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Neurolipofuscin is a Measure of Age in the Caribbean Spiny Lobster, Panulirus argus, in Florida

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NEUROLIPOFUSCIN IS A MEASURE OF AGE IN THE CARIBBEAN SPINY LOBSTER, *PANULIRUS ARGUS*, IN FLORIDA.

by

KERRY E. MAXWELL

Under the direction of Charles D. Derby

ABSTRACT

Accurate age estimates for the commercially-important Caribbean spiny lobster, *Panulirus argus*, would greatly enhance analyses of life history and population dynamics. Previous estimates of their age based on size and growth may be inaccurate because of variable growth in the wild. An established technique for aging crustaceans – histologically-determined lipofuscin content in the nervous system – was used on lobsters reared in the laboratory for up to five years. We verified the presence of lipofuscin in eyestalk neural tissue and described its distribution in cell cluster A of the hemiellipsoid body. Neurolipofuscin content of both sexes increased linearly over the five-year age range, with seasonal oscillations. Growth of these animals, on the other hand, showed sex differences and began to asymptote after three years. Neurolipofuscin concentrations in the two eyestalks from the same animal were similar. These results suggest that the neurolipofuscin technique will be valuable for estimating age of wild-caught *P.argus*.

INDEX WORDS: *Panulirus argus*, Neurolipofuscin, Spiny Lobster, Florida Keys, Growth

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LIST OF ABBREVIATIONS

ANCOVA – analysis of covariance

a_s - the fraction of the year that had elapsed at the point when neurolipofuscin accumulation is the highest

a_0 - the age, in years, at which neurolipofuscin concentration was zero

b – slope

C - amplitude

°C - Celsius

CL – carapace length

CNS – central nervous system

FWC – Florida Fish and Wildlife Conservation Commission

K – growth constant

L – length in mm CL

L_8 – asymptotic length

M- Molar

µm – micrometer

N – %VF of neurolipofuscin

NOAA – National Oceanic and Atmospheric Administration

Nm- nanometer

PaV1 – virus associated with *Panulirus argus*

S.E.M. – standard error of the mean

t – age in years, time

t_0 – age at size "0"

UV- ultraviolet

%VF – percent volume fraction

INTRODUCTION

The fishery for Caribbean spiny lobsters, *Panulirus argus*, is currently the second most valuable commercial fishery in Florida, annually worth on average US$ 35 million (Florida Fish and Wildlife Conservation Commission (FWC), unpublished data). Recent fluctuations in landings have highlighted deficiencies in our understanding of spiny lobster population ecology and in the data used for stock assessments. Many of these deficiencies are caused by a lack of accurate methods to estimate age of spiny lobsters. Age estimates are the foundation for computing growth rate, maturation, longevity, and mortality rate, all of which are vital for population analyses (Lyons *et al*., 1981; Davis and Dodrill, 1989; Forcucci *et al*., 1994; Muller *et al*., 1997). Thus, an accurate method of determining the age of *P. argus* individuals would be a useful tool for understanding the basic biology of this species and developing stock assessments.

Most age approximations of *P. argus* are based on size and growth data from tag-recapture studies (Forcucci *et al*., 1994; Muller *et al*., 1997) or modal analysis of size-frequency histograms (Eldred *et al*., 1972; Munro, 1983). Size, however, is generally considered to be a poor measure of age of crustaceans living in the field because growth is dependent on many environmental and density-dependent factors besides age (Davis and Dodrill, 1980; Davis, 1981; Lellis and Russell, 1990; Sheehy, 1992; James *et al*., 2001). Phillips *et al.* (1992) demonstrated the wide natural variation in the growth rate of *P. argus*, *Panulirus ornatus*, and *Panulirus cygnus*. Growth may be suppressed following injury as wounds heal and missing limbs regenerate (Davis, 1981; Hunt and Lyons, 1986). This is particularly important in Florida where intense recreational and commercial fisheries handle millions of sublegal-sized spiny lobsters (*i.e.*, animals below the minimum harvest size of 76 mm carapace length, CL), causing numerous

injuries (Davis and Dodrill, 1980; Lyons and Kennedy, 1981; Hunt, 1994). Growth may also be impaired by the fishery practice of using sublegal-sized animals as live bait in traps to exploit the gregarious behavior of spiny lobsters and lure legal-sized spiny lobsters into traps. Spiny lobsters used as bait may starve when they are confined in traps for extended periods of time, which can result in death or a reduction in growth rate (Lyons and Kennedy, 1981; Hunt *et al*., 1986; Matthews, 2001). For these and other reasons, size may be a poor indicator of age for wild spiny lobsters in Florida.

Some animals have structures such as otoliths, spines, vertebrae, scales, or shells that can be used for age determination (Lai *et al*., 1996; Campana, 2001; Lomovasky *et al*., 2002). Spiny lobsters and most other crustaceans, conversely, carry no permanent hard structures from which age can be determined, because they repeatedly shed their exoskeleton to grow larger. An alternative aging technique has been developed for crustaceans based on the concentration of the age pigment, lipofuscin, in soft tissues. The concentration of lipofuscin located in the central nervous system (CNS), hereafter referred to as neurolipofuscin, has been shown to be a good index of age in several crustaceans, including freshwater crayfish, *Cherax quadricarinatus* (Sheehy, 1990a) and *Pacifastacus leniusculus* (Belchier *et al*., 1998), American lobster, *Homarus americanus* (Wahle *et al*., 1996), European lobster, *Homarus gammarus* (Sheehy *et al*., 1996), Western Australian rock lobster, *P. cygnus* (Sheehy *et al*., 1998), Antarctic shrimp, *Notocrangon antarcticus* (Bluhm and Brey, 2001), Antarctic amphipods, *Waldeckia obesa* (Bluhm *et al*., 2001a), and mantis shrimp, *Oratosquilla oratoria* (Kodama *et al*., 2005).

The process of lipofuscin formation is thought to occur continuously throughout the life of all aerobically respiring cells (Terman and Brunk, 2004a). Lipofuscin is not a particular chemical compound but a heterogeneous granular aggregate of oxidatively or otherwise damaged

or superfluous macromolecules that are generated during normal metabolism. These are mainly proteins (30-70%) and lipids (20-50%) that appear to be resistant to lysosomal recycling (Eldred and Lasky, 1993; Jolly *et al*., 2002; Szweda *et al*., 2003). In at least some cell types, lipofuscin may be exocytosed (Fonseca *et al*., 2005a), but minimal turnover is generally thought to be the reason for lipofuscin's characteristic age-related accumulation in post-mitotic tissues. It is the predictability of this accumulation that makes lipofuscin a unique and outstanding biomarker of physiological ageing (Fonseca *et al*., 2005b) and a powerful ecological tool for age determination (Sheehy, 2002b).

