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Individual age and connective tissue lipofuscin in the hard clam *Eurhomalea exalbida*

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Abstract

In the hard clam *Eurhomalea exalbida*, autofluorescent granules were detected in high concentrations in the connective tissue around the intestine and in other tissues. Autofluorescence combined with Sudan black B and PAS positive reactions suggested that these granules were lipofuscin-like. The concentration of this material in the connective tissue (CT) around the intestine was quantified by image analysis and expressed as total area fraction occupied by lipofuscin granules. Lipofuscin concentration was distinctly better related with individual age as determined from stable isotope-validated shell growth bands than with any morphometric parameter. This relationship was described best by a Gompertz model: $\text{Lipofuscin}_t = 24.79\text{e} - \text{e}^{-0.029(\text{Age}\ t - 58.578)}$ ($N = 38$; $r^2_{\text{nl}} = 0.882$). Age was predicted from $\text{lipofuscin}_{\text{CT}}$ concentration by a von Bertalanffy model: $\text{Age}\ t = 68.00(1 - \text{e}^{-0.146(\text{Lipofuscin}_t + 0.028)})^{0.664}$ ($N = 38$; $r^2_{\text{nl}} = 0.933$). Our findings suggest that $\text{lipofuscin}_{\text{CT}}$ concentration in *E. exalbida* is a function of individual age. If this holds true for bivalves in general, lipofuscin may be a suitable proxy for age in species with less clear shell growth band patterns.
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Keywords: Bivalve; Growth; Age; Lipofuscin

1. Introduction

Lipofuscin in situ has been detected in different tissues and organs of various invertebrates and vertebrates (e.g. Donato and Sohal, 1978; Moore et al., 1980; Bassin et al., 1982;

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Sheehy, 1989; Mathew and Damodaran, 1997; Sheehy et al., 1998; Bluhm et al., 2001a). The rate of lipofuscin formation is assumed to depend mainly on cellular oxygen consumption (Sohal, 1981; Zs-Nagy, 1988; Katz et al., 1984). Hence, the amount of lipofuscin may be proportional to physiological age and can be suitable as a proxy of chronological age, too, as shown in many studies on crustaceans (e.g. Sheehy, 1992; Sheehy et al., 1995, 1999; O'Donovan and Tully, 1996; Wahle et al., 1996; Belchier et al., 1998; Bluhm and Brey, 2001). Molluscs studies of lipofuscin in situ have focused primarily on the effect of stress such as exposure to heavy metals or anoxic conditions (e.g. Krishnakumar et al., 1990, 1994; Viarengo et al., 1990; Sarasquete et al., 1992; Hole et al., 1992, 1993, 1995; Mathew and Damodaran, 1997). The few studies relating lipofuscin to age in molluscs (Clarke et al., 1990; Zielinski and Pörtner, 2000; Sukhotin et al., 2002) are based on solvent extraction of autofluorescent substances. As there is increasing evidence that such autofluorescence is not related to lipofuscin in situ (Nicol, 1987; Hill and Womersley, 1991; Sheehy and Roberts, 1991; Sheehy 1996, 2002; Jolly et al., 2002; Palmer et al., 2002, Porta 2002; Schmucker and Sachs, 2002), we will limit the discussion to studies based on lipofuscin in situ measured in situ in histological sections.

Individual age of bivalves is usually estimated from physical or chemical growth marks formed in their $CaCO_3$ shells by regularly occurring external (e.g. day–night cycle, tides, annual cycle) or internal (e.g. spawning) events. Molluscs, however, may fail to produce identifiable growth marks in systems where the seasonal signal is weak compared to "background noise", such as in tropical seas (e.g. Etim and Brey, 1994) or in beach surf zones (e.g. McLachlan et al., 1996). Under those conditions, a physiological age marker such as lipofuscin may be the best alternative for age estimation.

This study analyses (i) whether candidate granules found in various tissues of the long-lived clam *Eurhomalea exalbida* are likely to be lipofuscin, (ii) how the concentration of these granules is related to morphometric measures and individual age, and (iii) whether the relation between individual age and lipofuscin concentration is suitable for age determination.

