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Evidence of lipofuscin accumulation in the deep-water red shrimp *Aristaeomorpha foliacea* (Risso, 1827)

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Abstract

Lipofuscin, a non-degradable, degenerative fluorescent pigment which accumulates in post-mitotic cells, represents a promising method for ageing marine crustaceans. The presence and accumulation of lipofuscin has been studied in the deep-water red shrimp Aristaeomorpha foliacea (Risso, 1827) to assess its use as a tool for ageing larger (i.e., older) specimens and thus improve knowledge of the growth and longevity of this species. Specimens, gathered during experimental trawl surveys carried out in the Strait of Sicily (Mediterranean Sea), were stored directly on-board in 10% buffered formaldehyde solution; their brain was thereafter removed, prepared with various current histological techniques and examined with a binocular microscope. Thin sections of the olfactory lobe cell mass were also analyzed using fluorescence microscopy, and the lipofuscin concentration was measured through image analysis. Various indices were computed for each individual by pooling data from many images: number and coverage of the lipofuscin granules per unit area, and mean individual area of the granules. Lipofuscin was detected in all specimens investigated with characteristics (grain typology and dimension) strictly resembling those already described in other crustacean species. The present preliminary results encourage further studies to develop and validate a methodology based on the use of lipofuscin for improving the relative ageing of large A. foliacea shrimps.

Keywords: Ageing; Lipofuscin; *Aristaeomorpha foliacea;* Deep-water red shrimps; Crustacea; Mediterranean.

Introduction

The red shrimp *Aristaeomorpha foliacea* is a highly-prized catch of deep-water bottom trawl fisheries in the Mediterranean, the Atlantic and the Pacific. The life history of this species has been examined in numerous contributions (BIANCHINI & RAGONESE, 1994; BIANCHINI, 1999) and recently reviewed by CAU *et al.*

(2002) and POLITOU *et al.* (2004).

Establishing absolute ages is a critical item not only in growth studies, but also in all other evaluations which depend on growth parameters. Until now, size-based methods have been the elective tool used for ageing red shrimps, since they lack permanent hard parts and lose all the integumental structures at moulting (HARTNOLL, 2001). Still, despite the presence of well-defined modes in the length frequency distributions (4-5 and 2-3 modes in females and males of *A. foliacea,* respectively), estimates of growth rates and longevity parameters of the red shrimp are still hampered by the piling-up of larger and older specimens in the last mode, a phenomenon more pronounced in males, whose growth slows down after sexual maturity. Moreover, the trend in the most recent research into red shrimps is to assign them a longer life span, and obviously slower growth and lower natural mortality rates, than previously believed (CAU *et al.,* 2002). In the search for more precise aging methods for crustaceans, the classic alternatives are rearing and tagging, whenever they are feasible; novel methods involve radionuclide ratios, examination of the ovoid bodies and lipofuscin accumulation (HARTNOLL, 2001).

Tagging and rearing, besides the risk of altering the natural growth process (BIANCHINI & RAGONESE, 2007), are difficult to implement with deep-water red shrimps; also the use of the radionuclide ratio is difficult in the high seas and moreover it provides only the time interval from the last moult. The 'ovoid body', a durable hard structure in the stomach, has been so far observed only in Norway lobsters (HARTNOLL, 2001). Consequently, lipofuscin accumulation seems the most promising alternative method for ageing red shrimps, given its broadly demonstrated association with senescence (KATZ & ROBISON, 1986; ANONYMOUS, 2002; BRUNK and TERMAN, 2002).

Lipofuscin, a mixture of brown-yellow auto-fluorescent pigments (ELLEMER, 1981), is the final product of the auto-oxidation of molecular components of cells (mainly unsaturated lipids), which originates from the beginning of cell life but accumulates more quickly in post-mitotic cells following senescence (BRUNK & TERMAN, 2002). Different hypotheses have been proposed to explain the increase of these pigments over time (KATZ & ROBISON, 1986; BRUNK *et al.,* 1992; BRUNK & TERMAN, 2002); even if it is possible to slow or reverse the age-related increase in lipofuscin content by either inhibiting its formation or enhancing its disposal (TERMAN and BRUNK, 1999), it is generally accepted that the amount of lipofuscin might reflect, if not the chronological (absolute) age, at least the relative physiological age of specimens (BRUNK & TERMAN, 2002).