Lipofuscin accumulation reflects biological rather than chronological age, because its deposition represents the interplay between the cellular production of harmful metabolic byproducts such as free radicals and the imperfect systems that have evolved to eliminate such byproducts or repair the damage caused by them (Fonseca *et al*., 2005a). Consequently, for the purpose of age determination, neurolipofuscin concentration must be calibrated to the passage of calendar time under relevant environmental conditions. Neurolipofuscin accumulation rate has been altered experimentally, either directly or indirectly, by varying environmental temperature (Sheehy, 2002b), restricting physical activity (Sohal and Donato, 1979), restricting caloric intake (Moore *et al*., 1995), supplementing dietary antioxidants (Castro *et al*., 2002), or injuring the CNS (Fonseca *et al*., 2003). However, the ecological relevance of many of these treatment effects is unknown. A recent key finding from studies on known-age microtagged *H. gammarus* recaptured at up to 10 years of age is that, under complex and changeable natural environmental conditions, at least 93% of the individual variation in neurolipofuscin concentration is explained by only two variables, chronological age and average environmental temperature, or unknown covariates of temperature (Sheehy and Bannister, 2002).

Lipofuscin deposition is a manifestation of imperfect catabolism (Terman and Brunk, 2004b). Comparisons of neurolipofuscin concentrations in laboratory-reared crustaceans of differing sizes but identical chronological age (Sheehy, 1990a, Wahle *et al*., 1996, O'Donovan and Tully, 1996) and in adult insects of differing ages (*e.g.*, Fonseca *et al.*, 2005b) suggest that the age related accumulation of neurolipofuscin is decoupled from growth. The growth rate of most animals is individually variable and slows or stops at maturation as energetic resources are diverted from somatic development to reproduction; however, free radical-generating respiratory catabolism and lipofuscin accumulation continue. Consequently, in crustaceans, neurolipofuscin content is normally much more highly correlated with age than is size (Sheehy, 1990a; Sheehy *et al*., 1996; Belchier *et al*., 1998; Sheehy *et al*., 1998; Bluhm *et al*., 2001a; Bluhm and Brey, 2001; Kodama *et al*., 2005).

Spatial localization of neurolipofuscin within various regions of the brain has been considered to reflect a higher level of cellular metabolic activity in such areas (Friede, 1962). In crustaceans, neurolipofuscin is particularly conspicuous in a cluster of cell bodies (termed the lateral cluster) associated with the olfactory lobe of the central brain (Sheehy 1989) and a cluster of cell bodies (termed 'cluster A') associated with the hemiellipsoid body of the eyestalk (Sheehy *et al*., 1996). Neurolipofuscin granules are conspicuous by their small size (mostly 1-2 µm diameter), irregular shape, and autofluorescence (Sheehy, 1989; Sheehy and Wickins, 1994; Sheehy *et al*., 1996). Quantification of neurolipofuscin in the eyestalk is particularly advantageous because eyestalks are easily collected and there are two eyestalks from which replicate measurements can be made from each animal.

Our study was designed to examine whether neurolipofuscin concentration can be used as an effective measure of age in *P. argus*. First, we verified the presence of lipofuscin in the

lobster's nervous system, specifically that associated with the hemiellipsoid bodies of the eyestalks. Second, we attempted to streamline the existing neurolipofuscin quantification methodology to reflect the distribution and abundance of neurolipofuscin in *P. argus*. Third, we examined the neurolipofuscin accumulation pattern in known-age animals up to four years of age under conditions approximating those in the field, assessing both bilateral variation in the eyestalks and sex differences. Age determination for *P. argus* has many implications for fisheries management. Future studies on wild-caught *P. argus* will allow the estimation of growth and the development of age-structured population analyses to better describe spiny lobster population dynamics.

MATERIALS AND METHODS

Animal rearing

Spiny lobsters of known age were used to determine the rate of neurolipofuscin accumulation. To obtain animals of known age, we raised spiny lobsters between August 1999 and August 2004 under thermal conditions similar to those in the Florida Keys. Groups of recently settled pueruli and first stage juveniles were collected every 3-4 months from modified Witham collectors (Witham *et al*., 1968; Phillips *et al*., 2005) located 100 m offshore of Long Key (24° 48' N, 80° 50' W) and 100 m offshore of Big Munson Island (24° 37' N, 81° 23' W) in the Florida Keys (Acosta *et al*., 1997). For the purposes of this study, we consider the pueruli to be age 0 at settlement, when they are around 6 mm CL; however, *P. argus* has multiple planktonic phyllosoma stages lasting approximately 6 to 9 months (Lyons, 1980; Farmer *et al*., 1989). Each group of pueruli was communally raised in 1,500-liter aquaria and transferred to 9,500-liter aquaria as they grew at the Fish and Wildlife Research Institute, South Florida

Regional Laboratory in Marathon, Florida. Six spiny lobsters from the group collected in September 2001 were sacrificed every 4-5 months to collect neurolipofuscin-based age data for animals 12 to 35 months old. A total of 39 animals from the group collected in September 2001 were included in the neurolipofuscin-based age data. Twelve additional animals from groups collected earlier than September 2001 were also available to collect neurolipofuscin-based age data for animals up to 49 months old.

Water in the aquaria was pumped from nearby Florida Bay through a sand filter and partially recirculated through an 18 or 25 Watt ultraviolet sterilizer to prevent transmission of a naturally occurring lethal-pathogenic virus, PaV1 (Shields and Behringer, 2004). Temperature in the aquaria was maintained within 1 °C of the mean-monthly near-surface temperatures in a typical nursery habitat, Florida Bay, as measured by NOAA's National Data Buoy Center C-Man station located at Long Key, FL (24° 50' N, 80° 51' W, http://www.ndbc.noaa.gov/Maps/Florida.shtml), for the first year and subsequently within 1 °C of temperatures recorded at a typical adult habitat, Sombrero reef, as measured at Sombrero Key C-Man station (24° 37' N, 81° 06' W) for the remainder of the experiment. Mean monthly temperatures ranged from 21 to 31 ^{o}C (av. 26.4 ^{o}C) in Florida Bay, and from 23 to 30 ^{o}C (av. 26.7 ^{o}C) at Sombrero reef (Fig. 1).

