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Identification and Quantification of the Age-pigment, Lipofuscin, in Brains of the Deep-water Rose Shrimp *Aristeus antennatus* (Risso, 1816)

by

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Abstract

Aristeus antennatus is a demersal penaeid mainly living at depth of 400-800 m in Mediterranean Sea. Since this specie is a high economic important fishery of the Spanish Mediterranean waters, the studies of age structure of the population are essential for an efficient assessment of the resource. Exist a inability to accurately estimate the age through conventional methods in crustacean and a new methodology have been developed on basis of the gradual deposition of lipofuscin in post-mitotic tissues for the ageing of animals. The identification of the age-pigment have been realized on transversal sections of the olfactory lobe cells mass (OLCM) in brain of wild-caught *A. antennatus*, observed under epifluorescence microscopy and quantified using image analysis techniques. Three different measurements of lipofuscin levels (%area fraction, granule density and granule mean size) were recorded in ten distinct sections of OLCM by individuals, body size class and sex. Relationship between body size and lipofuscin concentration (% area fraction and density granule) increased significantly linear, however, values of r^2 were low. The resolution of age structure from the lipofuscin distributions was more successful than for body size distribution. Growth parameters using Von Bertalanffy function were derived for this specie on basis of the lipofuscin. The present results confirm the potential of the lipofuscin method for the resolution of cohorts in deep-water pennies and suggested that the application of this methodology can be useful in studies of the age structure in wild populations.

Introduction

Aristeus antennatus (Risso, 1816) is important target species of the Spanish Mediterranean bottom trawl fisheries, the knowledge of the age structure of the population being essential for a reliable assessment of the resource. In the absence of accurate methods to estimate the age in crustaceans, the quantification of lipofuscin in nervous tissue has been used satisfactorily in the estimation of the physiological age (Sheehy, 1990; Sheehy *et al*., 1995; Vila, *et al*., 2000). This technique is based on the progressive accumulation of lipofuscin in animal cells with age (Brunk *et al*., 1992). Thus, the amount of the pigment recorded in non-dividing cells may serve as a valuable marker of the individuals' physiological age and hence contribute to the knowledge of the age structure of wild populations.

The aim of this study is the quantification of the lipofuscin content in the olfactory lobe cell mass (OLCM) of the *A. antennatus* brains and the validation of the lipofuscin method as an useful tool for the determination of the structure age in wild shrimp populations.

Material and Methods

A. antennatus were fished by trawl in Mediterranean waters (eastern Spain) during the MEDIT-ES 0597 research cruise. The total catch was 331 males and 565 females. Heads were fixed in 10% formaldehyde in seawater and subsequently brains were dissected, dehydrated and embedded in paraffin wax. Quantification of the relative content of lipofuscin was carried out on cross sections of the OLCM (Figures 1 and 2) by epifluorescence microscopy and image analysis. Lipofuscin levels were quantified by three types of measurements: area fraction (percentage of the OLCM occupied by pigment granules), granule density (number of lipofuscin granules per 100 μm^2) and granule mean size (mean diameter of the lipofuscin granules). A total of 74 males and 100 females were analysed with length ranges of 18-34 mm carapace length (CL) and 17-51 mm CL, respectively.

A mixed model 3-level nested ANOVA was used to detect variations in the lipofuscin contents in the OLCM between sexes, size classes within sex, individuals within size classes and sections within individuals. The percentage variance at each level was also calculated.

The Pearson's correlation coefficient was used to test the relationships between CL and lipofuscin measurements and among lipofuscin measurement. The relationships between CL and lipofuscin (area fraction and granule density) were fitted to linear regression models.

Both length and lipofuscin frequency distributions were analyzed by the Bhattacharya's (Bhattacharya, 1967) and NORMSEP iterative method (Hasselblad, 1966). A separation index (SI) >2 was used for separate normal components.

