



Occurrence and quantification of the autofluorescent pigment neurolipofuscin in the brains of red shrimp *Pleoticus muelleri* (Bate, 1888) (Decapoda: Solenoceridae)

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Abstract. Due to the lack of structures that allow direct age determination in crustaceans, length-based methods are widely used to estimate age. However, such methods assume that there is a direct correlation between size and age, which is often not true. The amount of neurolipofuscin pigment in crustaceans' brains is related to physiological age and may represent an alternative age marker. The goal of this study was to verify the accumulation of neurolipofuscin in the nervous tissues of *Pleoticus muelleri* and to evaluate its potential use as an age marker. Six specimens were collected in the State of Santa Catarina, Southern Brazil. In the laboratory, the brains were dissected and prepared for observation under a microscope with epifluorescence. Samples of different size classes were analyzed. No significant difference was observed in the granule diameter of neurolipofuscin among the class sizes ($p > 0.05$; $R^2 = 0.07$). However, a significant difference was observed in concentrations of neurolipofuscin according to size ($p < 0.05$; $R^2 = 0.84$), in such a way that the largest group accumulated a higher concentration of the pigment. Thus, based on the results obtained in the present study, we can conclude that neurolipofuscin is a potential index of age in *P. muelleri*.

Keywords: Crustacea, age marker, lipofuscin granules

Resumo. Ocorrência e Quantificação do Pigmento Auto fluorescente Neurolipofuscina em Cérebros do Camarão Vermelho *Pleoticus muelleri* (Decapoda: Solenoceridae). Devido à falta de estruturas que permitam a determinação direta da idade em crustáceos, métodos baseados em comprimento são amplamente utilizados. Entretanto, tais métodos assumem que há uma relação direta entre tamanho e idade, o que nem sempre é verdadeiro. A quantidade do pigmento neurolipofuscina, nos cérebros de crustáceos, está relacionada com a idade fisiológica, representando um marcador alternativo da idade. O objetivo deste estudo foi verificar o acúmulo de neurolipofuscina no tecido nervoso de *Pleoticus muelleri*, e avaliar o seu uso potencial como marcador de idade. Seis indivíduos foram coletados no estado de Santa Catarina, sul do Brasil. No laboratório, os cérebros foram dissecados, e preparados para observação sob microscopia de epifluorescência. Amostras de diferentes classes de tamanho foram analisadas. Não foi observada uma diferença significativa no diâmetro dos grânulos de neurolipofuscina entre as classes de tamanho ($p > 0.05$; $R^2 = 0.08$). Entretanto, uma diferença significativa foi observada nas concentrações de neurolipofuscina de acordo com os tamanhos ($p < 0.05$; $R^2 = 0.84$), de tal forma que a maior classe de tamanho acumulou a maior concentração do neuropigmento. Assim, baseado nos resultados obtidos no presente estudo, nós concluímos que a neurolipofuscina é um potencial marcador de idade para a espécie *Pleoticus muelleri*.

Palavras-chave: Crustáceo, marcador de idade, grânulos de lipofuscina

Introduction

The red shrimp *Pleoticus muelleri* (Bate, 1888) (Decapoda: Solenoceridae) is endemic to the South American coast, appearing from Rio de Janeiro (20° S), Brazil, to Santa Cruz (50° S), Patagonia, Argentina. In Argentina, the red shrimp fishery concentrates its operations in the Patagonia region, and occupies an important place in the fishing sector of the country (Boschi 1997). Along the Brazilian coast, recently, the demand for red shrimp has increased (D'Incao *et al.* 2002; Castilho *et al.* 2008), due to a considerable decline in the stocks of more traditional and profitable marine shrimps, such as the pink shrimp *Farfantepenaeus brasiliensis* (Latreille 1817) and *F. paulensis* (Perez Farfante 1967). The marked fluctuations in landings of red shrimp raise several questions about its population ecology. Among the most important population parameters, age determination deserves attention, since it provides the background for the models that assess the exploitation state of a certain stock (Muller *et al.* 1997). The precise determination of the age structure in crustacean populations, especially those of commercial importance, such as red shrimp, is essential for an appropriate stock assessment (Vila *et al.* 2000, Fonseca & Sheehy 2007).