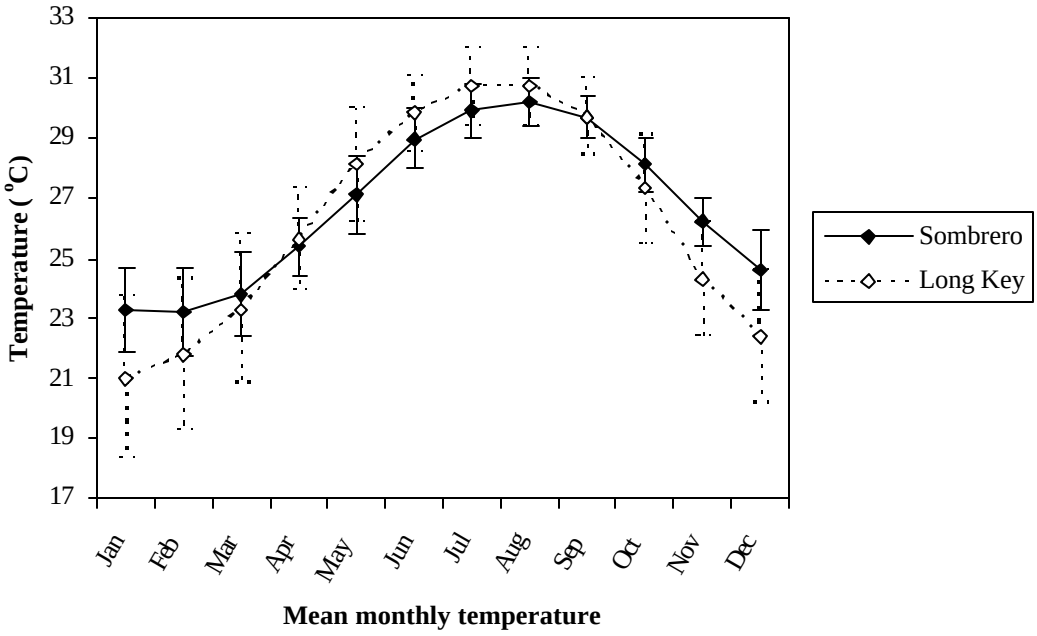

Figure 1. Mean monthly water temperature from 1999 to 2004 (± 1 S.E.M.) at lobster nursery habitat in Florida Bay (dashed line and open diamonds) and adult habitat at Sombrero reef (solid line and solid diamonds). From http://www.ndbc.noaa.gov/Maps/Florida.shtml. Water temperatures in our laboratory aquaria were adjusted as needed to match these temperatures within 1 ^{o}C.

Spiny lobsters were fed frozen fish, shrimp, or squid each day, and this was supplemented at least once per week with live or fresh fish, shrimp, snails, or crabs. The species of live snails and crabs used were those normally consumed by spiny lobsters in their natural environment (Cox *et al*., 1997) but could only be provided once per week because of limited availability. We made an effort to drop a piece of food in front of each animal so that all animals had equal access to food. Food was provided daily *ad libitum*; excess food was removed the

following day, and the subsequent volume of food was adjusted appropriately to avoid the accumulation of uneaten food.

Tissue preparation

Tissue was prepared for histology in 51 laboratory-reared animals; however 2 samples did not have replicates, since one eye was damaged. Our choice of tissue, the methods used to process the tissue, and the quantification of neurolipofuscin were based on Sheehy *et al*. (1996) with some modifications. Spiny lobsters were anesthetized by refrigeration for 1 hr at –10 ^{o}C prior to eyestalk ablation. Both eyestalks were removed from animals, immediately placed into 4% paraformaldehyde for 48 hr, transferred into 0.2 M phosphate buffer plus azide, and transported to Georgia State University for processing.

The internal neural tissue of the eyestalk was dissected away from the cuticle and dehydrated in increasing concentrations of ethanol, cleared in xylene, and embedded in paraffin. Eyestalk tissue was sectioned at 6-µm thickness using a microtome, and the resulting wax ribbon was placed on slides. The proximal ganglion of the eyestalk (the terminal medulla), including the hemiellipsoid body (see Fig. 6C in Blaustein *et al*., 1988, or Fig. 1 in Sheehy *et al*., 1996) was sectioned. This usually resulted in several hundred sections of which several dozen contained the area of interest – neurons in cluster A (see Fig. 11A in Blaustein *et al*., 1988). These neurons include local interneurons with neurites that branch in the hemiellipsoid bodies. This neural region has the highest concentrations of neurolipofuscin granules, which typically are in the extracellular spaces neighboring the somata of cluster A neurons (Sheehy *et al*., 1996, 1998).

Fluorescence microscopy

All sections of cluster A, beginning at the proximal end of the nerve tract connecting the hemiellipsoid body to cluster A, and continuing distally through the nerve tract (Blaustein *et al*., 1988, Sheehy *et al*., 1996), were sampled. Sections were excited with 450-490 nm blue light, and emission was detected at 515-585 nm using a Zeiss Axioplan fluorescence microscope with a 100x oil immersion objective. In each section, the area of cluster A that contained the highest neurolipofuscin concentration was imaged. Depending on the size of the animal, 15-40 (usually 25-30) images were captured per eyestalk using a Carl Zeiss ZVS-3C75DE camera fitted to the microscope. Field width of captured images was 125 µm. Brightness, contrast, and sharpness of the images were adjusted so that the neurolipofuscin granules were most obvious and distinct. Images were acquired using Digital Camera Acquire (Interactive Acquisition Utility, version 3.0) software and saved as 800 x 600 resolution bit map files.

Neurolipofuscin quantification

Images were analyzed using Adobe Photoshop 6.0. Color images were converted to a grey scale and, for each tissue section, a region of interest was selected and the cross-sectional areas of lipofuscin and background tissue within it were quantified as follows. The number of pixels representing fluorescent neurolipofuscin granules was quantified using automatic thresholding, which is a technique that selects all pixels above a user-determined brightness. All fluorescing artifacts that were clearly not neurolipofuscin granules, based on their size and shape, were deselected from the image (*e.g.*, Sheehy, 1989; Sheehy *et al*., 1996). Pixels representing tissue were selected using a similar threshold and manual editing approach. Pixels representing holes and other areas without tissue were excluded. Because captured images were centered on

the area in cluster A with highest concentrations of neurolipofuscin and because neurolipofuscin was often deposited along the edge of cluster A in *P. argus*, a few images had up to 50% of their area with no tissue. To correct for potential measurement biases associated with this variation and the shrinkage associated with poor tissue fixation, we used a weighted geometric average of the area fractions of neurolipofuscin in all images as a measure of the neurolipofuscin concentration in the individual (Sheehy *et al.*, 1998). Following stereological convention, this average area fraction is reported as a percent volume fraction (%VF).

Evaluation of methods

Because preparing and analyzing tissue for neurolipofuscin quantification is very labor intensive, strategies to reduce effort or increase precision were explored. One strategy to reduce effort involved the number of tissue sections used for image analysis. The second strategy addressing precision explored the use of one eye as opposed to paired eyes. We evaluated if time was better spent collecting an average neurolipofuscin value from two eyes and eliminating those few samples with high variability, or collecting samples of one eye from twice as many animals. The latter sampling strategy does not allow for sample replication and verification of neurolipofuscin concentration but does allow for the collection of twice as many neurolipofuscin-based estimates of age from the same effort in the laboratory.

To evaluate the number of sections necessary for precise measurements of neurolipofuscin, a random sub-sample of 5, 10, 15, and 20 sections was tested against the total number of sections in which the nerve tract was present. The selected precision level for this analysis was one standard error from the mean obtained by using all the possible sections.

To evaluate the use of paired eyes or single eyes, we used a Monte Carlo simulation on the data from pairs of eyes. In the simulation, randomized pairs of eyes with sample sizes of 20, 30, 40, and 50 pairs were generated by computer then analyzed using the paired-eye strategy. Then, randomized single eyes of 40, 60, 80, and 100 eyes were generated by computer and analyzed using the single-eye strategy. In the paired-eye strategy, pairs of eyes deviating by more than 2 standard errors from an expected slope of 1 were excluded from further analyses. In the single-eye strategy, all single eyes are included in the analysis. For this analysis, we sub-sampled from 49 laboratory-reared animals and 91 wild animals collected near the Dry Tortugas National Park (24°38' N, 82°51' W, 70 miles west of Key West FL) from which both eyes had neurolipofuscin concentration determined.

