization of vitamin E in crustaceans. In any case, it is believed that the ratio of α-tocopherol to PUFA in tissues is probably critical in protection against lipid oxidation, and may modulate the vitamin E requirement. According to Buettner [10], one molecule of α-tocopherol can protect approximately 1000 molecules of PUFA from oxidation. The molar ratio of α-tocopherol to PUFA is normally between 1:100 and 1:500 in human low density lipoproteins [18], between 1:400 and 1:2000 in rat tissues [48] and between 1:350 and 1:3400 in Atlantic salmon [26]. In the present study, values of molar ratio of α-tocopherol to PUFA in brain tissues from male and female *A. antennatus* ranged from about 1/180 to 1/300 indicating a level of protection 3–5 times higher than the minimum suggested by Buettner [10], and also indicated a sufficient level of vitamin E was ingested by this species in the wild. In females, a significant decrease of molar ratio of α-tocopherol to PUFA from stage I to stage IV was shown with increasing size–age (Table 1).

To investigate whether age-related accumulation of oxidative damage in *A. antennatus* was to due to a decline in the level of antioxidative defences, correlational studies relating antioxidant enzyme activities in brain tissue and size/age were performed. CAT is a major primary antioxidant defence component that works primarily to catalyse the decomposition of H_2O_2 to H_2O, sharing this function with GPX. Therefore, both these enzymes detoxify H_2O_2 derived from SOD activity. In the presence of low H_2O_2 levels, organic peroxides are the preferred substrate for GPX. However, at high H_2O_2 concentrations, they are metabolized by CAT [70]. As a result, it is commonly assumed that any significant increase in SOD must be accompanied by a comparable increase in CAT and/or GPX [67] to prevent excessive build up of H_2O_2, such as in aged oxidative damaged mitochondria [62]. In the present study, SOD activity in brain of males increased significantly with size–age, indicating a higher presence of superoxide anion and hydrogen peroxide production with increasing size–age. Since GPX activity showed an upward trend but CAT activity followed the opposite trend, it may be that the rate of production of hydrogen peroxide due to increasing SOD activity with size–age in male *A. antennatus* brain seems to be mainly dealt with by GPX Se-containing enzyme, whereas organic hydroperoxides, such as lipid peroxides, can be reduced to their corresponding alcohols by means of non-Se GPX activity. In females, no clear trends were apparent with the exception of CAT that decreased with size–age. Similar results have been described in insects for male fruit flies and house fly indicating that SOD and/or CAT inhibition perturbs the antioxidant defense system which could be counterbalanced by adaptive changes in antioxidant defence [38]. In any case, the relationship between SOD and the age of an organism is not clear, as the age-related changes in SOD activity are often species-, gender- and tissue-specific [67]. GST activity, which catalyzes both conjugation of GSH with lipophilic electrophiles and reduction of oxidants by GSH, also showed different patterns in brain of male and female *A. antennatus* with size–age. The decreasing trends in CAT activity shown in male and female brain with size–age seem to be counterbalanced by the increasing trends in SOD and GPX activities in males, and GST and GR activities in females.

There is very little information in the literature about the lipid content and the lipid composition of the brain in crustaceans [53] and only a few studies in fish [33,41,64]. The lipid class composition of brain from wild-caught males and females of *A. antennatus* is very similar to that of the pink shrimp *P. longirostris* [40]. In comparison to fish, both species 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 [41,64]. 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 [41,64]. It is perhaps noteworthy, that there were significant differences observed between the levels of cerebrosides, which increased sig-

nificantly with size–age in both males and females of the pink shrimp [40] but only in males of *A. antennatus*, in the present study. The proportion of cerebrosides in brain total lipids were correlated with carapace length in males ($r = 0.93$; $P < 0.0001$) but not in females. The significant increase in cerebrosides may be indicative of myelination processes [34,53]. The significant increase of cerebrosides (glycosphingolipids) during neural maturation may increase glycation reactions between the reducing sugar of the cerebroside and amino compounds and this could generate age-dependent pigment-like fluorophores, not related to the oxygen-dependent analogues of secondary lipid peroxidation products [69].

An interesting characteristic of the total lipid fatty acid composition from *P. longirostris* and *A. antennatus* brain lipids is the generally lower level of unsaturation in comparison to total lipid fatty acid acids from fish brain. The proportion of 22:6(n-3) in brain lipids from these species is approximately one-fifth 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 brain lipids of the shrimps [41,64].

The proportion of total monoenes, as also shown for *P. longirostris* [40], was higher than in fish brain and the levels of 24:1(n-9) and 24:1(n-7) showed upward trends in brain total lipids with increasing size–age. These fatty acids are particularly abundant in PC (data not shown) as observed in fish [34,41,42,64]. The presence of significant levels of 24:1 fatty acids in brain phosphoglycerides, although common in cerebrosides, has not been reported in mammals. However, most of the omissions of 24:1 from mammal brain may be the result of old or inadequate gas-liquid chromatography [53], and the role(s) that molecular species of phosphoglycerides containing 24:1 may have in fish and crustacean neural tissues remains to be elucidated.

It is noteworthy that the fatty acid composition of total lipids in the brain of *P. longirostris* [40] and *A. antennatus* showed an increase in monounsaturated fatty acids and a decrease in PUFA with increasing size–age, a fact that has also been observed in fish [41] and mammals [36]. There are several hypothesis to explain the disappearance of PUFA and the increase of monoenes in the brain during aging: (1) decreases in $\Delta 6$ and $\Delta 5$ desaturase activities and increase of $\Delta 9$ desaturase activity; (2) deficiency in PUFA uptake; (3) a change in the cellular make-up of the brain during aging, possibly involving a reduction in the number of dendrites (which are rich in PUFA); (4) increased lipid peroxidation processes during aging [36,60].

The presence of significant proportions of DMA was the result of synthesis and accumulation of PE-plasmalogens which are implicated in myelin membrane composition [53]. In brain lipids of *A. antennatus*, total DMA showed a significant increasing trend in both males and females in contrast to brain DMA in the pink shrimp (*P. longirostris*) that did not vary between the different size–age classes [40]. This fact and the increase of cerebrosides observed probably reflect maturation processes of the neural system. Plasmalogens are formed by oxidation of the corresponding alkyacylglycerophospholipid, produced in peroxisomes, by a microsomal enzyme requiring NADPH and molecular oxygen [50,53,63]. In addition, 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 [23,53]. This situation could be prone to generate free radicals and oxidative stress capable of inducing lipid peroxidation and generating aldehydes (TBARS, MDA) which are precursors of fluorescent peroxidation products and age pigment-like fluorophores [68].

In conclusion, the present study has shown that there are differences in brain antioxidant system, oxidation status and lipid composition between male and female *A. antennatus* with size–age. Males appear to be more prone to oxidative stress than females and as a consequence more suitable for ageing studies since the different variables measured showed more marked differences between size–age classes in males than in females. The increase in the level of oxidative stress with aging it is most likely due to an increase in the rates of oxidant generation than to a decline in the level of antioxidant defences, since the level of vitamin E in brain of *A. antennatus* did not show significant differences between size–age classes and presented enough antioxidant protection to membrane lipids. The levels of LSFP are partially dependent on MDA produced which may be partially dependent on increasing oxidant generation and decreasing antioxidant activity (vitamin E, CAT and GST in males, and vitamin E, CAT, SOD, GPXs in females) with size–age.

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