The relationship between area fraction and age-lipofuscin was fitted to simple von Bertalanfffy growth functions (VBGF):

$$CL_t = CL_\infty(1-e^{(t-t0)})$$

where CL_t is the carapace length at age t, CL_∞ is the mean asymptotic carapace length of the oldest shrimps, k is the curvature parameter defining how rapidly CL_∞ is reached, and t_0 is the theorical age at which CL=0.

Results

Effect of carapace length and sex in the lipofuscin content

Results of the mixed model 3-level nested ANOVA sowed no sexual differences for area fraction and granule density (Table 1) ($p>0.05$ y $p>0.01$, respectively). In contrast, significant differences in the lipofuscin content were found among size classes within sexes, and among individuals within size-classes ($p<0.0001$).

Components of the total variance (%) for the three-lipofuscin measurements are showed in Table 1. The highest component of variation was explained by the differences among individuals within size-classes, followed by the differences among size-classes within sexes. The contribution of sex to the total variance was low (7%). Variations caused by the error of the method, which is due to differences among sections within individuals, were 13% for both variables.

Relationships between lipofuscin content and carapace length

Relationships between carapace length and lipofuscin content, expressed as area fraction and granule density were correlated in both sexes, but the coefficients were higher in males than in females (Table 2). However, the correlation between granule mean size and CL was low in males (r=0.41) and negative in females (r=-0.05).

The granule density was strongly correlated with the area fraction in males and females (r=0.87 and r=0.92, respectively). The granule mean size was not correlated with the area fraction and granule density in both sexes (see table 2).

Relationships between lipofuscin levels (area fraction and granule density) and carapace length were fitted to linear models in both sexes (Figure 3). Determination coefficients were higher in males in both lipofuscin measurements.

Length frequency distribution analysis (LFDA)

Length frequency distribution of *A. antennatus* is shown in figure 4. Bhattacharya and NORMSEP analyses discriminated 4 age components in males and 5 in females with SI>2 (table 3). Relative age to each component was assigned on the basis on knowledge of the biology of the species.

Lipofuscin frequency distribution analysis

Figure 5 shows lipofuscin frequency distribution histograms, expressed as area fraction (AFD) and granule density (DFD) in both sexes. In males 5 physiological age groups were discriminated by NORMSEP in AFD and DFD. However, in females NORMSEP method discriminated 4 physiological age group in AFD and 3 in DFD. Results are summarised in Table 4. Mean size for each physiological age group based on lipofuscin are also showed.

Relationship between age based lipofuscin and lipofuscin

Relationships between lipofuscin levels and lipofuscin age based fitted well into a linear model in both sexes (r^2=0.95 in males and r^2=0.87 in females; Figure 6). The lipofuscin accumulation rate with age was more constant in males.

Growth rate

Growth rate has been separately estimated from through sizes resulting from length frequency distribution analysis and lipofuscin (area fraction) frequency distribution analysis (Table 5). Length increment for each age component increased progressively in LFDA, in special in females. However, results of ADFA show a decrease in growth rate in both sexes.

Discussion

Unlike other decapods (Sheehy, 1990; Sheehy *et al*., 1994; Vila *et. al*., 2000), the relative lipofuscin content in the OLCM was independent of sex. A positive linear increase in lipofuscin accumulation with size occurs in *A. antennatus*. However, a high variability in lipofuscin levels has been observed among animals of the same size. The slopes for female regressions of lipofuscin content vs carapace length were lower than those for males, indicating that for the same size the males are older. In *A. antennatus*, lipofuscin-based age classes were better discriminated than in the length frequency distribution analysis. When the lipofuscin content was expressed as area fraction, the results were more reliable than from using granule density as variable. The granule mean size proved as a poor indicator of age. These observations are in agreement with those made in *Marsupenaeus japonicus* (Vila *et al*., 2000). The mean size at age obtained from AFDA and DFDA showed a higher dispersion than those estimated from LFDA, indicating that individuals of the same size could be of different ages. On the other hand, the increment in size of each lipofuscin based age component decrease with ageing. On the contrary, when the age is estimated with length frequencies the growth rate increases with the age.