Validation of methods

The lipid content of the autofluorescing granules that we considered to be neurolipofuscin was confirmed by Sudan Black stain for lipids using standard methodology (Sheehy, 1989; Sheehy and Wickins, 1994; Sheehy *et al*., 1996). Selected sections of fluorescing neurolipofuscin in cluster A were photographed, then stained with Sudan Black and reimaged with identical orientation under bright-field illumination.

We validated our neurolipofuscin quantification technique by having two independent analyses performed on a subset of the data from animals caught near the Dry Tortugas National Park. For this analysis, 20 pairs of eyes were imaged and analyzed at the University of Leicester laboratory and compared to 96 eyes imaged and analyzed at Georgia State University. We found no significant differences ($p > 0.05$) in the results of the two independent analyses in either the bilateral variability of eyestalk neurolipofuscin concentration or the relationships between

neurolipofuscin concentration and CL, as assessed by Pearson correlation and ANCOVA, respectively; thus, this validation showed the consistency of our methodology with previous studies (Sheehy *et al*., 1996, 1998).

RESULTS

Neurolipofuscin in the spiny lobster

Autofluorescent granules in cluster A of the hemiellipsoid body of the eyestalk (Fig. 2a and 2b) stained positive for Sudan Black (Fig. 2 c and 2d), indicating their lipid content. The granules were also resistant to non-polar solvent (xylene) extraction during histological processing, possibly indicating that the lipids were bound. These findings help confirm the identity of the autofluorescent granules as neurolipofuscin. These granules were mainly extracellular and approximately 1-2 μm in diameter, although the more neurolipofuscin present in an individual, the greater the size of the individual granules and clusters of granules. Neurolipofuscin was often not evenly distributed throughout cluster A, but rather tended to aggregate along its edge, especially in animals with high neurolipofuscin concentrations (*i.e.*, in older animals). Autofluorescent granules also occurred in the lateral cluster of somata associated with the olfactory lobe of the central brain (data not shown).

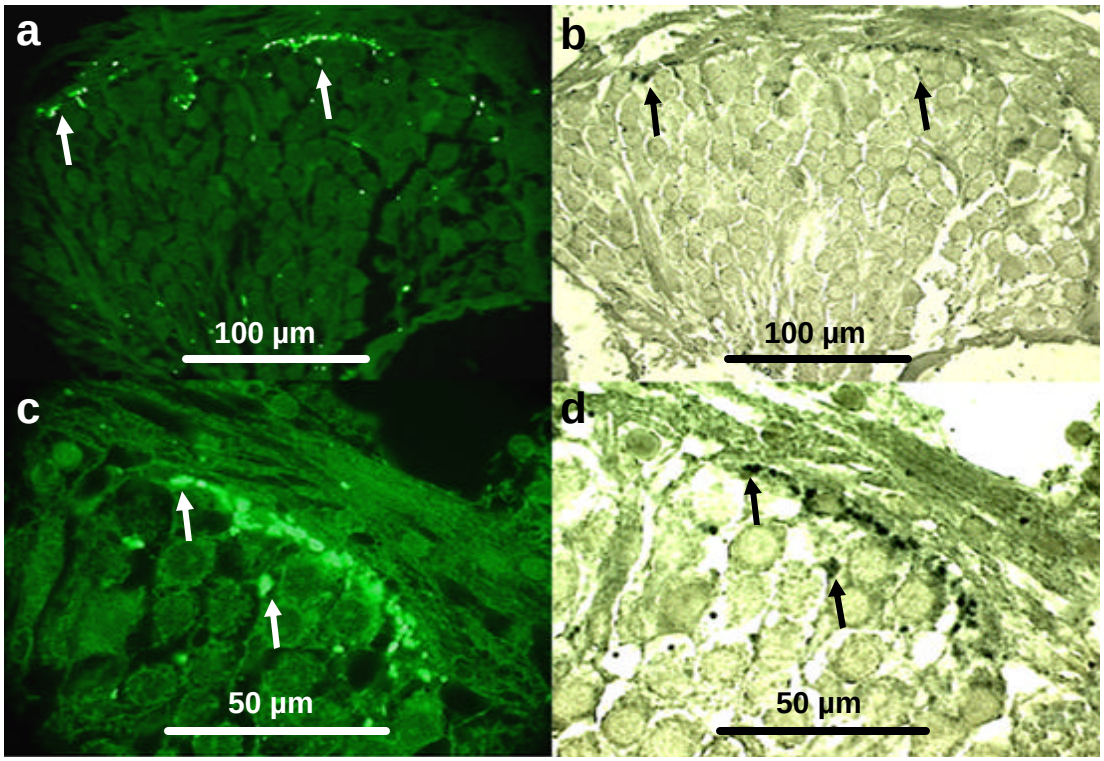

Figure 2. Autofluorescence (a and b) and Sudan black staining (c and d) of a single section of cell cluster A of the eyestalk hemiellipsoid body in *P. argus.* (a) and (c) magnified 40x. (b) and (d) magnified 100x. Arrows indicate selected granules exhibiting both autofluorescence and Sudan Black staining, confirming their identity as neurolipofuscin.

Evaluation of methods

Neurolipofuscin concentrations in the left and right eyestalks of sampled lobsters did not differ significantly (Wilcoxon signed rank test: $n = 49$, $p = 0.434$) (Fig. 3). A strong bilateral correlation existed even over the relatively narrow range of neurolipofuscin concentrations encountered in the study (Pearson correlation: $n = 49$, $r = 0.85$, $p < 0.0001$). Bilateral variability increased for neurolipofuscin concentrations over 1.0 %VF (*i.e.*, in older animals), although such values were rare in our population. Three of the oldest animals with high variability between

eyestalks were identified as outliers (defined as points deviating by 2 standard errors from the expected slope of 1). These samples were excluded from further analysis so as not to exert undue influence on the regression of neurolipofuscin concentration on age. Although this level of bilateral variability was not different from that seen previously in *H. gammarus* (Sheehy, 2002a) (Fig. 4), the maximum neurolipofuscin concentrations observed in our *P. argus* samples were much lower than those seen in *H. gammarus*.