Crustaceans present unique challenges for age determination, due to their lack of aging structures (otoliths, scales and fin spines) caused by the periodic loss of their hard parts during molting (Hartnoll 2001, Smith & Addison 2003). All the age approximations of *P.muelleri* are based on modal analysis of size-frequency histograms (Castilho *et al.* 2012). Size, however, is generally considered to be a poor method for aging crustaceans living in the field, since growth is dependent on environmental and density-dependent factors besides age (Sheehy 1992, James *et al.* 2001).

Thus, to solve this problem, alternative methods of determining age for crustaceans have been tested. Recently, a new method was developed by Kilada *et al.* (2012) to directly determine the age of individual crustaceans. This technique relies entirely on counting annual growth bands deposited on the eyestalks of shrimps and crabs and the gastric mill of lobsters, and it has recently been tested in other groups of crustaceans. Although it is a promising technique, it still needs more studies to validate the method, since one of the restrictions of

the method is that the growth of bands has an influence from the environment and the season. Thus, the seasonally driven growth variations in subtropical taxa, for example, can be relatively less marked, resulting in reduced readability (Fowler 1990) or bi-annual periodicity (Ledland *et al.* 2015).

However, another method of age determination in crustaceans introduced by Etterhank (1983), and first tested with the antarctic krill *Euphasia superba* (Dana 1852), has proven to be effective in solving age groups in crustaceans. This method relies on accumulation over time of neurolipofuscin pigments in the brain or eyestalk neural tissue, which provides a measure of physiological age (Sheehy *et al.* 1998, Bluhm & Brey 2001, Kodama *et al.* 2006, Sheehy & Prior 2008). Lipofuscin occurs as fluorescent granular pigments in postmitotic tissue (neural tissue). It is a product of free radical-mediated lipid peroxidation and the accumulation of nondegradable oxidized macromolecules in lysosomes (Brunk & Terman 2002, Chowdhury *et al.* 2004). Morphological lipofuscin occurs in all cell masses of the brain and eyestalks of decapod crustaceans (Sheehy *et al.* 1996), being particularly conspicuous in the *globuli* cell masses associated with the olfactory lobe. The amount of pigment increases with age and senescence, often showing a positive relationship with chronological age (Brunk & Terman 2002, Chowdhury *et al.* 2004).

Hence, neurolipofuscin quantification may represent an important alternative in determining the age structure of short-lived species of commercial interest. The reason is based on the prediction that the cohorts for this method have a better resolution than the length-frequency method (Sheehy *et al.* 1994). According to Fonseca & Sheehy (2007), in the species *Pacifastacus leniusculus* (Dana 1852) (lobster), the growth rate declines more with age than neurolipofuscin accumulation rate (Sheehy 1992), and, consequently, the overlap of cohorts at asymptotic length in the length-frequency distribution is not a problem in the neurolipofuscin method. Another advantage of the method is that the age-specific variation in neurolipofuscin concentration is less variable than size-at-age (Belchier *et al.* 1998, Sheehy & Bannister 2002), generating normal components with lower standard deviations and thus higher separation indices (Sheehy *et al.* 1994). Thus, since *P. muelleri* is a

short-lived species, one that has a longevity of approximately 2.5 years (Castilho *et al.* 2012), the use of the length-frequency method may lead to a failure in detecting older cohorts, since a vast variety of sizes is associated to a determined age group. It may also create mortality and a growth underestimation rate, resulting in an erroneous assessment and leading to the overexploitation of the resource (Campana 2001).

So, the global importance of shrimp fisheries, and the successful application of the neurolipofuscin technique for studies of population age structure in other crustaceans, especially in short-lived species, prompted us to provide the first evidence of the age pigment lipofuscin accumulation in the nervous tissues of *P. muelleri*, and the content variation of the pigment according to different class sizes, showing the potential of image analysis of morphological neurolipofuscin as a quantification procedure for determining the age of this important commercial species.