Many factors are known to affect the amount of lipofuscin in cells: antioxidants (CASTRO *et al.,* 2002), temperature (SHEEHY, 1990a; O'DONOVAN & TULLY, 1996), population density (GIRVEN *et al.,* 1993), pollutants (TOTARO *et al.,* 1985a, b; KRISHNAKUMAR *et al.,* 1995; GUARINO *et al.,* 1995), hypoxia (MATHEW & DAMODARAN, 1997).

The first attempts at ageing crustaceans using lipofuscin accumulation were carried out by ETTERSHANK (1983, 1984, 1985) on the krill shrimp *Euphausia superba;* the association between lipofuscin concentration and age was

enhanced when histological techniques replaced the solvent-based methods (SHEEHY, 1996; CASTRO *et al.,* 2002).

While many studies on lipofuscin accumulation in penaeid shrimps exist (PEIXOTO *et al.,* 2002), and even on shrimps of the same family Aristeidae (*Aristeus antennatus*: SOBRINO *et al.,* 2001; VILA GORDILLO, 2005), until now lipofuscin formation and accumulation have not been studied in *A. foliacea.* Therefore the aim of the present paper is present evidence of lipofuscin accumulation with age in this deep-water shrimp.

Materials and Methods

Specimens of red shrimp were gathered in the spring of 2004, during the experimental bottom trawl survey carried out in the framework of the international project Medits (BERTRAND *et al.,* 2000). Each specimen was measured (carapace length, CL mm), sexed and its sexual maturity condition was established on board. The selected size range for the collected specimens was 33-38 mm and 32-64 mm CL for males and females, respectively. The shrimps were individually stored in containers filled with 10% buffered saline water formaldehyde solution. Once in the laboratory, they were removed from the fixative solution, washed in running water, weighed (whole body weight, in g), and the cephalothorax was separated from the abdomen.

The brain was removed under a stereomicroscope, leaving the circum-esophagus *commissura* longer than the optical nerves, in order to allow the proper orientation of the piece during the inclusion (SHEEHY, 1989; ENCARNACÃO & CASTRO, 2001). The sample was thereafter embedded in Bio Plast-extra paraffin. The block was sliced perpendicularly to the anterior-posterior axis, obtaining serial sections of 6 μm thickness. In order to highlight the brain topography and better identify the olfactory lobe, serial sections along all the brain were colored by standard methods and observed with a binocular stereomicroscope.

The target structure for lipofuscin identification was the olfactory lobe cell mass (OLCM), a.k.a. 'globuli cells', clusters of small cells with prominent nucleus. Serial sections always including the OLCM, uncolored, were employed for lipofuscin identification and quantification by fluorescence microscopy. A microscope (Olympus) equipped with epi-fluorescent light (100 W) and a DAPI filter with a 409 nm wavelength characteristic was used. Images were captured at different magnifications using a high-resolution digital camera; trials proved that 40x was the most suitable for estimating the lipofuscin concentration.

The images were treated using the Image Pro Plus software (Media Cybernetics, Inc; version 6 for Windows 95), integrated with an *ad hoc*-developed macro (by the software house Immagini & Computer of Milan, Italy). The total number of granules and the total surface covered by the granules per unit area were quantified in each image and considered as indices of lipofuscin quantity. Three indices were thereafter calculated for each individual (by pooling the information of all the used images), in accordance with: (A) the percentage of the surface covered with lipofuscin; (B) the mean individual area of the lipofuscin granules, expressed as μm^2, calculated directly by the macro; and (C) granule density, i.e. the number of lipofuscin granules per 100 μm^2 (respectively

defined as A%, Ag and GD in CASTRO *et al.* [(2002]). Basic statistics on the lipofuscin indices (mean and standard deviation) were computed.

The estimated age is derived for the females from the vBGF curve $CL_f = 65.5 * (1 - e^{(-0.669*age)})$, while for males it is derived from the linear function $CL_m = 28.17 + 3.84 * age$ (RAGONESE *et al.,* 1994).

Results

The basic features of the brain of *A. foliacea* have been reconstructed on the basis of a complete series of consecutive slices and are schematically shown in Figure 1. The present examinations show that the red shrimp brain conforms to the typical decapod brain as indicated also by the presence of the 'olfactory lobe cell masses' (OLCMs) in the deuterocerebrum (SANDEMAN *et al.,* 1993).