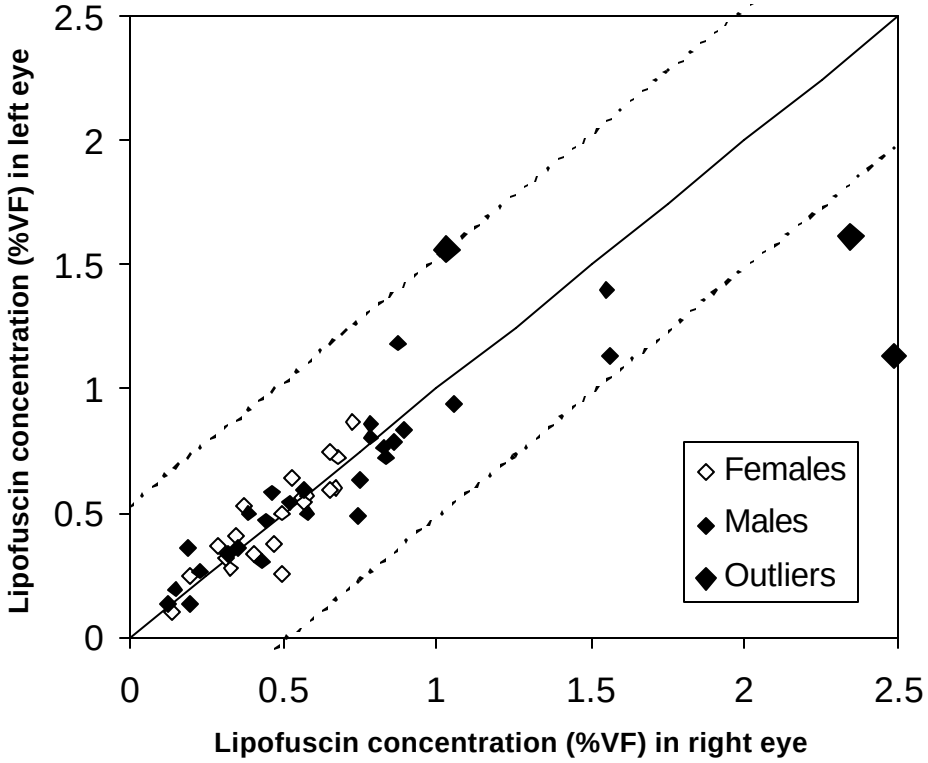

Figure 3. Left and right eyestalk neurolipofuscin concentrations were strongly correlated (Pearson correlation: n = 49, $r = 0.85$, $p < 0.0001$). There was no significant bilateral difference in the neurolipofuscin concentrations (Wilcoxon signed rank test: n = 49; $p = 0.434$). Solid reference line has a slope of 1. Dotted line represents the 95% prediction interval. Solid diamonds represent males, and open diamonds represent females. Large diamonds represent

outliers (defined as points deviating by 2 standard errors from the expected slope of 1) that were not included in further analysis.

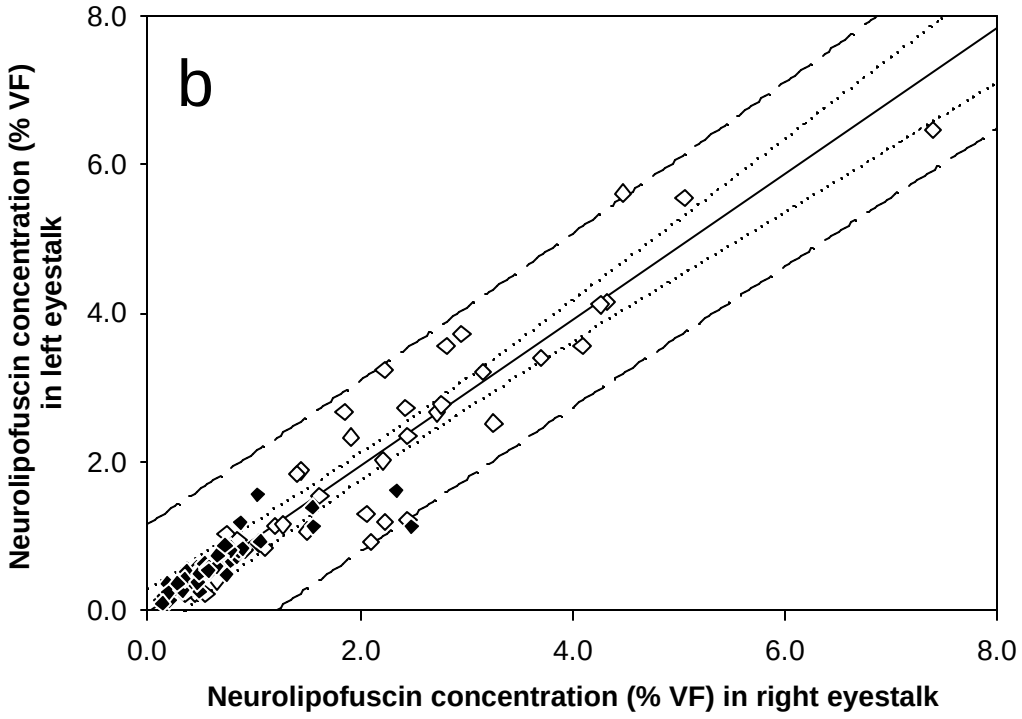

Figure 4. Bilateral comparison of neurolipofuscin concentrations in paired eyestalks in *P. argus* (this study) and in *Homarus gammarus* (from Sheehy 2002a). Bilateral variability is similar for the two species, but the range of neurolipofuscin concentrations (physiological ages) in the *P. argus* samples (solid diamonds) is much lower than that for *H. gammarus* (open diamonds). Linear regression and its 95% confidence and prediction limits (solid, dotted, and dashed lines, respectively) for the *H. gammarus* data only.

The analysis evaluating whether fewer paired eyes or more single eyes provides better neurolipofuscin values for a hypothetical population demonstrated that a large sample of single

eyes is superior. The mean %VF of neurolipofuscin is lower in the population generated from paired eyes because those with high variability are discarded, and the majority of the highly variable eyes has high concentrations of lipofuscin (Fig. 3). The difference in mean % VF of neurolipofuscin between the two strategies amounts to less than 2 months of neurolipofuscin accumulation. However, the standard deviation decreases as the sample size increases, and is lower in the population generated from single eyes (Table 1).

Table 1. Mean neurolipofuscin values and standard deviation for samples of lobsters generated by Monte Carlo techniques. The top half of the table represents a sample generated from single eyes, and the bottom half represents samples generated from paired eyes with values differing by more than 2 standard deviations removed. Standard deviation decreases as the sample size increases and is lower in the samples generated from single eyes.

Number of samples	**40**	**60**	**80**	**100**
Mean %VF of neurolipofuscin for single eyes	0.5325	0.5362	0.5375	0.5317
SD %VF of neurolipofuscin for single eyes	0.0510	0.0418	0.0366	0.0294
Number of samples	**20**	**30**	**40**	**50**
Mean %VF of neurolipofuscin for paired eyes	0.4904	0.4876	0.4889	0.4876
SD %VF of neurolipofuscin for paired eyes	0.0547	0.0486	0.0421	0.0342

The analysis evaluating the number of sections necessary to achieve precise measurements of neurolipofuscin demonstrated that a random sub-sample of 20 sections from the

total number of sections imaged provided sufficiently precise measurements of neurolipofuscin 92% of the time, whereas using 15, 10, or 5 sections yielded precise measurements of neurolipofuscin only 82%, 61%, or 44% of the time, respectively.