Material and Methods

Sampling and Sample Preparation: A total of six red shrimp specimens were sampled in the spring of 2012. Each individual was measured (Cephalotorax

length, CL – mm), and the neurolipofuscin content was verified and quantified in six samples from three different size classes, based on the CL (class I – 10 to 15mm; class II – 15-25mm and class III – >25mm). The samples were collected in shallow coastal waters (7-20 m) off the Balneário Camboriú coast, Santa Catarina, Southern Brazil (Fig. 1).

The brains were carefully dissected, in order to preserve the area of interest, and thus, all nerves originating from the brain were removed, except for the circumesophageal commissure, to facilitate handling and orientation in paraffin wax, which was made perpendicularly. The brains were dehydrated in increasing ethanol concentrations (30 min in 70% ethanol, and one hour each in 80%, 90% and 100% ethanol) and then transferred to a half ethanol, half xylene bath, and to a xylene bath for 30 min, and embedded in paraffin wax on a vertical plane, with the supra-esophageal ganglion facing the cutting edge of the wax block. Serial sections (6µm) of the brains were dewaxed through three xylene changes (2 minutes in each). During sectioning, only the sections containing the olfactory lobe (OL), neurite tracts (NT) and the olfactory lobe cell mass (OLCM) – the region which was standardized as the location for neurolipofuscin quantification – were used.

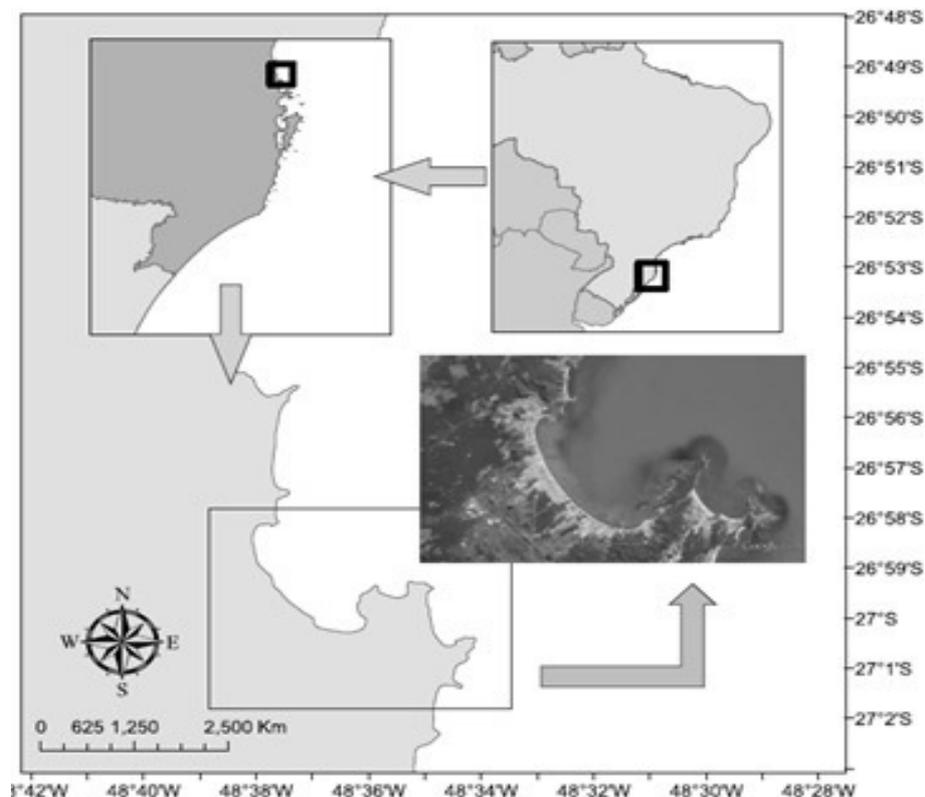


Figure 1. Map of the region of Camboriú Beach, State of Santa Catarina, South Brazil.