The results here in presented confirm the potential of the lipofuscin method for resolving cohorts in deep-water shrimps.

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Table 1. Mixed model 3-level nested ANOVA for lipofuscin area fraction (%), granule density (nºgran./100 µm^2) and granule mean size (µm). Variances components for each source of variation is indicated.

Source of variation	gl	SS	MS	F	*p*	Variance components (%)
Area fraction (%)						
Sex	1	3.47	3.47	2.00	0.16	2
Size class within sex	49	83.94	1.71	3.31	<0.0001	36
Individuals within size class	123	63.62	0.52	38.49	<0.0001	49
Sections within individuals	1565	21.03	0.01			13
Granule density (nºgran./100 µm^2)						
Sex	1	8.14	8.14	4.72	0.04	7
Size class within sex	49	83.56	1.71	3.03	<0.0001	33
Individuals within size class	123	69.16	0.56	37.48	<0.0001	47
Sections within individuals	1565	23.48	0.01			13
Granule mean size (µm)						
Sex	1	0.18	0.18	4.48	0.04	3.6
Size class within sex	49	1.97	0.04	1.13	0.29	4
Individuals within size class	123	4.35	0.03	29.74	<0.0001	68.7
Sections within individuals	1565	1.86	0.001			23.7

Table 2. Pearson's correlation coefficients (r_p) for lipofuscin measurements and carapace length, and for comparison lipofuscin variables.

Variables	Pearson's correlation coefficients (r_p)	
	Males	Females
Area fraction vs carapace length	0.79**	0.41**
Granule density vs Carapace length	0.62**	0.43**
Granule mean size vs Carapace length	0.41**	-0.05
Area fraction vs Granule density	0.87**	0.92**
Area fraction vs Granule mean size	0.33**	0.10**
Granule density vs Granule mean size	-0.11*	-0.23**

** High significantly correlation ($p< 0.01$).
* Significantly correlation ($p<0.05$).

Table 3. Size frequency distribution analysis.

Males				
Component	Relative age (months)	N	Mean±sd	Separation index
1	11	55	20.38±1.40	-
2	23	92	23.80±1.52	2.35
3	35	139	27.90±1.94	2.37
4	47	45	32.55±1.66	2.58
Females				
1	11	90	23.94±2.41	-
2	23	134	29.24±2.06	2.37
3	35	135	33.79±2.34	2.07
4	47	105	39.54±2.60	2.32
5	59	83	48.31±3.82	2.73
6	71	10	56.87±1.09	3.49

Table 4. Lipofuscin frequency distribution analysis.

	Component	Relative age (months)	N	Mean±sd	Separation index	Mean CL estimated (mm)
Males						
Area fraction (%)	1	23	38	0.57±0.21	-	23.55±2.63
	2	35	17	1.14±0.23	2.54	27.63±1.91
	3	47	12	1.76±0.19	2.95	32.12±1.59
	4	59	5	2.24±0.12	3.06	33.60±1.10
	5	71	2	2.72±0.10	4.32	32.90±0.00
Granule density (nº gran./100 μm^2)	1	23	31	0.43±0.17	-	23.82±2.76
	2	35	26	0.95±0.15	3.22	27.71±3.44
	3	47	13	1.30±0.14	2.46	32.17±1.63
	4	59	2	1.75±0.09	4.08	32.10±2.60
	5	71	2	2.21±0.10	4.82	31.75±1.15
Females						
Area fraction (%)	1	23	32	0.36±0.63	-	28.61±5.52
	2	35	57	0.87±0.20	2.87	35.37±8.14
	3	47	9	1.61±0.21	3.61	40.17±6.67
	4	59	3	2.81±0.37	4.36	42.70±2.07
Granule density (nº gran./100 μm^2)	1	23	44	0.35±0.17	-	30.31±7.09
	2	35	54	0.77±0.67	2.00	38.12±6.72
	3	47	2	1.77±0.07	5.92	42.75±3.05

Table 5. Growth rate estimate through results of length frequency distribution analysis and lipofuscin frequency distribution analysis expressed as area fraction.