Neurolipofuscin content increases with age

The neurolipofuscin concentration in *P. argus* eyestalks increased linearly with age between years and had a strong seasonal component. There was no difference in neurolipofuscin concentration (ANCOVA with age as the covariate, $p > 0.05$) related to the sex of these lobsters. In the absence of sex differences, sexes were pooled in subsequent analyses. For all lobsters raised in the laboratory up to 4 years of age under thermal conditions similar to those in the Florida Keys, there appears to be an average annual neurolipofuscin accumulation rate of 0.29 %VF based on the linear regression: N = 0.290t - 0.119, where t = age in years and N = %VF of neurolipofuscin (n = 48, $r^2 = 0.61$, $p < 0.0001$) (Fig. 5). The intercept of the linear model represents a delay of several months post settlement before the onset of microscopically detectable neurolipofuscin accumulation. The annual neurolipofuscin accumulation rate appears consistent between years with seasonal oscillation observed each year. Examination of the seasonal patterns of neurolipofiscin accumulation was limited to lobsters collected in September of 2001 because this collection period included most of the lobsters that were raised in the laboratory and avoided any effects of including lobsters that were initially collected during different seasons. This eliminated all lobsters over age 35 months and several younger lobsters from the season analysis. We used the seasonalized linear model of neurolipofuscin concentration on age developed by Sheehy (2002b):

$$N = b(A-a_0)bc(2p)^{-1}\sin 2p(A-a_s) + bc(2p)^{-1}\sin 2p(a_0-a_s)$$

where N = neurolipofuscin concentration (%VF) at age A (years), b = 0.297, c (amplitude) = 0.081, a_s (the fraction of the year that had elapsed at the point when neurolipofuscin accumulation is the highest) = 1.036, and a_0 (the age, in years, at which neurolipofuscin concentration was zero) = 0.511 (n = 38, r^2 = 0.81, $p < 0.0001$) (Fig. 5). The underlying slope of this model (b) is consistent with the linear model applied to the larger database which included older lobsters. Two of the animals collected in September 2001 were excluded from the seasonalized linear model because they deviated from the model by more than two standard errors.

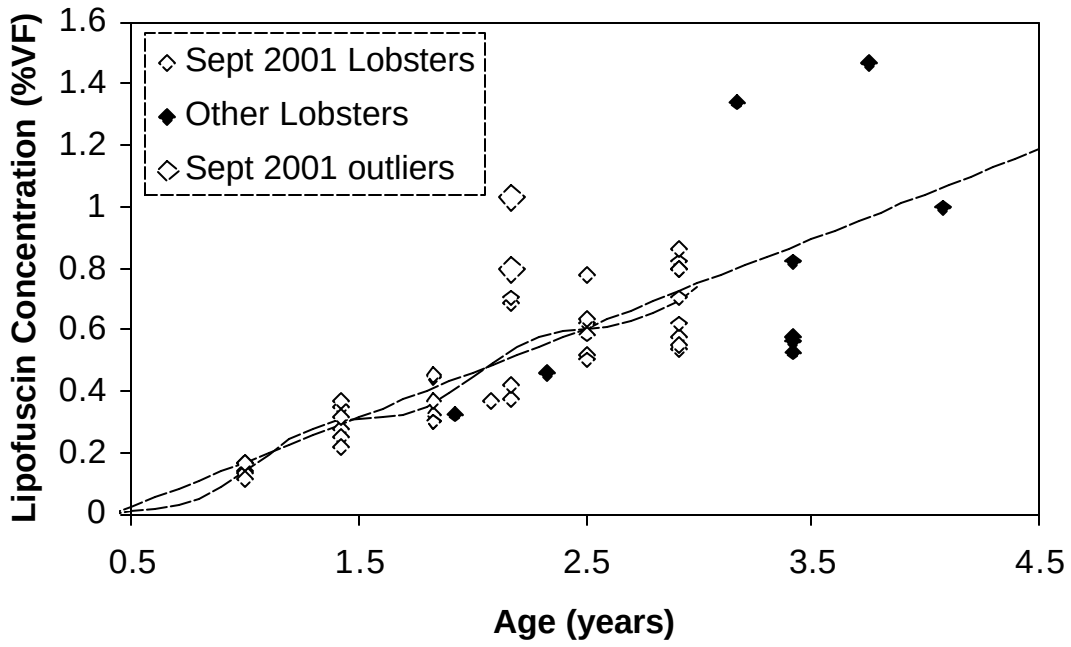

Figure 5. Relationship between age and neurolipofuscin concentration (average of both eyestalks) in *P. argus* reared in the laboratory at ambient sea temperatures. The linear regression of neurolipofuscin concentration on age is N = 0.290t - 0.119, where t = time in years, and N = %VF of neurolipofuscin (r^2 = 0.61). Seasonal oscillation in neurolipofuscin concentration was detected in lobsters collected in September of 2001 (open diamonds). Other animals (solid

diamonds) were excluded from this analysis because they were collected during other times of the year, as were two animals collected in September 2001 because they deviated from the seasonalized linear model by more than 2 standard deviations (large diamonds).

Growth and age

Spiny lobsters in our study grew rapidly and averaged 63 mm CL at one year. By year two, male and female growth rates differed (ANCOVA, using age as a covariate: $p < 0.05$), and their average size was 109 and 100 mm CL, respectively. Growth for both males and females appears to begin to asymptote (Fig. 6), but the limited age range of the lobsters examined herein probably does not fully reflect the decrease in growth rate for either males or females. The length-at-age data were well described by a von Bertalanffy growth model $L(t) = L_\infty*(1-\exp(-K*(t-t_0)))$, where asymptotic length $L_\infty = 182$ mm CL, K (growth constant) = 0.47 years^{-1}, and $t_0 = 0$ for males ($r^2 = 0.95$, n = 23, $p < 0.05$), and $L_\infty = 143$ mm CL, K = 0.62, and $t_0 = 0$ for females ($r^2 = 0.84$, n = 22, $p < 0.05$) (FiSAT II for growth models, Table Curve 2D for calculation of r^2 and p). These L_∞ values closely match the 95th percentile of the CL distribution of 3600 spiny lobsters captured from the Dry Tortugas National Park by divers between 1996 and 1998 for both males (180 mm CL) and females (145 mm CL) (Taylor, 1958; Bertelsen and Matthews, 2001). The Dry Tortugas National Park was closed to lobster fishing in 1973 and likely represents the most undisturbed population in Florida; hence, length data from there should generate a realistic estimate of L_∞.