The OLCM, the structure investigated for the lipofuscin quantification, looks like a lateral back-ventral expansion of the olfactory lobe. At small magnification, the OLCM appears as a bunch of small cells with its apex inside the olfactory lobe (which is partially covered by the external component of the bunch). On the section, the wider part of the bunch shows a typical fan shape. Cells look rotund (with a diameter around 6 μm)

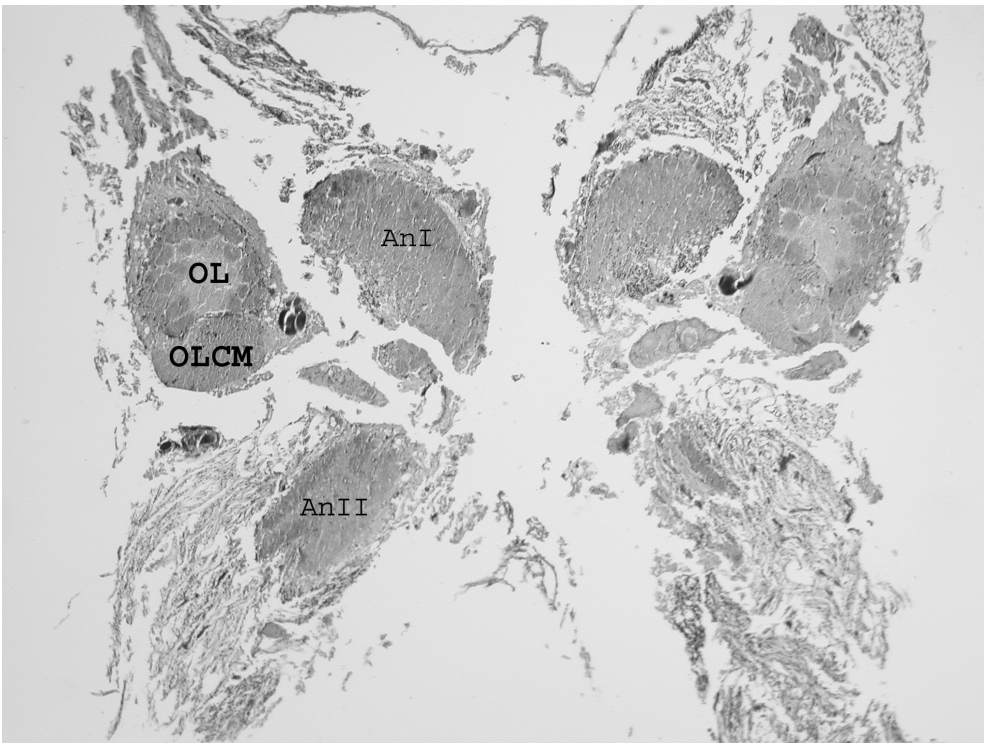


Fig. 1: Section of the brain of *Aristaeomorpha foliacea.* The arrow indicates the areas used for lipofuscin quantification (olfactory lobe cell mass, OLCM).

and almost filled by the nucleus, with the cytoplasm confined to the peripheral portion of the cell.

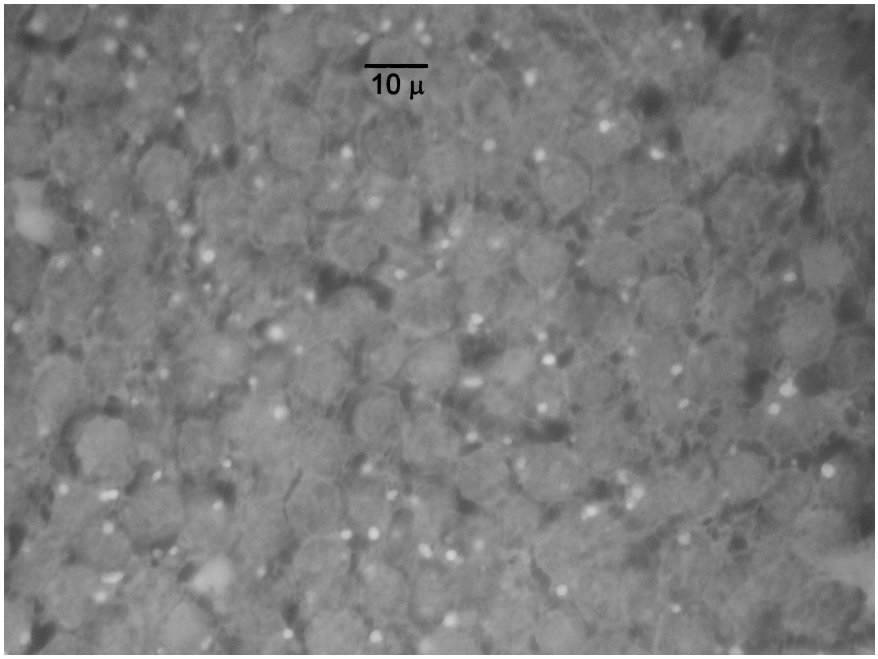
Lipofuscin was observed in every preparation of the OLCM, appearing as roundish granules of variable dimension (normally with a diameter of less than 1 μm, but sizes up to almost 3 μm were also measured) uniformly distributed in the peripheral cytoplasm (Fig. 2). These granules contain a variable amount of membrane residues embedded in a more homogeneous material. Lipofuscin was also detected in other portions of the brain and in the nerve cords (antennal neuropilum and esophageal connectives), but in these structures the lipofuscin granules were not so clearly observed. These observations are in accordance with results already published for other shrimp species (SHEEHY, 1990b; SHEEHY *et al.,* 1995; SOBRINO *et al.,* 2001; CASTRO *et al.,* 2002; VILA GORDILLO, 2005).