2. Materials and methods

2.1. Clam collection, morphometrics and age

Hard clams *E. exalbida* (Chemnitz, 1795) were collected in Ushuaia Bay (54°50′18″ S, 68°16′25″ W; Fig. 1) by SCUBA diving at 3 to 5 m depth between October 1998 and September 1999. The salinity during the study period fluctuated around 30.9‰ and 32.9‰ and monthly mean sea water temperature ranged between 8.7 °C in summer and 4.5 °C in winter (Schroeder, unpublished).

In all individuals collected, we measured shell height H (umbo to the ventral margin), length L (anterior–posterior axis) and width W (precision ± 0.1 mm), and determined shell mass (SM) as well as soft body wet mass (M) (precision ± 0.01 g).

Individual age was inferred from shell growth bands. Examination of polished shell cuts by stereo microscope using reflecting light showed a pattern of alternating broad opaque and narrow translucent bands. Stable oxygen isotope shell profiles ($\delta^{18}O$)

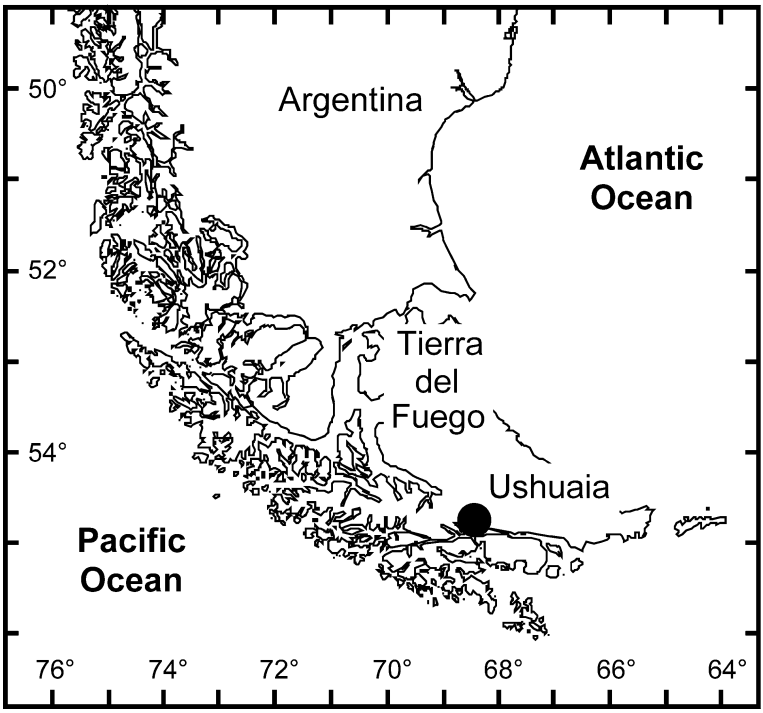

Fig. 1. Sampling site near Ushuaia, Tierra del Fuego, South America.

(Lomovasky et al., 2002) confirmed that one translucent band was formed each winter and one opaque band during each spring and summer. *E. exalbida* was found to be a slow growing species with a probable life span up to 70 years. Repeated ring counts by two independent observers showed that variability of age estimates was about 1 year on average but could be as much as 4 years in animals with more than 50 rings. For further details, see Lomovasky et al. (2002).

2.2. Lipofuscin identification and quantification

For lipofuscin analysis, a transverse piece of the anterior visceral mass was fixed in Bouin and embedded in paraplast. We checked unstained thin sections (5 μm) for autofluorescent lipofuscin-like granules using a Zeiss III RS fluorescence microscope with Plan-Apochromat objective (25 ×) and an HBO 50W AC super pressure mercury lamp with 365 nm as well as 450 nm excitation filters. The histochemical properties of candidate granules were assessed using the PAS–Alcian blue–hematoxylin (Moore et al., 1980) and the Sudan black B (see Bluhm et al., 2001a) techniques. The first of two subsequent sections was stained with the former, the second section with the latter. Autofluorescence and positive staining with both PAS (indicating aldehyde groups) and Sudan black (indicating compound lipids) at the same position were considered indicative of lipofuscin granules.

