

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

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Abstract. Accurate age estimates for *Panulirus argus*, the commercially important Caribbean spiny lobster, would greatly enhance life history and population analyses. Most age approximations of *P. argus* are based on size and growth data, but size is generally considered a poor proxy for age of crustaceans in the field because of numerous environmental, density-dependent, and fishery-related factors. An established technique for aging crustaceans, employing histologically determined lipofuscin content in the nervous system, was investigated using known-age lobsters reared in the laboratory at ambient temperatures. We verified the presence of lipofuscin in eyestalk neural tissue by using autofluorescence and Sudan black staining and described its distribution in cell cluster A of the hemiellipsoid body. Neurolipofuscin accumulated with age; the overall trend was linear with indications of seasonal oscillation, whereas growth began to approach an asymptote after 3 years. Differences in the neurolipofuscin concentrations in the two eyestalks from the same animal were statistically insignificant. There was also no difference in the neurolipofuscin concentrations of males and females of the same age. The present data suggest a maximum potential lifespan for *P. argus* of about 20 years. These results also suggest that the neurolipofuscin technique will be valuable for estimating age of wild-caught specimens of *P. argus*.

Introduction

The fishery for Caribbean spiny lobsters, *Panulirus argus* (Latrielle, 1804), is currently the second most valuable commercial fishery in Florida, annually worth on average US\$35 million (Florida Fish and Wildlife Conservation Commission, unpubl. 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 for estimating the 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 individuals of *P. argus* would be a useful tool for understanding the basic biology of this species and for 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 analyses of size-frequency histograms (Eldred *et al.*, 1972; Munro, 1983). Size, however, is generally considered to be a poor measure of the 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 rates 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

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Abbreviation: %VF, percent volume fraction.

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), 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. The animals used as bait may starve when they are confined in traps for extended periods of time, which can result in a reduction in growth rate or in death (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). But spiny lobsters and most other crustaceans carry no permanent hard structures from which age can be determined, because they repeatedly shed their exoskeleton to grow larger. An alternative technique for aging crustaceans has been developed on the basis of the concentration of the age pigment, lipofuscin, in soft tissues. The concentration of lipofuscin in the central nervous system, hereafter referred to as neurolipofuscin, has been shown to be a good index of age in several crustaceans, including two 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); an Antarctic shrimp, *Notocrangon antarcticus* (Bluhm and Brey, 2001); an Antarctic amphipod, *Waldeckia obesa* (Bluhm *et al.*, 2001a); and a 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 are apparently resistant to lysosomal recycling (Eldred and Lasky, 1993; Jolly *et al.*, 2002; Szwedka *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 postmitotic tissues. It is the predictability of this accumulation that makes lipofuscin a unique and outstanding biomarker of physiological aging (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 in-

terplay 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. The rate of neurolipofuscin accumulation 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 central nervous system (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 individuals of *Homarus 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 temperature, where temperature may be the environmental average 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 different sizes but identical chronological age (Sheehy, 1990a; Wahle *et al.*, 1996; O'Donovan and Tully, 1996) and in adult insects of different 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 more highly correlated with age than is size (Sheehy, 1990a; Sheehy *et al.*, 1996, 1998; Belchier *et al.*, 1998; Bluhm *et al.*, 2001a; Bluhm and Brey, 2001; Kodama *et al.*, 2005).

Localization of neurolipofuscin within various regions of the brain has been considered to reflect a higher level of cellular metabolic activity in those areas (Friede, 1962). In many decapod 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

each animal has two eyestalks from which replicate measurements can be made.

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 the lipofuscin associated with the hemiellipsoid bodies of the eyestalks. Second, we attempted to streamline the existing methodology for quantifying neurolipofuscin to reflect the distribution and abundance of neurolipofuscin in *P. argus*. Third, we examined the pattern of neurolipofuscin accumulation in known-age animals up to 4 years of age under thermal conditions approximating those in the field, assessing both bilateral variation in the eyestalks and sexual differences. The ability to determine the ages of *P. argus* has many implications for fisheries management. Future studies on wild-caught individuals of *P. argus* will allow us to estimate growth and develop age-structured population analyses, which will in turn allow us to better describe spiny lobster population dynamics.

Materials and Methods

Animal rearing

We determined the rate of neurolipofuscin accumulation in spiny lobsters of known age. 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 to 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 about 6 mm in carapace length (CL); however, *P. argus* has multiple planktonic phyllosoma stages lasting a total of about 5 to 9 months (Lyons, 1980; Farmer *et al.*, 1989; Goldstein *et al.*, 2006). Each group of pueruli was communally raised in 1500-liter aquaria and transferred to 9500-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 to 5 months so that we could collect data on neurolipofuscin concentration-at-age for animals aged 12 to 35 months. A total of 39 animals from this group were analyzed; 12 additional animals up to 49 months old from groups collected before September 2001 were also analyzed.