Age component	Growth rate			
	Length frequency distribution analysis		Lipofuscin frequency distribution analysis	
	Males	Females	Males	Females
1-2	3.4	5.3	4.1	6.8
2-3	4.1	4.6	4.5	4.8
3-4	4.7	5.8	1.5	2.5
4-5	-	8.8	0	-
5-6	-	8.6	-	-

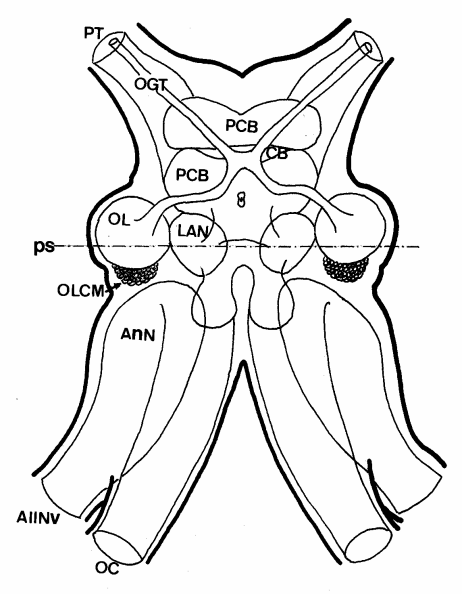

Fig. 1. Penaeid brain anatomy (dorsal view) based on the brain of *Penaeus monodon* (from Sandeman *et al*., 1993, and Sandeman & Scholtz,1995). Abreviations: AnN, antenna II neuropil; AllNv, antenna II nerve root; CB, central body; LAN, lateral antenna I neuropil; OC, oesophageal connective; OGT,olfactory globular tract; OL, olfactory lobe; OLCM, olfactory lobe cell mass; PCB, median protocerebrum; PT, protocerebral tract. Plane of sectioning through the olfactory lobe cell mass is shown by the ps line.

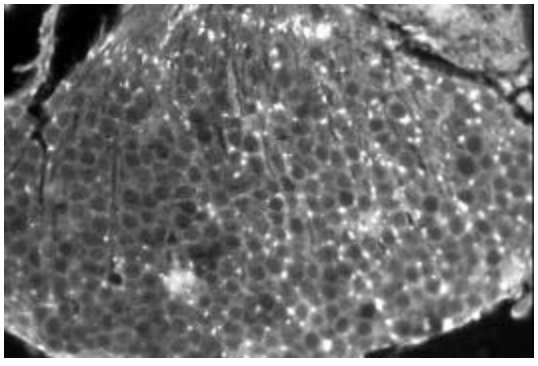

Fig. 2. Olfactory lobe cell mass in Aristeus antennatus showing lipofuscin granules (x20).

