Occurrence of lipofuscin age pigment in *Chasmagnathus granulatus* (Decapoda, Varunidae)

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Abstract

The occurrence of *in situ* neurolipofuscin was investigated in the supra-oesophageal ganglion (brain) of the crab *Chasmagnathus granulatus* found in the estuarine region of the Patos Lagoon (Southern Brazil). Histological procedures were tested to assess the most suitable methodology to observe *in situ* neurolipofuscin. The standard histological protocol used for studies in crustaceans was slightly modified. Orientation of the brain permitting longitudinal sectioning was the most appropriate to visualize neurolipofuscin in the olfactory lobe cell mass. The results of this investigation provide the first evidence that neurolipofuscin occurs at quantifiable amounts in this grapsoid, and they raise the prospect of application of the neurolipofuscin approach for age determination of *C. granulatus*.

Key words: Neurolipofuscin, age pigment, age determination, *Chasmagnathus granulatus*, Crustacea

Introduction

Chasmagnathus granulatus Dana, 1851 (Decapoda, Varunidae) is an estuarine crab found in salt marshes and mangroves on the Southern Atlantic coast, from Brazil (Rio de Janeiro) to Argentina (Patagonia) (Boschi, 1964). Populations of *C. granulatus* inhabiting estuaries (40° 46'S, 64° 50'W; Rio Negro Province, Argentina) near the southernmost limit of occurrence of the species experience temperatures ranging from 7.8°C (winter average) to 22.6°C (summer average) (Bas *et al.*, 2005), while populations at the northermost limit of occurrence (23° 12'S, 44° 43'W; Paraty, Brazil) experience an average annual temperature around 23°C (Negreiros-Fransoso, per. com.). The largest individuals of *C. granulatus* are commonly males, and the maximum sizes (carapace width, CW) reported for this crab vary from 25.6 mm (D'Incao *et al.*, 1993; Lagoa dos Patos, Brazil) to 41.1 mm (Barutot, per. com.; Lagoa do Peixe, Brazil). This species has a wide salinity tolerance due to an efficient mechanism of hyper and hypo-osmoregulation of the extracellular fluid (Santos and Bianchini, 1997). Although *C. granulatus* is not commercially relevant, this species is well studied, e.g., carbohydrate and lipid metabolism, and responses to hypoxic conditions, becoming a good model for the study of crustacean physiology (e.g., Santos and Nery, 1987; Santos *et al.*, 1987).

In the estuarine region of the Patos Lagoon (Southern Brazil), previous studies on individual growth of *C. granulatus* suggested that males grow faster than females, and that the maximum longevity of this species is *ca.* 2 years, (D'Incao *et al.*, 1993). The mean size at the onset of maturation of females (16.5 mm CW) (Ruffino *et al.*, 1994) is thought to be approximately 6 months of age. In both of these previous studies, however, age was estimated indirectly though standard size-based methods.

The quantification of lipofuscin age pigment in nervous tissues (i.e., neurolipofuscin), by microscope-based approach, has been successfully established as a reliable and more precise methodology to resolve age groups in natural populations of crustaceans than size-based approaches (Sheehy, *et al.*, 1998; Bluhm and Brey, 2001; Kodama *et al.*, 2005). As this methodology is based on analysis of images captured from unstained sections, it is necessary to understand the morphology of the structures in which neurolipofuscin will be estimated (images should be consistently captured at the same region), and to set an appropriate histological procedure in order to have samples suitable for quantitative analysis.

To date, there is no information about the accumulation of neurolipofuscin in *C. granulatus*. The present investigation provides the first evidence that the age pigment lipofuscin occurs at quantifiable amounts in the nervous tissue of *C. granulatus,* and that the accumulation of lipofuscin is age dependent, raising prospects for application of the neurolipofuscin approach for age determination of populations of this grapsoid in the wild.

Material and methods

Chasmagnathus granulatus of unknown age collected in the estuarine region of Patos Lagoon were taken to the laboratory. In addition, ovigerous female broodstock were obtained from the estuarine region and held in laboratory tanks until hatching of larvae in order to obtain known age individuals for ongrowing. Larvae and early juveniles were mass-reared in 5 l containers, on a 12L: 12D room lighting cycle at controlled water temperatures of 20°C ± 2°C, and salinity 30. When hatchlings moulted to the crab 5 stage they were transferred to a 50 l tank, with the same controlled photoperiod and temperature, and salinity 20. Fifty of these hatchlings have been maintained in the laboratory since April/2004. They have been fed *ad libitum* with shrimp, crab meat, and dry food pellets. These known age crabs were sampled twice (at 8 months and 12 months after hatching) for neurolipofuscin analysis. In the first sample, four males and four females were taken, while in the second sample two males and two females were taken.