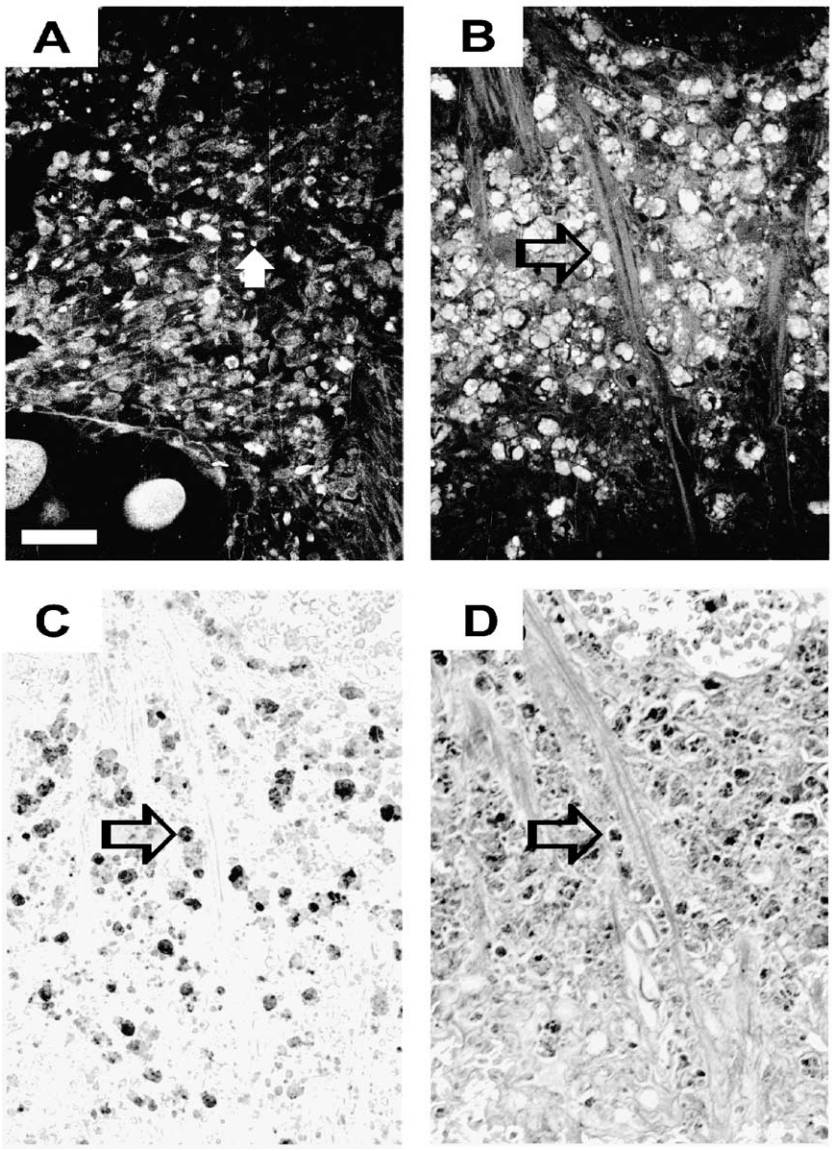

Fig. 2. Lipofuscin-like granules in the connective tissue of *E. exalbida*. (A) Autofluorescence in an 8-year-old individual. Single lipofuscin granules appear as white dots (white arrow indicates one example), larger spots represent aggregated granules. (B–D) Three subsequent sections from a 60-year-old individual. Most of the lipofuscin is in the form of aggregated granules. (B) Autofluorescence, (C) Sudan black B, (D) PAS–Alcian blue–hematoxylin. Arrow indicates identical aggregate of lipofuscin granules. White bar in A = 100 μm.