The basic indices of the individual lipofuscin contents are presented in Table 1. From Table 1 and Figure 3, taking into account the paucity of the data, it seems evident that lipofuscin accumulation is positively related to body size, i.e. larger animals show higher amounts of lipofuscin in the OLCM. This is represented by a greater area covered by the granules and apparently also by their size, but this phenomenon is not as clear when considering the number of the granules, which varies without an apparent pattern.

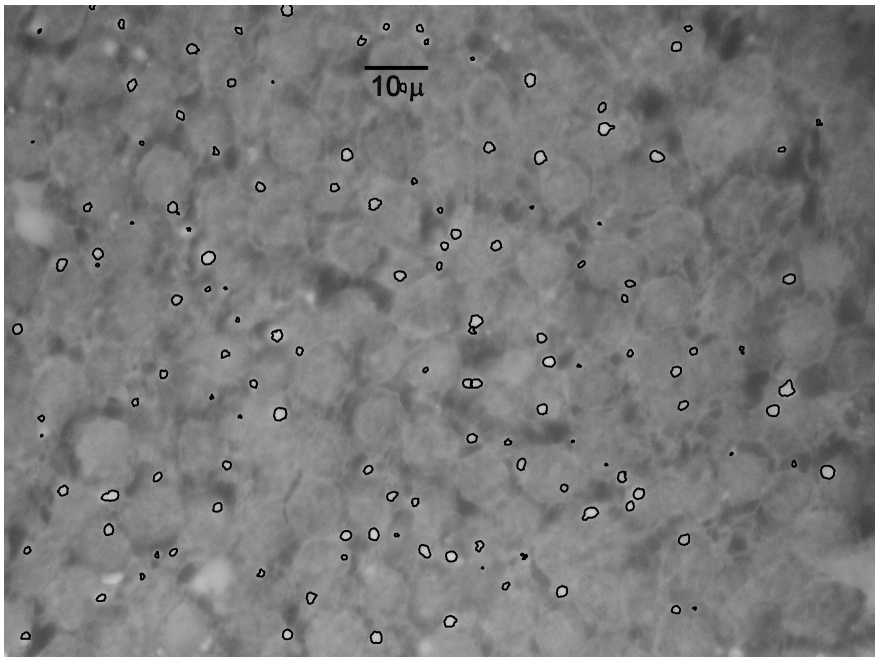
The gender of the shrimps seems to play a role in the lipofuscin accumulation pattern (Fig. 3). Males presented levels of lipofuscin (percentage area covered) similar to that of larger, and older, females, with males of a putative age of 2-3 years reaching the same values of lipofuscin

Table 1
Area covered (%) with lipofuscin granules, mean individual size (in μm^2) of lipofuscin granules and number of lipofuscin granules per mm^2 in the olfactory lobe cell mass (OLCM) of males and females *Aristaeomorpha foliacea*.