In the laboratory, crabs (from the field and from the laboratory rearing) were cryoanaesthetized in iced water for 10 minutes prior to brain (supra-oesophageal ganglion) removal. A piece of the carapace was then removed by cutting the frontal margin (from an orbit to another) and, then cutting toward the anterior margin of the cardiac region. The removal of this piece exposed the stomach, which was also removed. Afterwards, a piece of the anterior-ventral region of the crab containing the mouthparts, the esophageal canal, and the brain was removed (pictures of this procedure are available at http://homepage.mac.com/dbf1/), and fixed in 10% formaldehyde (1 part of formalin: 3 parts of water). After 48 h in the fixative, the brain was dissected out, dehydrated and embedding according to the following protocol: ethanol series (70%; 85%; 95%; 100%; 1h each); 100% ethanol: 100% xylene (1:1) (1 h); 1st xylene (30 min); 2nd xylene (30 min); 1st infiltration in 58°C melting point wax at 60°C (30 min); 2nd infiltration (30 min). Sectioning was performed using a rotary microtome set at 6-µm section thickness. The brain was orientated to permit a longitudinal and horizontal (transverse) sectioning. The supra-oesophageal ganglion has three main regions (protocerebrum, forebrain; deuterocerebrum, midbrain; and tritocerebrum, hindbrain). The deuterocerebrum, has a pair of olfactory lobes (OL) which lie on each side of the brain. Each lobe has a large cell mass associated with it (olfactory lobe cell mass, OLCM) called cluster 10, which is connected to the OL by axon tracts (AT). During sectioning, only the sections containing the OL, AT, and OLCM (region which has been standardized as the location for lipofuscin quantification) were utilized. Serial sections ("ribbons") were collected and floated in a water bath (40°C) to allow stretching. After floating onto a standard microscope slide (normally 3 ribbons of 11 sections per slide) sections were allowed to dry overnight. Mounting was performed after three dewaxing baths in xylene 100% (two minutes each). Coverslips were mounted with Entellan mountant.

No staining was utilized. Unstained sections were observed, at 40 X and 100 X objectives, using an Olympus BX-50 microscope with epifluorescence attachment (BX-FLA) set either at green (514 nm) or at blue (450 nm) excitation filters. Images of the OLCM were acquired using a CCD camera.

Results

The anatomy of the brain of *C. granulatus* is similar to that described for other grapsoids (Sandeman *et al.*, 1992). Cluster 10 (OLCM) is well developed and it is located posterior, and slightly dorsal, to the OL (fig. 1). Cluster 10 is composed of small globuli cells, which have a large nucleus filling most of the cell body. In longitudinal sections, the paired OLCM was observed in up to 25 sections (depending on the brain size). The axon tract connection to the OL is identified with ease in longitudinal sections (fig. 1), and it was observed that the number of sections containing the axon tract connection to the OL varied from 7 to 15.

Neurolipofuscin was identified in histological sections by its diagnostic yellow autofluorescence, irregular granular appearance, intracellular accumulation, and age-related accumulation (see below). Neurolipofuscin deposits were observed in many cell masses, such as anterior cluster, cluster 3, and cluster 10 (OLCM) (fig. 1). Most of lipofuscin granules observed had diameter varying from 1 to 5 μm. The intensity of the fluorescence of the lipofuscin granules allowed a good discrimination from the background of cells. Neurolipofuscin granules were brighter, and the contrast sharper, when slides were observed under blue excitation.

Quantification of neurolipofuscin in known age crabs demonstrated an age dependent accumulation. The mean value ± standard error of neurolipofuscin loading in 8 months old crabs was 0.12% ± 0.01, while for 12 months old crabs the figure was 0.27% ± 0.08. These values were statistically different ($t = 3.07 > t_{0.05(1)} = 1.76$; $P = 0.004$; $DF = 14$). Young crabs, reared in the laboratory, had much less lipofuscin granules in the OLCM than the largest crabs (presumably the oldest) caught in the field (fig. 2a, b).