For each individual clam, 10 digital color images of autofluorescent activity covering 110200 μm^2 each were taken randomly from the region around the intestine. In these images, yellow autofluorescent granules were discriminated from the surrounding tissue by light intensity range selection using Pro Plus 3.0 image analysis software (Media Cybernetics). The total area fraction of lipofuscin granules in the connective tissue (CT) around the intestine was estimated by dividing the cross-sectional area of lipofuscin granules by the total area of tissue analysed, multiplied by

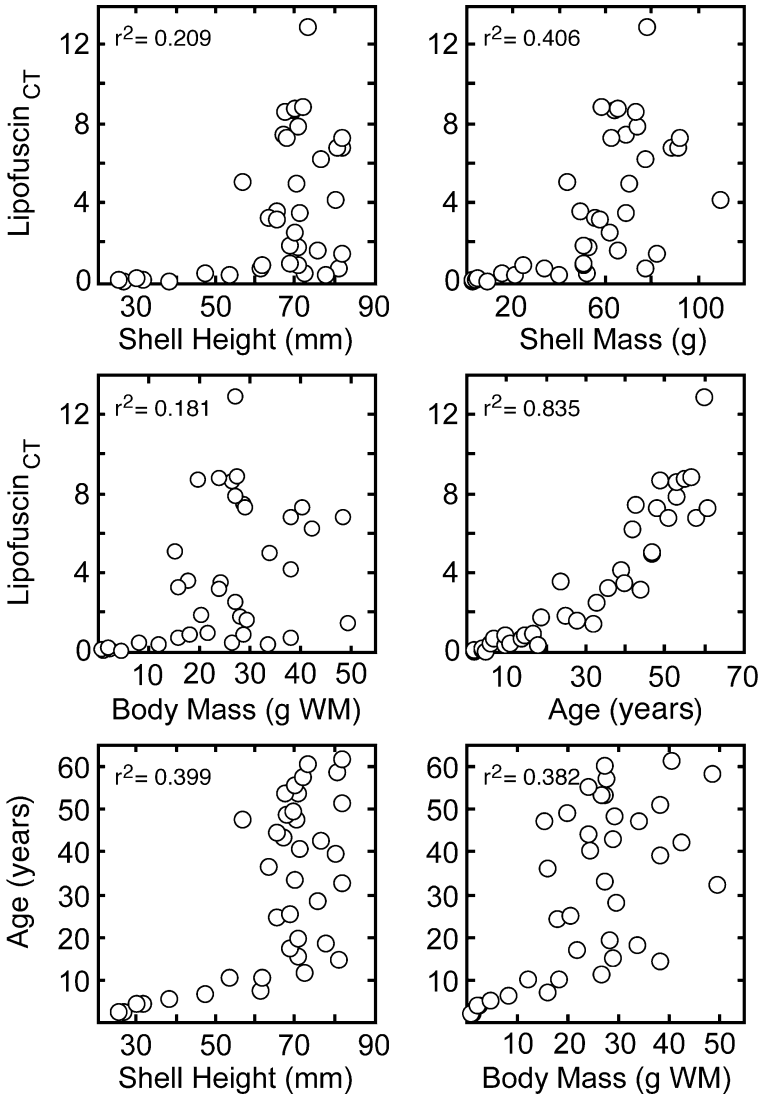

Fig. 3. Correlations between shell height, shell mass, body mass, individual age and lipofuscin$_{CT}$ concentration in *E. exalbida*. All correlations are significant at $P=0.05$.

100, for each image. Average individual lipofuscin$_{CT}$ concentration was obtained by averaging total area fraction of the 10 images taken.

Relationships between lipofuscin$_{CT}$ concentration, morphometric parameters (*S*, *W*, *H*, SM, *M*) and individual age were analysed by correlation analysis and the iterative nonlinear fit of various models.

3. Results

3.1. Lipofuscin properties and relationships to clam age and size

We examined 38 *E. exalbida* individuals and found yellow autofluorescent granules (i) in connective tissue around the intestine, (ii) in the intestinal epithelium, (iii) in the digestive gland, (vi) in connective tissue among the gonadal alveolus and (v) inside the gonadal alveolus in males. The autofluorescent granules (Fig. 2A,B) showed resistance to