sex	CL (mm)	age class (approx)	coverage %	granule size μm^2	numerical density grains/mm^2
M	33	1	0.55	0.46	11970
M	34	1	0.67	0.31	21990
M	35	2	0.87	0.62	14110
M	36	2	0.74	0.51	14530
M	38	3	1.28	0.95	13460
F	32	1	0.28	0.29	9700
F	45	2	0.35	0.36	9750
F	45	2	0.28	0.36	7760
F	46	2	0.21	0.35	5860
F	53	3	0.15	0.39	3980
F	63	5	0.92	0.73	12600
F	64	6	1.28	1.41	9060

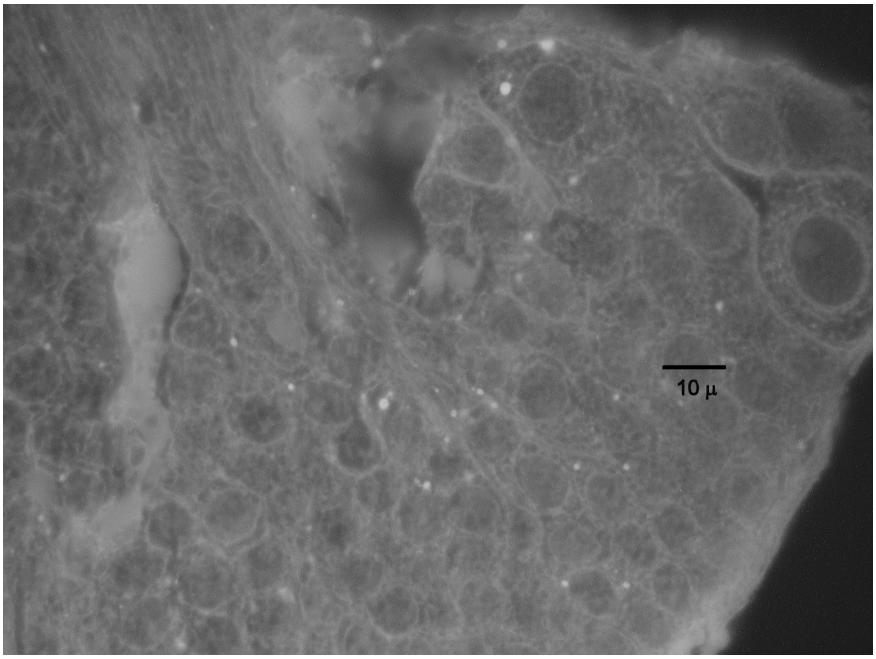


2a.

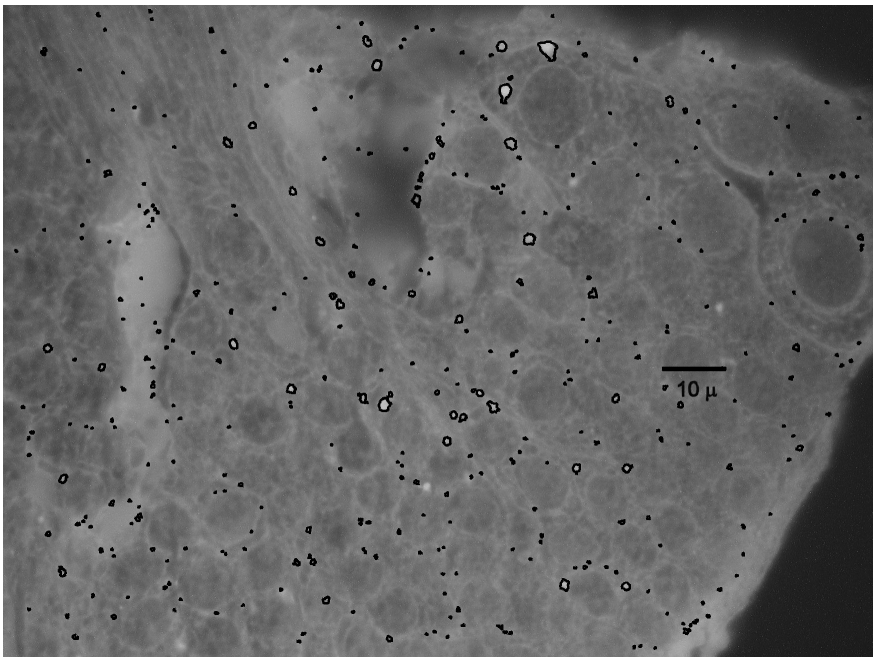


2b.