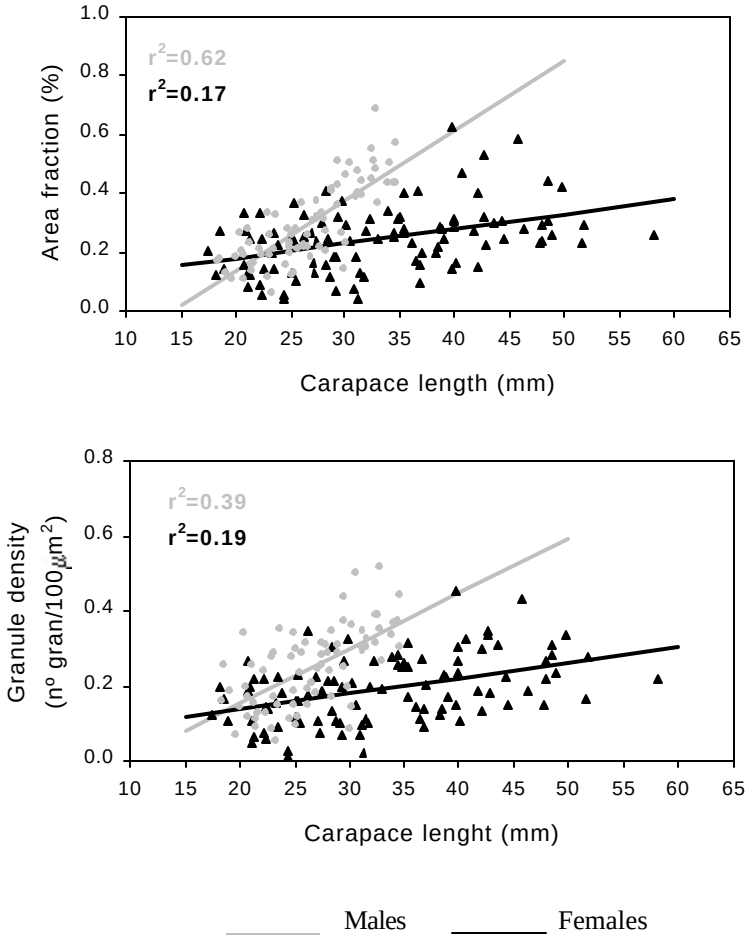

Fig. 3. Linear regressions for the relationship between area fraction and granule density and carapace lenght. Determination coefficients are showed in the top of the graph.

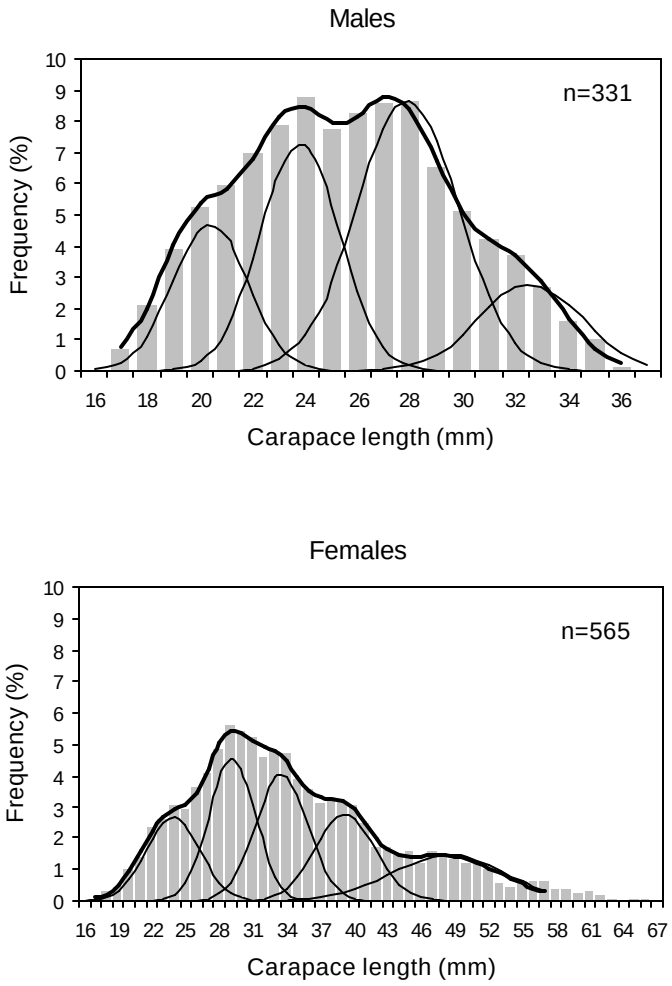

Fig. 4. Size frequency distribution histograms and results of the modal analysis.