Discussion

Determination of age in crustaceans using neurolipofuscin, quantified by microscope-based methods, was first proposed 15 years ago (Sheehy, 1990). Since them, increasing numbers of reports of a significant relationship between neurolipofuscin volume and chronological age have been published (e.g., Belchier *et al.*, 1998; Sheehy *et al.* 1996; Vila *et al.* 2000). Use of neurolipofuscin histograms to resolve age groups demonstrated that neurolipofuscin frequency distributions provide a better resolution of age groups, particularly older ones, than size frequency distributions (Sheehy, *et al.*, 1998; Bluhm and Brey, 2001; Kodama *et al.*, 2005).

The present investigation reports the first observation of *in situ* neurolipofuscin for *C. granulatus*. Although the histological procedure utilized for dehydration and embedding did not differ very much from the standard methodology proposed earlier (cf. Sheehy and Wickins, 1994), some issues are worthy noting. Firstly, tissues were fixed in 10% formaldehyde, instead of 10% seawater formaldehyde. Preliminary observations showed that the best histological condition was observed when tissue was fixed in the former fixative. Secondly, the wash in phosphate buffered saline was skipped, without any loss of histological quality. Thirdly, tissues were infiltrated using two baths of wax of 30 minutes each; vacuum was not utilized during embedding. In addition, some samples were dehydrated using reduced times for alcohol 70% and 85% (30 minutes), with no apparent loss of histological quality. The histological procedure adopted for *C. granulatus* also works for *Armases rubripes* (Fonseca, pers. obs.), which is another grapsoid found in the estuarine region of the Lagoa dos Patos.

Brain orientation for longitudinal sectioning provided excellent results. In these sections the OLCM appears larger than in transversal sections and the AT connection to the OL is easily identified. Even in the smallest brains, at least 7 sections containing the AT were observed. The procedure adopted involving removal of a small piece of the anterior region of the animal made dissections of brains easy. Trials have shown that this approach is also feasible for larger species such as the blue crab *Callinectes sapidus*. Eyestalks have been used in most of studies, but for penaeids, regarding age determination of crustaceans by the neurolipofuscin method. Eyestalks are often preferred because they are easy to get at the moment of sampling. However, because eyestalks of *Chasmagnathus granulatus* are very small, they are much more difficult to dissect out than a brain. For this reason, brains were used in the present investigation for the observation of *in situ* neurolipofuscin in *C. granulatus*.