Fig. 2: Brain sections of fully mature *Aristaeomorpha foliacea* (up: male, 34 mm CL; down: female, 64 mm CL): fluorescence image (100x) from the olfactory lobe cell mass (OLCM), with the lipofuscin granules evidenced according to conventional methods (left) and after the macro ad hoc developed (right).



2c.



2d.

Fig. 2: Brain sections of fully mature *Aristaeomorpha foliacea* (up: male, 34 mm CL; down: female, 64 mm CL): fluorescence image (100x) from the olfactory lobe cell mass (OLCM), with the lipofuscin granules evidenced according to conventional methods (left) and after the macro ad hoc developed (right).

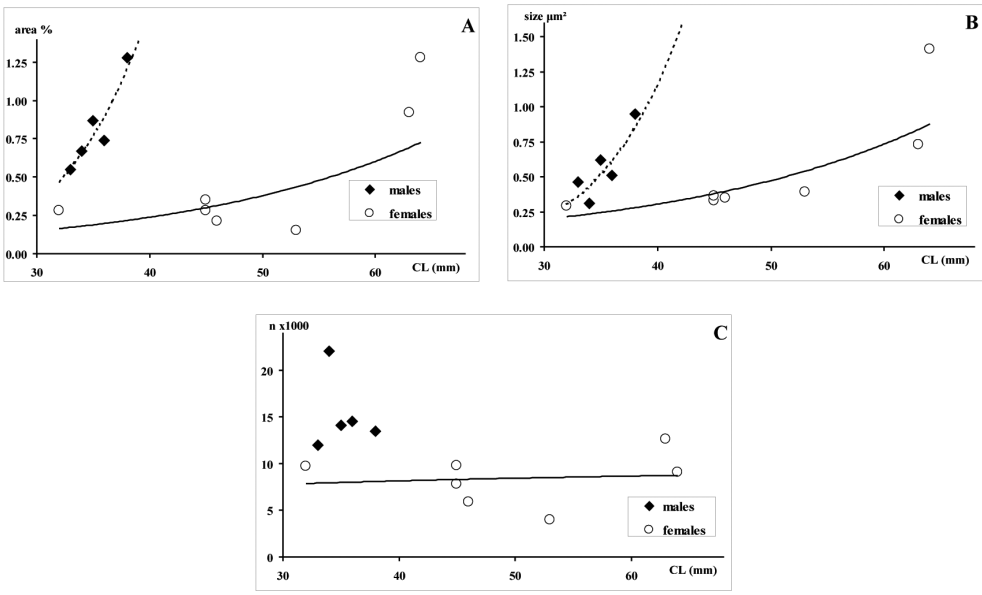


Fig. 3: Area (A, %), individual size (B; μm^2), numerical density (C; $N/\mu m^2$) of lipofuscin granules in sections of the olfactory lobe cell mass (OLCM) vs. shrimp size (CL; mm) in *Aristaeomorpha foliacea* (filled diamonds = males; empty circles = females).

coverage as females belonging to the 5-6 year age classes. Moreover, males have a higher numerical density of the granules, well above that of females of all sizes.

Discussion

Lipofuscin is a degenerative auto-fluorescent pigment, broadly diffused in the animal kingdom, whose amount shows a positive correlation with advancing age and maximum life spans (BRUNK & TERMAN, 2002). After the introduction of histological and image analysis methods, the suitability of lipofuscin accumulation for ageing has been widely recognized, especially in crustaceans, which loose all durable hard parts during the moult (HARTNOLL, 2001).

Despite the heterogeneous composition and genesis of lipofuscin, its relationship with aging has been demonstrated in many groups of crustaceans such as krill (NICOL *et al.,* 1991), amphipods (BLUHM *et al.,* 2001), clawed (O'DONOVAN & TULLY, 1996; SHEEHY *et al.,* 1996; WAHLE *et al.,* 1996; SHEEHY *et al.,* 1999; CASTRO *et al.,* 2002;) and spiny lobsters (SHEEHY *et al.,* 1998), crayfish (SHEEHY, 1990a; SHEEHY *et al.,* 1994; BELCHIER *et al.,* 1998) and shrimps, both caught in the wild or raised in aquaculture (SHEEHY *et al.,* 1995; VILA *et al.,* 2000; MEDINA *et al.,* 2000; SOBRINO *et al.,* 2001; PEIXOTO *et al.,* 2002; VILA GORDILLO, 2005). These studies, based also on animals kept in controlled conditions, allowed the improvement of extraction and methods of analysis, as well as the confirmation that, regardless the sex, lipo-

fuscin accumulation is almost linear with time (BLUHM *et al.,* 2001; VILA GORDILLO, 2005), resulting in a more precise and accurate descriptor of age than carapace length (SHEEHY *et al.,* 1994; VILA *et al.,* 2000; SOBRINO *et al.,* 2001). Nevertheless, when wild-caught animals are investigated many problems remain to be solved (PEIXOTO *et al.,* 2002).