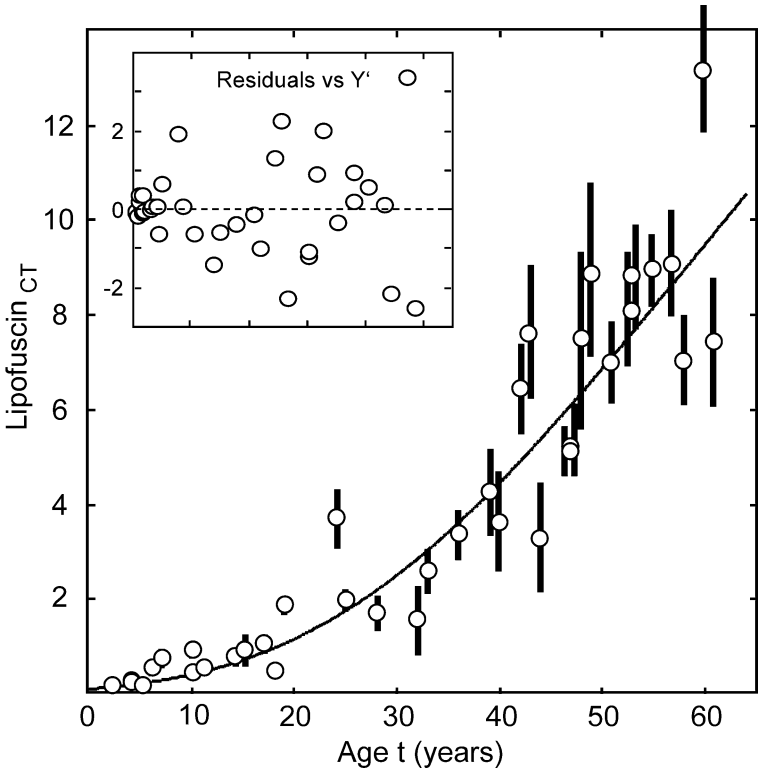

Fig. 4. Relation between measured lipofuscin$_{CT}$ concentration and age in *E. exalbida*.

$$\text{Lipofuscin}_t = 24.79e^{-e^{-0.029(\text{Age } t-58.578)}} \quad N = 38;\ r^2 = 0.882$$

Black bars indicate 95% confidence range of mean %AF. Inlay shows plot of residuals versus estimated values.

solvent extraction during histological processing and positive reactions with Sudan black B (Fig. 2C) and PAS (Fig. 2D), thus indicating that they consist of lipofuscin.

Granule concentration appeared to be highest in the connective tissue around the intestine, which was therefore used for lipofuscin quantification. Diameter of detectable granules ranged from 2.7 to 9.0 μm. In older clams, identification of single granules was hampered by their tendency to aggregate in dense clusters (Fig. 2B). In single images, lipofuscin$_{CT}$ concentration was between 0 and 18.62% area fraction, while in individual clams, average concentrations ranged from 0.02% (S.D. = 0.03) to 12.84% (S.D. = 2.97) area fraction.

Morphometric parameters, individual age and lipofuscin$_{CT}$ concentration were all significantly ($P < 0.05$) intercorrelated, but age was clearly the principal determinant of lipofuscin$_{CT}$ concentration (Fig. 3). This relation was described best by a Gompertz growth model with the parameter values (Fig. 4):

$$\text{Lipofuscin}_t = 24.79\text{e}^{-\text{e}^{-0.029(\text{Age}\ \ t-58.578)}};\ N = 38;\ r^2 = 0.882.$$

4. Discussion

4.1. Is the substance found in E. exalbida lipofuscin?

The granules found in various tissues of the hard clam *E. exalbida* show morphological, autofluorescent and histochemical properties similar to those described for lipofuscin in other molluscs (e.g. Nelson and Morton, 1979; Moore et al., 1980; Mathew and Damodaran, 1997), crustaceans (e.g. Sheehy, 1989; Wahle et al., 1996; Medina et al., 2000; Bluhm et al., 2001a,b) and vertebrates (Eldred et al., 1982). This supports the view that the connective tissue granules in *E. exalbida* are lipofuscin.