Fluorescence Microscopy and Neurolipofuscin Quantification: For determination of relative lipofuscin content in brains, images of the 10 to 15 central-most sections of the OLCM per animal were captured and digitized, and the unstained sections were observed at 40X 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. Brightness, contrast and sharpness of the images were adjusted so that the neurolipofuscin granules were most obvious and distinct. All fluorescing points that were clearly not lipofuscin granules (according to size and shape) were deselected on the image (Sheehy 1989, Sheehy *et al.* 1996). Pixels representing holes and other areas without tissue were excluded. After discrimination of the grey-scale levels, edited images of the lipofuscin granules were quantified, using the software Gimp 2.8. For the volume calculation of neurolipofuscin in each sample, a geometric average of the area fraction of neurolipofuscin was used in all images as a measure of the neurolipofuscin concentration in the individual (Sheehy *et al.* 1998). This average area fraction is reported as a percent volume fraction (%NF). An analysis of variance (ANOVA) was used to compare the content of neurolipofuscin, the mean granule diameter and the mean axon size among different size classes. A linear regression model ($y = ax + b$) was fitted to the relationships between

lipofuscin content and cephalotorax length and mean granule diameter. The quality of the fit was assessed by the coefficient of determination, r^2 , and the visual appearance of the plotted data.

Results

The Cluster 10 (OLCM) is well developed and is located posteriorly, and slightly dorsal, to the OL, composed of small *globulli* cells, which have a large nucleus filling most of the cell body. The neurite tract connection is easily identified. The OLCM contains a series of small neurons, which, in cross sections, show a semi-spherical shape, with a neurites tract which penetrates into the OL. Each lobe has a large cell mass (OLCM) associated with it called cluster 10, which is connected to the OL by neurite tracts (Fig. 2). Morphological neurolipofuscin was found in all samples, in considerable amounts, appearing as somewhat round granules of variable dimensions (normally with a diameter less than 1 μm , uniformly distributed in the peripheal cytoplasm (Fig. 3). The granules mean diameter increased according to the class size (Table I). These granules contain a variable amount of membrane residue embedded in a more homogeneous material. The analysis of variance (ANOVA) showed a significant difference between the accumulation rates between the class sizes ($p < 0.05$; $R^2 = 0.84$) (Fig.4).

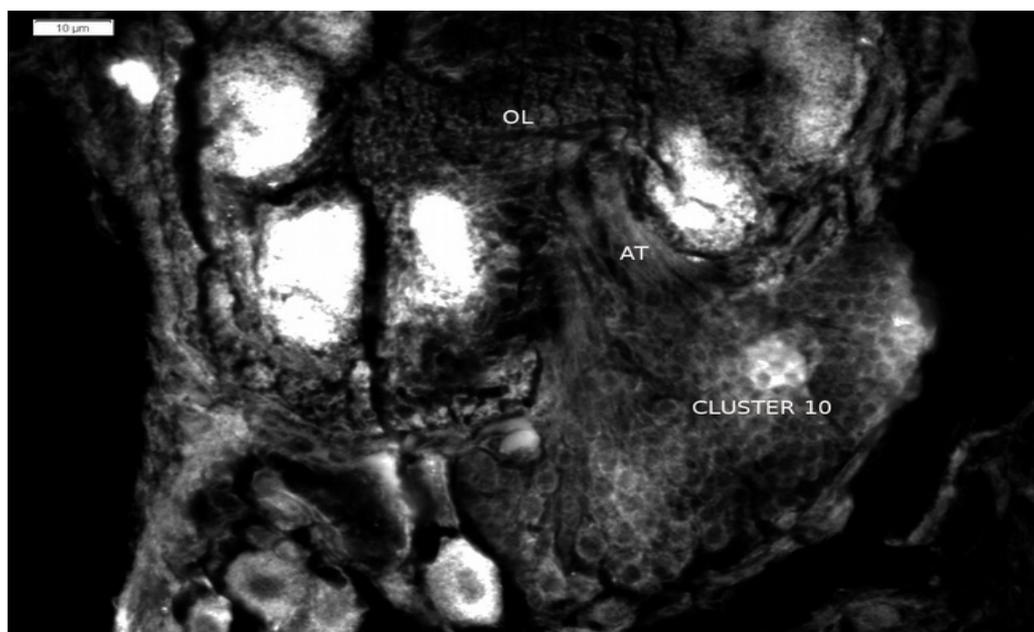


Figure 2. Autofluorescent histological section (6 μm -thick) of a region of the brain showing the olfactory lobe (OL), the axon tracts (AT) and the cell mass cluster 10, which contains *globulli* cells in which neurolipofuscin granules were found.