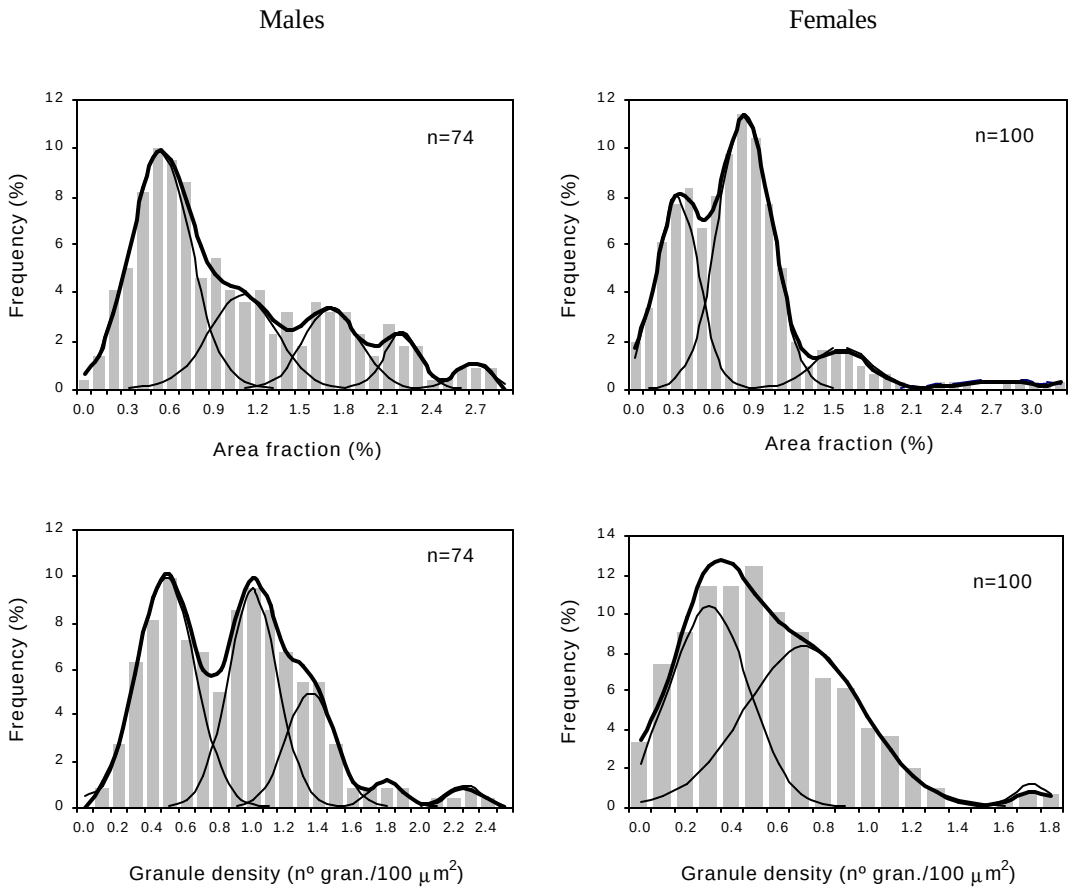

Fig. 5. Lipofuscin frequency distribution and results of the modal analysis.

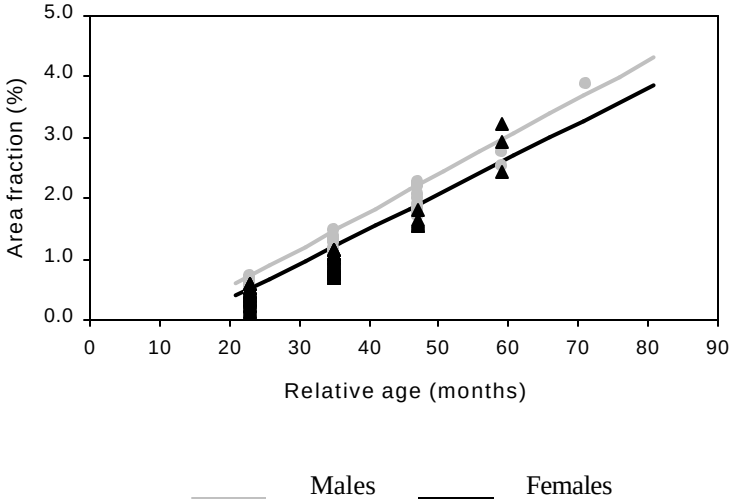

Fig. 6. Linear regressions for the relationship between area fraction and relative age based on lipofuscin.