Lipofuscin seems to be common in molluscs, as it has been reported in situ in various tissues and organs of several species such as the gastropods *Maoricrypta monoxyla* (Nelson and Morton, 1979) and *Aplysia punctata* (Taieb and Vicente, 1999) and the bivalves *Mytilus galloprovincialis* (Viarengo et al., 1987, 1990; Regoli, 1992; Sarasquete et al., 1992), *Mytilus edulis* (Moore et al., 1980; George et al., 1982; Lowe, 1988; Burbidge et al., 1994; Hole et al., 1995; Krishnakumar et al., 1994) and *Perna viridis* (Krishnakumar et al., 1990; Mathew and Damodaran, 1997). Only a few findings are reported from clams so far: In the alimentary tract epithelium, in gonadal connective tissue and in the renal epithelium of *Mya arenaria* and *Mercenaria mercenaria* (Brown and O'Toole, 1978); in the digestive gland of *Ruditapes phillipinarum* (Sarasquete et al., 1992) and of *Sunetta scripta* (Mathew and Damodaran, 1997); and in the kidney of *Tridacna gigas* (Reid et al., 1984). As in *E. exalbida*, these are all tissues containing mitotic cells, which is in contrast to crustaceans, insects and vertebrates, where lipofuscin seems to be concentrated in postmitotic cell tissues such as nerves (Hammer and Braum, 1988; Sheehy et al., 1999; Bluhm and Brey, 2001).

In *E. exalbida*, lipofuscin granule concentration was highest in the connective tissue around the intestine. Ordinary light- (Fig. 2) and confocal laser microscopic evidences point towards the extracellular location of these granules, as observed in alimentary tract

epithelium and gonadal connective tissue of *M. arenaria* and *M. mercenaria* (Brown and O'Toole, 1978).

4.2. What form does the relationship between lipofuscin concentration and age take?

There are few studies that deal with independent measurements of age and lipofuscin in situ concentration in invertebrates, and most of them refer to crustaceans (Sheehy, 1990, 1992; Sheehy et al., 1994; O'Donovan and Tully, 1996; Sheehy et al., 1996; Wahle et al., 1996; Belchier et al., 1998; Vila et al., 2000). If seasonal oscillations are not taken into account, the majority of these studies points towards a linear relationship between age and lipofuscin throughout the greater part of the lifespan. There is, however, evidence for nonlinearity during certain parts of the life history, e.g. for decreasing lipofuscin accumulation rates towards advanced age (Sheehy, 1992; Sheehy et al., 1995) or even during the whole life (Vila et al., 2000).

Our data show a distinct positive exponential relationship between lipofuscin and age during the first third of the life span of *E. exalbida* (Fig. 4), whereas >20 years, the relationship appears more linear. A zone of decreasing lipofuscin accumulation, however, is not clearly identifiable; the inflection point of the Gompertz model is situated at 59 years. This zone may be situated beyond the maximum age included in our study or may be obscured by the high variability among the few data from animals older than 55 years. As this is the first study of lipofuscin in situ accumulation in (i) a bivalve and (ii) across an age range of >50 years of life, it is rather difficult to say whether this shape of the lipofuscin to age relation reflects particular features of either the class Bivalvia or of the species investigated or methodical shortcomings. A significant part of the lipofuscin granules present in *E. exalbida* may have been smaller than 2.7 μm, the resolution limit of the low objective magnification employed (25 ×), as observed in many invertebrates (e.g. Bluhm et al., 2001a,b; Sheehy, 2002). The tendency for granules and aggregations to become larger in older individuals may have introduced an age-related bias in our measurements of lipofuscin_{CT} concentrations. Eliminating this bias could change the shape of the age-lipofuscin relation significantly.

4.3. Can we use lipofuscin as a proxy for individual age in E. exalbida?

The aim of the majority of recent studies on lipofuscin in crustaceans has been to assess its usefulness for age determination. Due to molting, most crustaceans do not have permanent hard structures that can be used for ageing (e.g. Sheehy et al., 1994, 1995; O'Donovan and Tully, 1996; Wahle et al., 1996; Belchier et al., 1999; Bluhm and Brey, 2001). In molluscs, however, lipofuscin accumulation has been studied primarily in relation to stress (Krishnakumar et al., 1990; Viarengo et al., 1990; Hole et al., 1992, 1993, 1995; Mathew and Damodaran, 1997). Our study is the first to show a clear relationship between age and lipofuscin in situ in a bivalve.