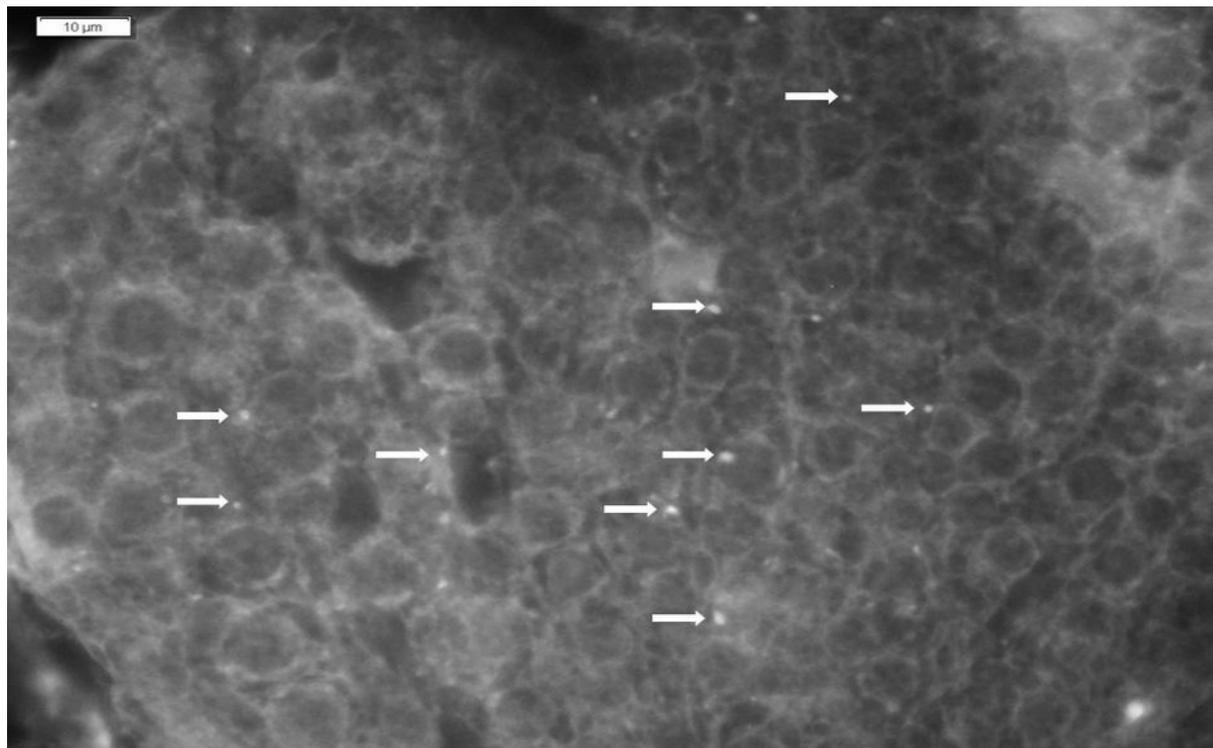


Figure 3. Autofluorescent histological section (6μm-thick) of the OLCM. Several neurolipofuscin granules are indicated (by arrows). Scale bar: 10μm

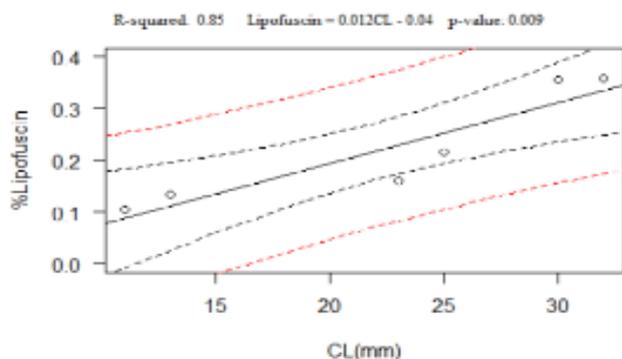


Figure 4. Linear regression between the neurolipofuscin concentration and cephalotorax length for *P. muelleri*, with the following parameters: R squared (R^2), equation ($y=ax+b$) and p value.