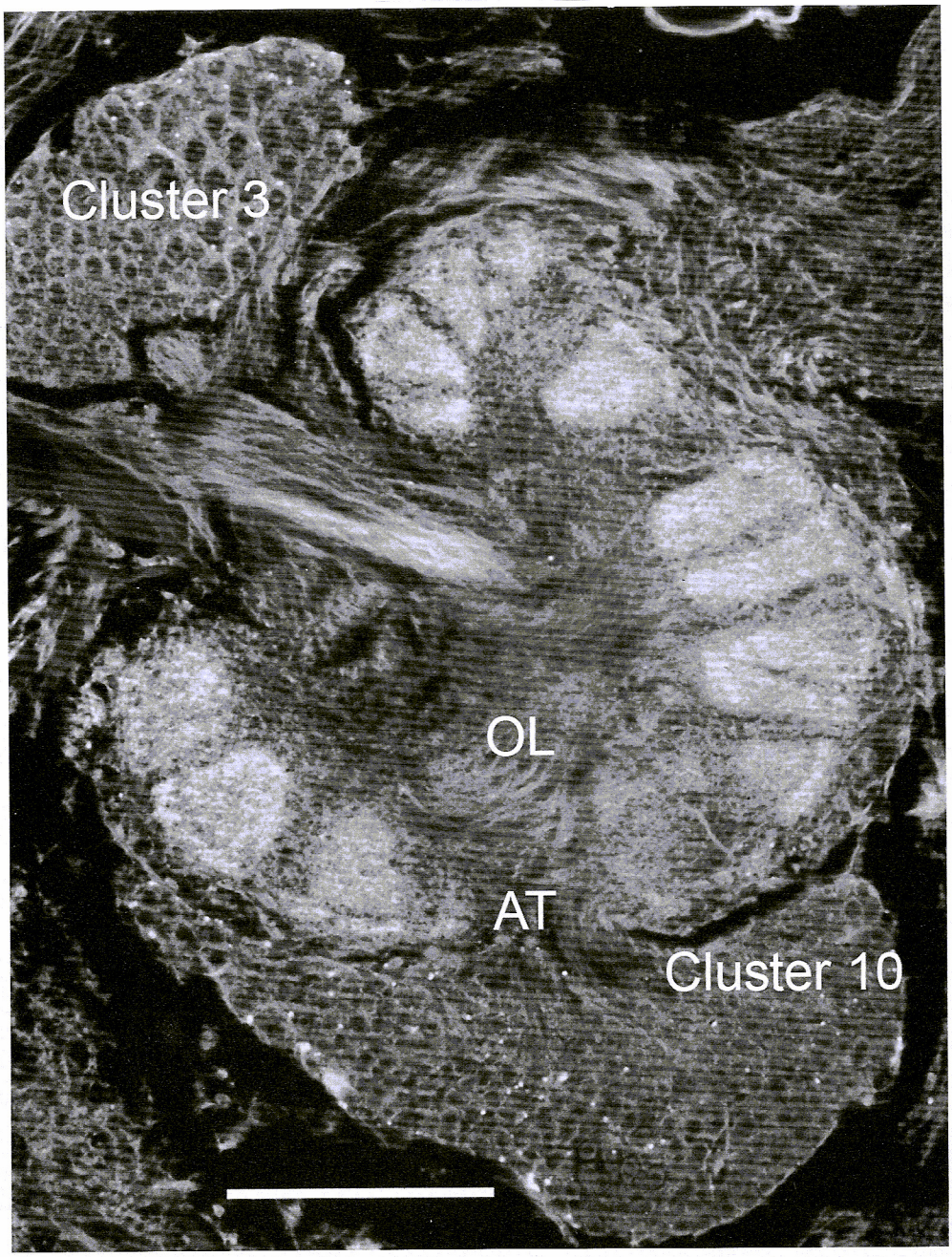

Figure 1: Autofluorescent histological section (6µm-thick) of a region of the brain showing the olfactory lobe (OL), the axon tracts (AT), and the cell mass cluster 3, and the olfactory lobe cell mass (OLCM, cluster 10). Cluster 3 and cluster 10 contain globuli cells associated with lipofuscin granules (the brightest points). Scale bar: 50 µm.