The pattern of lipofuscin accumulation with age may vary with the investigated brain structure. Lipofuscin was detected earlier in some species when using nerve cords in place of the OLCM (CASTRO *et al.,* 2002), whereas in other cases higher concentrations were found in the connection fiber tracts than in the OLCM (ENCARNACÃO & CASTRO, 2001). Moreover, various conditions could affect the lipofuscin accumulation, as it has been observed that oxidative stresses can promote accumulation (BRUNK & TERMAN, 2002), while antioxidants in the feed can reduce it (CASTRO *et al.,* 2002). Also spatial heterogeneity and water temperature may influence the lipofuscin accumulation rate; more realistic thermo-age models are thus required, in particular for long-living crustaceans gathered from their natural environment (SHEEHY *et al.,* 1994).

According to CASTRO *et al.* (2002), lipofuscin measurements in crustaceans should be treated as a method to highlight the relationship between size and physiological age, rather than chronological age. That seems the proper case for the red shrimps, in which the LFD interpretation becomes critical only in the last component among the 4-5 (females) or 2-3 (males) 'age' modes normally detected in the samples. Lipofuscin may be the covariable for testing the hypothesis of a piling-up effect, allowing the analysis to concentrate on a narrow range of size classes of apparently 'similar-age' specimens. The detection of different physiological ages (e.g., by analyzing lipofuscin concentration-frequency; SHEEHY *et al.,* 1994; BLUHM *et al.,* 2001) could improve the estimates of growth and longevity in the red shrimps, as already demonstrated for other synchronously-reproducing, slow-growing and long-living crustacean species (BLUHM *et al.,* 2001).

The lipofuscin indexes of the red shrimps can be compared with those previously published for other species and in other anatomical structures only in a broad and general way (VILA *et al.,* 2000; CASTRO *et al.,* 2002; VILA GORDILLO, 2005). The effect of age on lipofuscin accumulation is commonly evident in the covered surface and in the granule density, but not in the granule size, suggesting that the lipofuscin increase over time is mainly due to the formation of new granules (WAHLE *et al.,* 1996) and not to the size increment of the existing ones (i.e., the process is neoplasic and not hyperplasic).

Present results show that *A. foliacea* accumulates lipofuscin in an appreciable and quantifiable way, in particular in the OLCM. No other study on the lipofuscin of this species is known; nevertheless, it may be supposed that in the Mediterranean populations, living in a stable environment (CAU *et al.,* 2002) and feeding on an ample range of preys, food and temperature are not likely to play a substantial role in fogging the lipofuscin pattern, at least in adults of the same sex.

Contrary to results obtained from other shrimps (SOBRINO *et al.,* 2001; VILA GORDILLO, 2005), in *A. foliacea* the dimension of the lipofuscin granules seems related to the shrimp size rather than the numerical density of the gran-

ules. Unfortunately, data are insufficient for a significant statement on this subject.

Regarding the gender effect, the present preliminary results suggest, from a physiological point of view, that the age assigned to the males using length-based models is underestimated or that senescence is more rapid in males, due to their shorter life span.

Conclusions

Lipofuscin accumulation, given its broadly demonstrated association with senescence, seems a promising alternative method for ageing animals which lack durable hard parts, such as the decapod crustaceans, and the shrimps in particular.

The present preliminary results appear to show a quantitative relationship between the shrimp size and the OLCM area covered by lipofuscin, encouraging further studies to develop and validate the methodology for improving the relative ageing of large *A. foliacea* shrimps.

The discrimination of older age classes which are now confounded in the piling-up effect should have positive implications on the management of this resource, of primary importance for the fisheries in the Strait of Sicily and the eastern Mediterranean.

An effort to replicate the present experiment using a larger amount of individuals is therefore desirable, in order to quantify the relationship between lipofuscin accumulation and size (as a proxy of age) in the deep-water red shrimp *A. foliacea.*

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