Mean neurolipofuscin area fraction was 0.26 (± 0.07), and ranged from 0.1 to 0.35% (Table II). In

spite of analyzing only two samples per class size, a significant increase in neurolipofuscin concentration was observed as the individuals got larger. In the first size class, which included individuals with a size smaller than 15 mm CL, an average of 0.11% (± 0.04) neurolipofuscin accumulation was found, increasing to 0.18% (± 0.06) in individuals of a class size between 15-25 mm, and increased to 0.35% (± 0.07) for the largest animals (>25mm).

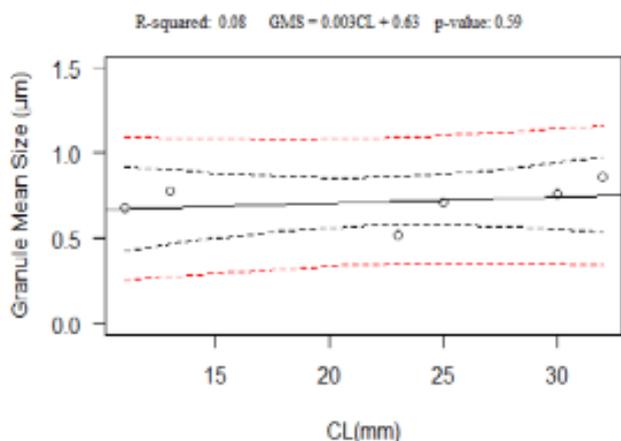
The diameter of lipofuscin granules showed no significant differences between different class sizes ($p > 0.05$, $R^2 = 0.08$) (Fig.5), with a mean diameter of 0.76μm (± 0.22), a minimum of 0.21 and a maximum size of 1.68. The intensity of the fluorescence of the neurolipofuscin granules allowed for good discrimination from the background of the cells (Fig. 3). The mean axon size was 4.85 (± 1.21), ranging from 2.09 and 6.97, and showed no apparent pattern.

Table I. Variation in the mean granule diameter of *Pleoticus muelleri* according to class size. Standard Deviation (SD) and confidence intervals of means (CI 95%).

Size (CL - mm)	Mean granule diameter	SD	95% IC	
10-15mm	0.67	0.17	0.55	0.76
15-25mm	0.73	0.21	0.60	0.81
>25mm	0.83	0.26	0.77	0.89

Table II. Variation in the mean neurolipofuscin area fraction (%) of *Pleoticus muelleri* brains by classe size. Standard Deviation (SD) and confidence intervals of means (CI 95%)

Size (CL - mm)	Mean neurolipofuscin area fraction (%)	SD	95% IC	
10-15mm	0.11	0.021	0.015	0.18
15-25mm	0.18	0.025	0.022	0.21
>25mm	0.35	0.034	0.033	0.39

**Figure 5.** Linear regression between the mean diameter of the lipofuscin granules (μm) and cephalotorax length for *P.muelleri*, with the following parameters: R squared (R^2), equation ($y=ax+b$) and p value.

Discussion

Our results report the first occurrence of morphological lipofuscin in *Pleoticus muelleri*. The position and morphology of the OLCM observed in *P. muelleri* brains were similar to those described for other Penaeid species, such as *Penaeus monodom* (Sheehy *et al.* 1995), *Marsupenaeus japonicus* (Vila *et al.* 2000), *Parapenaeus longirostris* (Medina *et al.* 2000) and *Farfantepenaeus paulensis* (Peixoto *et al.* 2002).

Regarding size, the mean granule diameter of lipofuscin in *P.muelleri* did not show significant differences among the class sizes. This pattern is similar to those found in other short-lived shrimps, such as *F. paulensis* (Peixoto *et al.* 2002) and *M. japonicus* (Vila *et al.* 2000, Vila Gordillo 2005). On the contrary, long-life span species, such as the deep shrimp *Aristaomorpha foliacea* (Mezzasalma *et al.* 2008) and the lobsters *Homarus americanus* (Wahle *et al.* 1996) and *Homarus gammarus* (Sheehy *et al.* 1996), have shown significant increases in granule size according to class size. Thus, it is likely that those individuals with very long life spans will

develop lipofuscin granules for a longer period of time and could result in larger granule sizes.