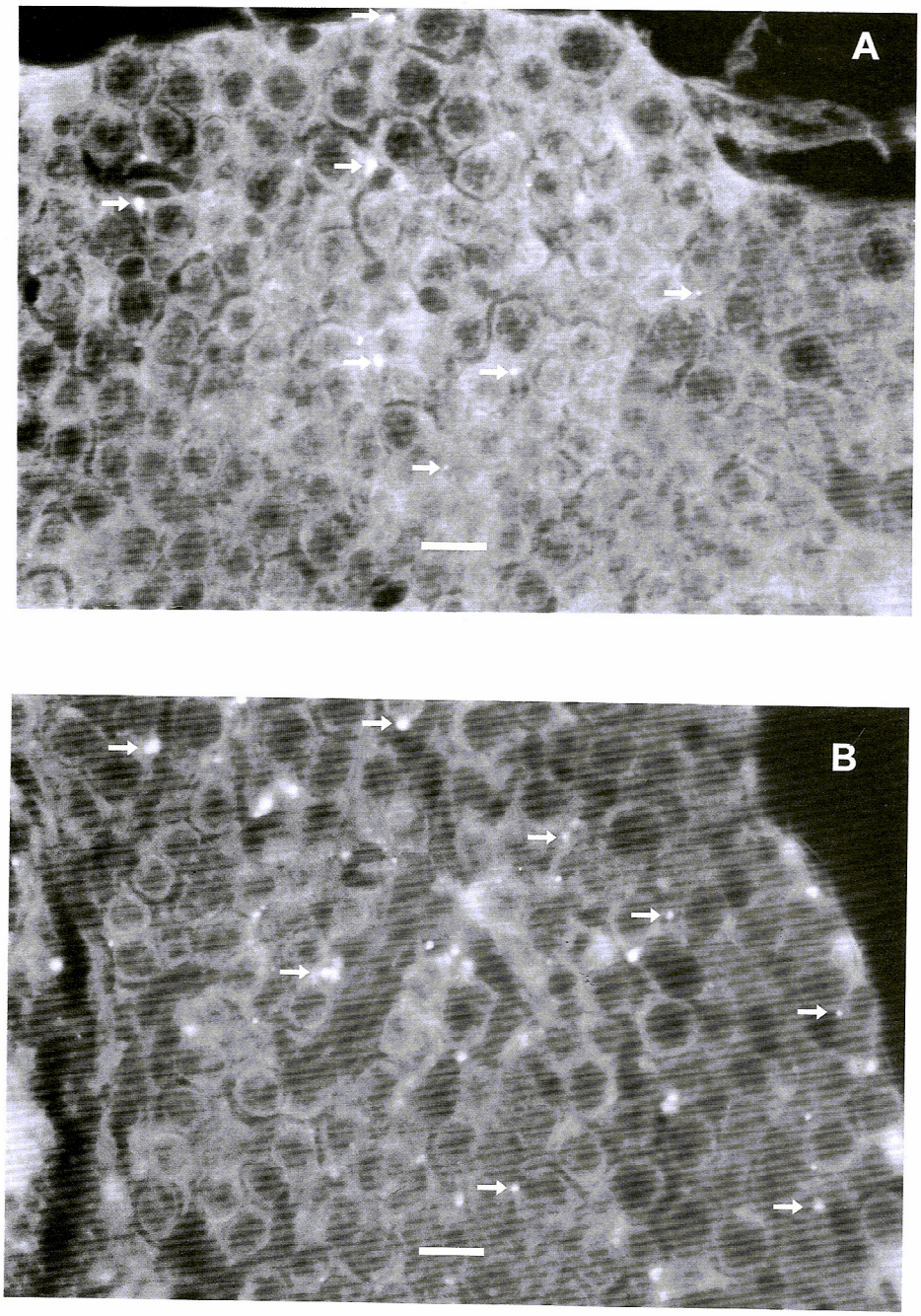

Figure 2: Autofluorescent histological section (6µm-thick) of the OLCM of a (a) 8 months old crab, reared in laboratory (male, 11.2 mm CW), and of an (b) unknown age male caught in the field (26 mm CW), presumably old. Several lipofuscin granules are indicated (arrows). Scale bar: 10 µm.

It is believed that lipofuscin has indeed been found in *C. granulatus*. Firstly, granules observed showed yellow autofluorescent emission when excited by blue light, which is a defining characteristic of lipofuscin (Katz and Robinson, 2002). Secondly, the deposits of lipofuscin seem to be mostly intracellular, which is consistent with the observations of neurolipofuscin in crustaceans. Thirdly, data from laboratory rearing reported in this investigation are indicative of age dependent neurolipofuscin accumulation in *C. granulatus*. Although this information is still preliminary, provided this rearing is planned to last for at least 30 months, it is nevertheless important because it shows a significant difference in neurolipofuscin loading between groups

of crabs of different ages. At the moment, it is preferred do not draw any conclusion about gender differences in neurolipofuscin accumulation, provided there is an insufficient sample size.

The results reported in this investigation suggest that the quantification of *in situ* neurolipofuscin is possible, and its use may be a reliable tool for age determination of *C. granulatus*. Consequently, the next steep is to obtain a calibration of the amount of neurolipofuscin in the OLCM of *C. granulatus* against chronological age. To perform it, a group of known age *C. granulatus* has been reared in our laboratory. Moreover, a neurolipofuscin frequency analysis is under way on a sample of 500 animals collected in the field. If neurolipofuscin approach proves to be reliable for age determination of *C. granulatus* in populations in the wild, interesting issues regarding the population dynamics of *C. granulatus* could be addressed. For example, populations of *C. granulatus* are found in the whole estuarine region of the Lagoa dos Patos, which are subject to different salinity regimes. No information is available on the effect of habitat salinity on neurolipofuscin accumulation in the Crustacea. Secondly, marked differences in maximum sizes of individuals (particularly of males) are observed in populations inhabiting two estuarine regions of the Southern Brazilian Coast (Patos Lagoon and Peixe Lagoon), which are just 140 Km apart. This raises the question as to whether this difference is related either with distinct growth rates or with distinct life spans (or both). Age determination by neurolipofuscin approach would be useful to clarify this issue.

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References

Bas, C.; Luppi, T. and Spivak, E. 2005. Population structure of the South American estuarine crab, *Chasmagnathus granulatus* (Brachyura: Varunidae) near southern limit of its geographical distribution: comparison with northern populations. Hydrobiologia, 537: 217-228.