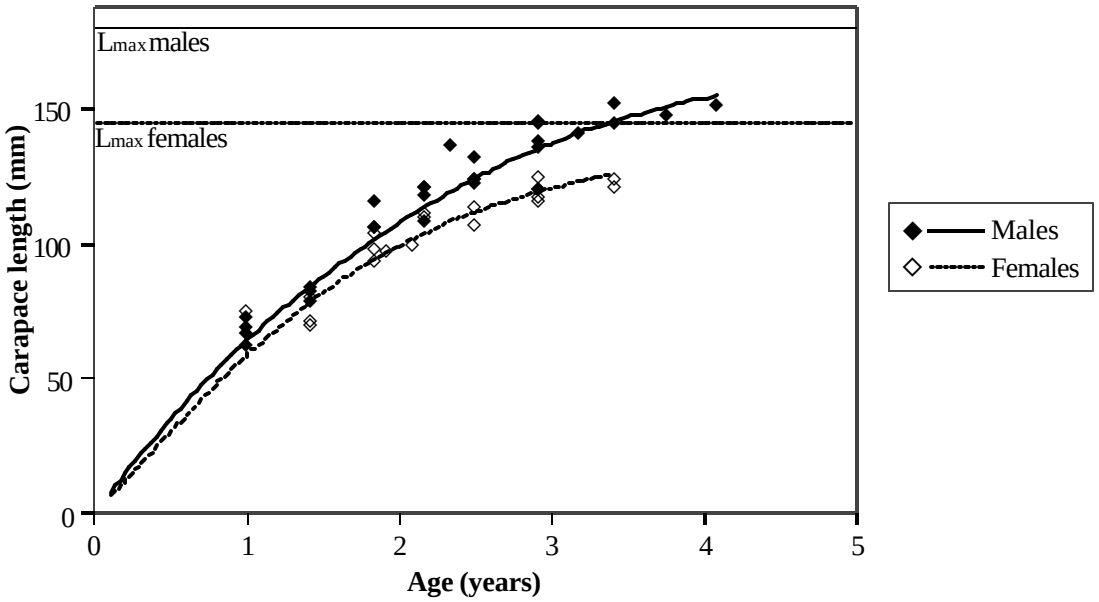

Figure 6. The length-at-age data for tank-reared *P. argus* was well described by a von Bertalanffy growth model $L(t) = L_\infty * (1-\exp(-K*(t-t_0)))$ with L_∞ = 182 mm CL, K = 0.47 years^{-1}, t_0 = 0 and r^2 = 0.95 for males, and L_∞ = 143 mm CL, K = 0.62, t_0 = 0 and r^2 = 0.84 for females. Horizontal reference lines indicate L_∞ for males and females.

DISCUSSION

We show that neurolipofuscin concentration, measured histologically in the central nervous system of laboratory-reared Caribbean spiny lobsters. *Panulirus argus*, is correlated with the chronological age of both males and females. These results suggest that the neurolipofuscin technique holds great promise for use in estimating age of wild-caught spiny lobsters.

Neurolipofuscin in the central nervous system of *P. argus* exhibits fluorescence, morphological and histochemical properties (Fig. 2), and age-related accumulation (Fig. 4) that is similar to that reported previously for other invertebrate and vertebrate species (*e.g.*, Sheehy, 1989; Sheehy and Wickins, 1994; Sheehy *et al*., 1996; Wahle *et al.*, 1996; Bluhm *et al*., 2001b; Lomovasky *et al*., 2002; Porta, 2002; Fonseca *et al*., 2005a). We observed that neurolipofuscin tended to cluster in *P. argus*, especially for lobsters with large quantities of neurolipofuscin. The clustering of neurolipofuscin is mentioned in previous studies of crustaceans including the freshwater crayfish, *Cherax cuspidatus* (Sheehy, 1989), *H. americanus* (Wahle *et al*., 1996), Antarctic amphipods, *W. obesa* and *Eurythenes gryllus* (Bluhm *et al*., 2001b), and Norway lobster, *Nephrops norvegicus* (Fonseca *et al*., 2005a), as well as in the hard clam, *Eurhomalea exalbida* (Lomovasky *et al*., 2002). We noted a previously unreported feature of neurolipofuscin granules in *P. argus* – the tendency for granules to aggregate along the outer margin of cluster A (Fig. 2). The cause and consequence of clustering and aggregations near the margin are uncertain.

As these marginal neurolipofuscin deposits were often the densest aggregations in a section, they were included in our measurements of lipofuscin according to our methodology. Although inclusion of the margins caused some histological sections to include less tissue, a comparison of our results with those obtained previously for *H. gammarus*, in which no marginal neurolipofuscin deposits were measured (Sheehy, 2002a) and which successfully aged animals to year-class using neurolipofuscin (Sheehy and Bannister, 2002), found no difference in bilateral variability (Fig. 4). However, evaluation of internal variability within cluster A indicated that we needed to sample more sections per individual to achieve the desired level of measurement precision, *i.e.*, 20 sections, than in several previous studies, which used only 5 to 10 sections

(Sheehy, 1989, 1990a, 1990b; Wahle *et al*., 1996; O'Donovan and Tully, 1996; Sheehy *et al*., 1998; Vila *et al*., 2000; Lomovasky *et al*., 2002). Most of these studies did not specifically evaluate the sample sizes needed to optimize precision, but DeKerros *et al.* (1995) found that in *H. americanus*, a random selection of 12 sections of the olfactory lobe lateral cluster provided a neurolipofuscin estimate within 10% of the true mean in 95% of cases. Our estimate of 20 sections per individual is, however, consistent with Sheehy's (2002a) recommendation of 20 to 30 sections.

Given the amount of time and effort required to determine neurolipofuscin concentration, it is important to evaluate sample size and potential outliers. For the range of ages that we examined in this study and those thought to occur in the fishery (Muller *et al.,* 1997), better estimates of the age structure of a group of lobsters would be obtained by sampling one eye from twice as many lobsters rather than collecting replicate samples to identify potential outliers. Outliers, though still present, would be less influential in a larger sample size. In addition, a larger sample size is beneficial for analyses used to identify cohorts (Sheehy *et al.*, 1998).

The lack of a statistical difference in neurolipofuscin concentrations between two eyestalks of a given animal does not reflect the bilateral variability associated with neurolipofuscin concentrations greater than 1.0 %VF. Even though there was some variation between the pairs of eyestalks of older spiny lobsters, it was within the range observed in other species (Fig. 4). Additional observations of older lobsters are required to evaluate if the bilateral variability observed in our oldest animals is a common feature.

Our results on neurolipofuscin accumulation and growth in laboratory-reared *P. argus* up to 4.1 years after settlement are similar to those reported previously for laboratory-reared individuals of the Western Australian rock lobster *Panulirus cygnus*, up to 6 years post-

settlement (Sheehy *et al.*, 1998). At an average environmental temperature of 26.6 ^{o}C, the approximate mean neurolipofuscin accumulation rate in *P. argus* was 0.29 %VF per year, while in *P. cygnus* it accumulated a little more slowly at a rate of 0.21 %VF per year at a lower average temperature of 20.6 ^{o}C (Sheehy *et al*., 1998). The observed age-specific variability in neurolipofuscin concentration, as indicated by r^2, was 0.61 using a linear regression and 0.81 using a seasonalized model for *P. argus,* and 0.80 using a linear regression for *P. cygnus* over a slightly greater age range (Sheehy *et al*., 1998). For both species, age-specific variability in size was relatively very low under laboratory conditions: r^2 = 0.95 for males and 0.84 for females of *P. argus*, and 0.99 for *P. cygnus*, sexes pooled (Sheehy, unpublished data). These results appear contrary to a number of previous studies in which neurolipofuscin variability was found to be markedly lower than size variability in known-age laboratory-reared or microtagged-and-recaptured individuals (*e.g.*, Sheehy, 1990a; Sheehy *et al.*, 1996; 1999; Belchier *et al*., 1998; Sheehy and Bannister, 2002). However, the present laboratory results require cautious interpretation when considering their implications for the use of either size or neurolipofuscin concentration for age prediction of *P. argus* from natural populations.