In order to establish a model that predicts individual age from lipofuscin concentration, we need (i) a sufficient amount of data to reduce statistical noise to tolerable levels, and (ii) data that cover as much of the lifespan of the species as possible, to avoid

the bias involved in extrapolating nonlinear models. Our study includes 38 data points only but covers about 6/7 of the lifespan of *E. exalbida* (Lomovasky et al., 2002). In *E. exalbida*, age was predicted best from lipofuscin$_{CT}$ concentration by a von Bertalanffy model (Fig. 5):

$$\text{Age } t = 68.00\left(1 - e^{-0.146(\text{Lipofuscin}_t + 0.028)}\right)^{0.664} \quad (N = 38;\ r^2 = 0.933).$$

Goodness-of-fit of this model is high, but the residuals (Fig. 5) indicate, nevertheless, that estimates of age range between as much as -10 and $+10$ years of true age, which is clearly less precise than annulus-based ageing for this species. Predictive reliability may be enhanced either by (i) adding more individuals to the data set, (ii) reducing variability of lipofuscin measurements by analysing more sections and/or by quantifying lipofuscin by more advanced techniques such as confocal laser microscopy (e.g. Bluhm and Brey, 2001), or (iii) establishing multiparameter models which include additional variables derived from shell size and/or mass. Despite the inferior precision of lipofuscin-based age estimates in clams, such models can still be very useful in a population dynamics context,

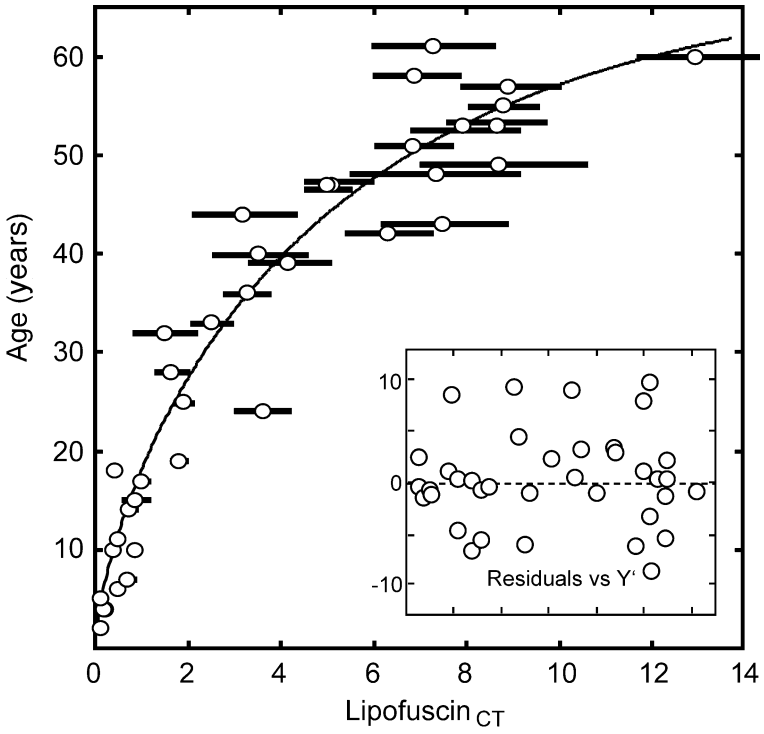

Fig. 5. Individual age predicted from lipofuscin$_{CT}$ concentration in *E. exalbida* by a von Bertalanffy model

$$\text{Age } t = 68.00\left(1 - e^{-0.146(\text{Lipofuscin}_t + 0.028)}\right)^{0.664} \quad (N = 38;\ r^2 = 0.933)$$

Black bars indicate 95% confidence range of mean %AF. Inlay shows plot of residuals versus estimated values.

for example, in indicating longevity or for the identification of age classes in frequency distributions of field populations (e.g. Sheehy et al., 1995; Bluhm et al., 2001a,b).

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