Belchier, M.; Edsman, L.; Sheehy, M. R. J. and Shelton, P. M. J. 1998. Estimating age and growth in long-lived temperate freshwater crayfish using lipofuscin. Freshwater Biology, 39: 439-446.

Bluhm, B. and Brey, T. 2001. Age determination in the Antarctic shrimp *Notocrangon antarcticus* (Crustacea: Decapoda), using the autofluorescent pigment lipofuscin. Marine Biology, 138: 247-257.

Boschi, E. E. 1964. Los crustáceos decápodos Brachyura del litoral bonarense (Republica Argentina). Boletin del Instituto de Biologia Marina, 6: 1-76.

D'Incao, F.; Ruffino, M. L.; Silva, K. G.; Braga, A. C. and Marques, L. H. C. 1993. Crescimento de *Chasmagnathus granulata* Dana, 1851, em um marisma do estuário da Lagoa dos Patos, RS (Decapoda: Grapsidae). Revista Brasileira de Biologia, 53: 637-643.

Katz, M. L. and Robison, W. G. 2002. What is lipofuscin? Defining characteristics and differentiation from other autofluorescent lysosomal storage bodies. Archives of Gerontology and Geriatrics, 34: 169-184.

Kodama, K.; Yamakawa, T.; Shimizu, T. and Aoki, I. 2005. Age estimation of the wild population of Japanese mantis shrimp *Oratosquilla oratoria* (Crustacea: Stomatopoda) in Tokyo Bay, Japan, using lipofuscin as an age marker. Fisheries Science, 71: 141-150.

Ruffino, M. L.; Telles, M. D. and D'incao, F. 1994. Reproductive aspects of *Chasmagnathus granulata* Dana, 1851 (Decapoda, Grapsidae) in the Patos Lagoon estuary - Brazil. Nauplius, 2: 43-52.

Sandeman, D.; Sandeman, R.; Derby, C. and Schmidt, M. 1992. Morphology of the brain of crayfish, crabs, and spiny lobsters: a common nomenclature for homologous structures. Biological Bulletin, 183: 304-326.

Santos, E. A. and Bianchini, A. 1997. Physiological adaptations in invertebrate and fish. P 47-50 In Seeliger, U.; Odebrecht, C. and Castello, J. P. Subtropical convergence environments: the coast and sea in the southwestern Atlantic. Springer-Verlag, Berlin.

Santos, E. A. and Nery, L. E. M. 1987. Blood glucose regulation in an estuarine crab, *Chasmagnathus granulata* (Dana, 1851) exposed to different salinities. Comparative Biochemistry and Physiology A - Physiology, 87: 1033-1035.

Santos, E. A.; Baldisseroto, B.; Bianchini, A.; Colares, E. P.; Nery, L. E. M. and Manzoni, C. G. 1987. Respiratory mechanisms and metabolic adaptations of an intertidal crab, *Chasmagnathus granulata* (Dana, 1851). Comparative Biochemistry and Physiology A - Physiology, 88: 21-25.

Sheehy, M. R. J. 1990. Potential of morphological lipofuscin age-pigment as an index of crustacean age. Marine Biology, 107: 439-442.

Sheehy, M. R. J.; Shelton, P. M. J.; Wickins, J. F.; Belchier, M. and Gaten, E. 1996. Ageing the European lobster *Homarus gammarus* by the lipofuscin in its eyestalk ganglia. Marine Ecology Progress Series, 143: 99-111.

Sheehy, M. R. J.; Caputi, N.; Chubb, C. and Belchier, M. 1998. Use of lipofuscin for resolving cohorts of western rock lobster (*Panulirus cygnus*). Canadian Journal of Fisheries and Aquatic Sciences, 55: 925-936.

Sheehy, M. R. J. and Wickins, J. F. 1994. Lipofuscin age pigment in the brain of the European lobster *Homarus gammarus* (L.). Microscopy and Analysis (March), 23-25.

Vila, Y.; Medina, A.; Megina, C.; Ramos, F. and Sobrino, I. 2000. Quantification of the age-pigment lipofuscin in brains of known-age, pond-reared prawns *Penaeus japonicus* (Crustacea, Decapoda). Journal of Experimental Zoology, 286: 120-130.

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