Our results suggest a slightly higher growth rate for *P. argus* than that estimated from most previous studies. The average growth rate for the first year was 63 mm CL/yr, and for the second year was 46 mm CL/yr in males and 37 mm CL/yr in females (Fig. 6). Previous studies using tag–recapture data from Florida estimated yearly growth in the first year at 22-66 mm CL/yr, with the vast majority being 22-40 mm CL/yr (Witham *et al*., 1968; Eldred *et al*., 1972; Davis and Dodrill, 1980; Davis, 1981; Hunt and Lyons, 1986; Forcucci *et al*., 1994). Growth estimates from tag-recapture studies, however, may underestimate growth due to growth inhibition caused by tagging injuries and possible interference with the molting process

(Forcucci *et al*., 1994). Additionally, spiny lobsters are most likely to lose their tags when they molt, so a disproportionate number of recaptures would be the animals that are molting at a slower rate. The higher growth rates in the laboratory might be attributed to the fact that our laboratory-reared animals had ready access food and did not experience any injuries.

We found relatively little individual variability in the growth rates of our laboratory-reared lobsters and, consequently, that their size was a good indicator of their age. However, as detailed in the Introduction, there is evidence that natural populations of spiny lobsters exhibit more variable growth rates due to a variety of causes and thus that size would be a poor indicator of age in the field. Reduced growth variability in laboratory populations compared with natural populations has been reported previously for *Homarus* spp. In individually-reared early benthic phase lobsters (< 3 years old), CL was more correlated with age than was neurolipofuscin concentration in both *H. gammarus* (r^2 = 46.8 and 90.3, respectively) and *H. americanus* (r^2 = 82.8 and 92.2, respectively) (O'Donovan and Tully, 1996; Wahle *et al*., 1996). The authors suggested that the unnatural availability of equivalent living spaces and the absence of social interactions resulted in similar-sized animals. In contrast, microtag recaptures of *H. gammarus*, ranging in age from 4.4 – 9.6 years of age, from various fisheries in the United Kingdom (Sheehy *et al.*, 1996; 1999; Sheehy and Bannister, 2002) indicated that, overall, age explains 80.2% of the variation in neurolipofuscin concentration and only 1.8% of the variation in CL in the field. Although a relatively strong association (r^2 = 0.78) between CL and age for microtag-recaptures of Norwegian *H. gammarus* was found for a selective sample of lobsters collected over two years (Uglem *et al.*, 2005), the relationship between size and age was considerably poorer if returns from a longer time period were analyzed (r^2 = 0.11, age: 3 - 13 years, hatched in 1988 - 1989, n = 877, unpublished data). This strong association between age and size in *H.*

gammarus in some cases might be a result of selective sampling and not necessarily an indication of a general pattern.

Another consideration in interpreting our results relates to the size of our 'experimental window' (Sheehy *et al.*, 1995) relative to the maximum size, neurolipofuscin concentration and age attainable by *P. argus*. Our experimental lobsters achieved maximum sizes of 152 mm CL for males and 124 mm CL for females. These are around 85% of the male and female maximum length (L_8) estimates of 182 mm CL and 143 mm CL, respectively. The greatest neurolipofuscin concentration and age of our experimental lobsters was 1.9 %VF and 4.1 years, respectively, which represent only around 25-30% of the 6-7% maximum possible neurolipofuscin values reported in other species (Sheehy *et al*., 1998; Sheehy, 2002a) and maximum age in *P. argus* (Kanciruk, 1980). The point of these comparisons is to illustrate that in Figure 6, we are observing almost the entire size range, while Figure 5 includes a only a very limited part of the potential range of neurolipofuscin values. Had we been able to compare age-specific variability across the full range of neurolipofuscin concentrations and ages that are possible in *P. argus*, we would almost certainly have seen higher correlations between neurolipofuscin concentration and age, as found in previous studies (*e.g.*, Sheehy, 1990a, Belchier *et al*., 1998).

There is a significant relationship ($r^2 = 0.81$, $p < 0.001$) between the mean neurolipofuscin concentration of a cohort and the standard deviation of the neurolipofuscin concentrations of individuals comprising that cohort. This relationship does not differ significantly across a wide range of decapod species (Sheehy and Bannister, 2002) and our *P. argus* data are no exception. Based on this conserved relationship, it is possible to estimate the precision achievable for neurolipofuscin-based age estimates for *P. argus* in Florida. The 95% prediction interval for an age estimate of 1 year, for example, is 0.5-2.0 years. For an age

estimate of 4 years, it is 3-6.5 years. Detection of annual cohorts in neurolipofuscin concentration frequency distributions, as has been achieved for *P. cygnus* (Sheehy *et al*., 1998), *W. obesa* (Bluhm *et al*., 2001), *N. antarcticus* (Bluhm and Brey, 2001), and *O. oratoria* (Kodama *et al*., 2005), remains to be tested for wild populations of *P. argus*.

The seasonal oscillation of neurolipofuscin accumulation (Figure 5) appears to describe the majority of the variation associated with age in this study. Similarly, there appears to be a seasonal component in neurolipofuscin accumulation rates for other crustaceans reared under ambient thermal conditions (Sheehy *et al*., 1994; Vila *et al*., 2000; Tully *et al*., 2000; Sheehy, 2002b). In our laboratory study, the influence of seasonality, based primarily on temperature, was initially obfuscated because of the inclusion of lobsters collected at different times of the year. Despite a more tropical distribution of most *P. argus* in the Caribbean, in Florida, spiny lobsters are subject to more temperate conditions and appear to have neurolipofuscin accumulation patterns similar to other temperate species. This may not be the case for *P. argus* from more tropical climates and we might expect to see latitudinal gradients in the neurolipofuscin accumulation patterns as has been observed in *H. gammarus* (Tully *et al*., 2000; Sheehy, 2002). Since the temperature of our tanks was maintained at levels found in Florida Keys waters, the neurolipofuscin deposition rates observed in this study should approximate those of wild lobsters in Florida and may be useful for calibrating the age of potential cohorts.

It is possible to estimate the maximum potential lifespan of *P. argus* from the present data. Recent findings suggest a strong inverse relationship ($r^2 = 0.92$, $p < 0.0001$) between mean neurolipofuscin accumulation rate and longevity in arthropods (Sheehy, 2002b; Fonseca *et al*., 2005b). Based on this relationship and our average neurolipofuscin accumulation rate (0.29 %VF), the maximum lifespan of *P. argus* in Florida can be calculated at roughly 20 years. This

estimate is based on accounts from other species whose life span and maximum neurolipofuscin, approximately 6-7% VF, appear correlated (Sheehy *et al*., 1998; Sheehy, 2002a). Perhaps fortuitously, this life expectancy is identical to a previous length-based estimate (Kanciruk, 1980).

Our future aim is to use the findings from this study as a guide for neurolipofuscin-based age estimation and identification of potential cohorts for lobsters in the Florida Keys and the Dry Tortugas. In so doing, we should be able to more accurately calculate the growth rate, age at maturation, and mortality rate of Caribbean spiny lobsters. These types of data are fundamental for understanding the basic ecology of lobsters and developing population analyses. These basic population parameters are essential for the proper management and creation of regulations to ensure a sustainable fishery for this ecologically important and commercially valuable species.

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