The unstained OLCM sections showed fluorescent granules that increased their concentration according to the class size. In the present study, there was a linear increase in the neurolipofuscin content according to the increase of class size, varying from 0.1% to 0.35%. Peixoto *et al.* (2002), with the shrimp *Farfantepenaeus paulensis*, a coastal species that has similar growth parameters, showed a higher accumulation rate of neurolipofuscin, ranging from 0.24% to 0.68%. However, this study used reared specimens under controlled conditions, which may explain this difference in the neurolipofuscin accumulation pattern between the two species. Previous investigations comparing the growth performance of shrimps, between wild and reared conditions, suggest that the shrimps under controlled conditions and unlimited food supply have shown a significant increase in growth rate. The age determination of the spiny lobster *Panulirus argus* using the neurolipofuscin method (Matthews *et al.* 2009), using specimens reared, showed that the combination of unlimited amounts of food that is higher in energy, with minimal energy expenditures, determined a 31% greater growth rate in the laboratory than in the wild during the first year (Sharp *et al.* 2000), leading to an erroneous interpretation of the population structure. In addition, laboratory animals, with larger cephalotoax lengths as a result of a higher energy supply, determine unrealistically large Von's Bertalanffy k coefficients and asymptotic lengths smaller than those observed in nature (Maxwell *et al.* 2007).

If we compare the lipofuscin accumulation level with other shrimp species from high latitude environments, we see a change in this pattern. The study done by Bluhm & Brey (2001), with the Antarctic shrimp *Notocrangon antarcticus*, in the Wedell Sea, where the temperature is below 0°C , the species has a $L_{\infty}=25$ mm cephalotorax length, and

their longevity can reach 10 years for females and the the accumulation levels of neurolipofuscin ranged from <0.001 and 0.22%, which is lower compared to those obtained for *P.muelleri* (0.1% to 0.35%). This can be explained by: 1) the food availability, which is lower in this area, compared to continental-shelf areas of lower latitudes (Arntz & Gorny 1991); consequently, the energetic intake affects the metabolism and the lipofuscin formation process and 2) temperature, since the combination of low temperatures and slow metabolism leads to a slow growth, slow accumulation rate and high longevity of polar species. (Chapelle & Peck 1995). Thus, formation and accumulation of lipofuscin may be affected by spatial and temporal environmental variability (Sheehy *et al.* 1996).

Recently, a new method of aging was proposed by Kilada *et al.* (2012), based on the counting of growth bands observed in the endocuticle layer in thin longitudinal sections of the meso-cardiac ossicles of the gastric mills in the two squat lobster species and in the eyestalks of nylon shrimp. This method has been used in other crustaceans, such as freshwater crayfish (*Cherax quadricarinatus*) (Ledland *et al.* 2015), crabs (*Portunus pelagicus*) (Kilada & Ibrahim 2016) and lobsters (*Homarus americanus*) (Leland *et al.* 2015). Although it is a promising methodology in determining the age structure of crustaceans, we must consider that it still needs studies to definitively validate this method, especially due to the mixing of bands in tropical and high latitude environments, where seasons are not well marked (Leland *et al.* 2015).

Thus, the ubiquitous cellular deposition of neurolipofuscin represents an attractive and valuable aging technique with potentially widespread use (Sheehy 1990, Belchier *et al.* 1998). Lipofuscin is a degenerative auto-fluorescent pigment, broadly diffused in the animal kingdom, and its accumulation shows a positive correlation with advancing age and maximum life spans (Brunk & Terman 2002). Furthermore, this is a method that seems to show good resolution for short-lived life cycles of species, such as *P. muelleri*, since the length-frequency distribution method is very limited for age determination, resulting in nondistinguishable cohorts. This study confirms the occurrence of morphological lipofuscin in the red shrimp *P. muelleri* for the first time, and the results of the present study suggest that the quantification of neurolipofuscin is possible, and that its use can be a helpful tool for age determination of this species.

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