Water in the aquaria was pumped from nearby Florida Bay through a sand filter and partially recirculated through an 18- or 25-W ultraviolet sterilizer to prevent transmission of a naturally occurring lethal 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 the National Oceanic and Atmospheric Administration's National Data Buoy Center (NDBC) C-Man station located at Long Key, Florida (24°50'N, 80°5'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 °C (average = 26.4 °C) in Florida Bay, and from 23 to 30 °C (average = 26.7 °C) at Sombrero reef (Fig. 1).

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 be provided only 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 from 51 laboratory-reared animals was prepared for histology; however, two samples did not have replicates because 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

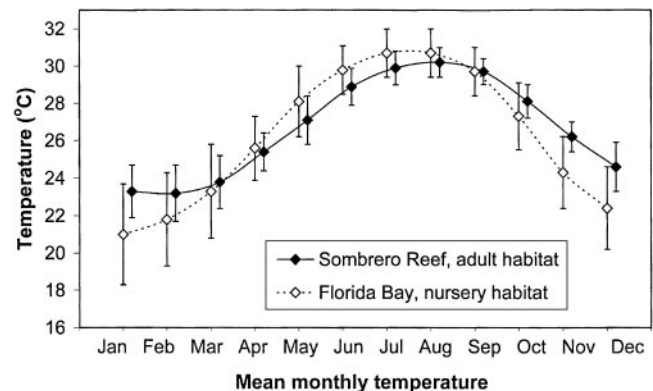


Figure 1. Mean monthly water temperature from 1999 to 2004 (± 1 SEM) at lobster nursery habitat in Florida Bay (dotted line and open diamonds) and adult habitat at Sombrero Reef (solid line and solid diamonds). From <http://www.ndbc.noaa.gov/Maps/Florida.shtml>. Water temperatures in our laboratory aquaria were adjusted as needed to match these temperatures within 1 °C.

refrigeration for 1 h at -10°C prior to eyestalk ablation. Both eyestalks were removed from animals, immediately placed into 4% paraformaldehyde for 48 h, transferred into 0.2 mol l^{-1} phosphate buffer plus azide, and then transported to Georgia State University for processing.

The internal neural tissue of the eyestalk was dissected from the cuticle and dehydrated in increasing concentrations of ethanol, cleared in xylene, and embedded in paraffin. Eyestalk tissue was sectioned at $6\text{ }\mu\text{m}$ 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, several dozen of which 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 ipsilateral hemiellipsoid body. 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 blue light at 450–490 nm, and emission was detected at 515–585 nm by using a Zeiss Axioplan fluorescence microscope with a $100\times$ 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 by using a Carl Zeiss ZVS-3C75DE camera fitted to the microscope. Field width of captured images was $125\text{ }\mu\text{m}$. 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, ver. 3.0) software and saved as 800- \times 600-resolution bitmap files.

Neurolipofuscin quantification

Images were analyzed using Adobe Photoshop 6.0. Color images were converted to gray 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 by 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, judging from 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 thresholding 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 showed as much as 50% of their area having 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

We explored strategies for increasing the precision with which neurolipofuscin can be measured, and because preparing and analyzing tissue for neurolipofuscin quantification is labor-intensive, for reducing the effort involved. Our effort-reduction strategy involved varying the number of tissue sections used for image analysis. The strategy addressing precision explored the use of one eye rather than both eyes. We evaluated whether 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 samples to be replicated or neurolipofuscin concentrations to be verified, but it does allow twice as many neurolipofuscin-based estimates of age to be determined from the same effort in the laboratory.

To evaluate the number of sections necessary to precisely measure neurolipofuscin, random subsamples of 5, 10, 15, and 20 sections were 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. We used 35 single eyes from wild animals in the Florida Keys to complete this analysis.

To evaluate the efficacy of using paired eyes or single eyes, we used a Monte Carlo simulation on the data from all 49 pairs of eyes analyzed in this paper plus 86 pairs of eyes from wild animals collected near the Dry Tortugas National Park ($24^{\circ}38'\text{N}$, $82^{\circ}51'\text{W}$, 70 miles west of Key West, FL). In the simulation, 20, 30, 40, and 50 randomized pairs of eyes were subsampled by computer and then analyzed *via* the paired-eye strategy. Randomized samples of 40, 60, 80, and 100 single eyes were also subsampled 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 were included in the analysis.

Validation of methods

We confirmed the lipid content of the autofluorescing granules that we considered to be neurolipofuscin by using Sudan black stain for lipids, following the standard methods (Sheehy, 1989; Sheehy and Wickins, 1994; Sheehy *et al.*, 1996). Selected sections of fluorescing neurolipofuscin in cluster A were photographed and 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 86 eyes imaged and analyzed at Georgia State University. We found no significant differences ($P > 0.05$) between the findings of the two laboratories in the bilateral variability of eyestalk neurolipofuscin concentration, as assessed by a multivariate ANCOVA with size as the covariate; thus, this validation showed the consistency of the results of our methods with those of 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, c) stained positive for Sudan black (Fig. 2b, d), indicating their lipid content. The granules were also resistant to nonpolar solvent (xylene) extraction during histological processing, possibly indicating that

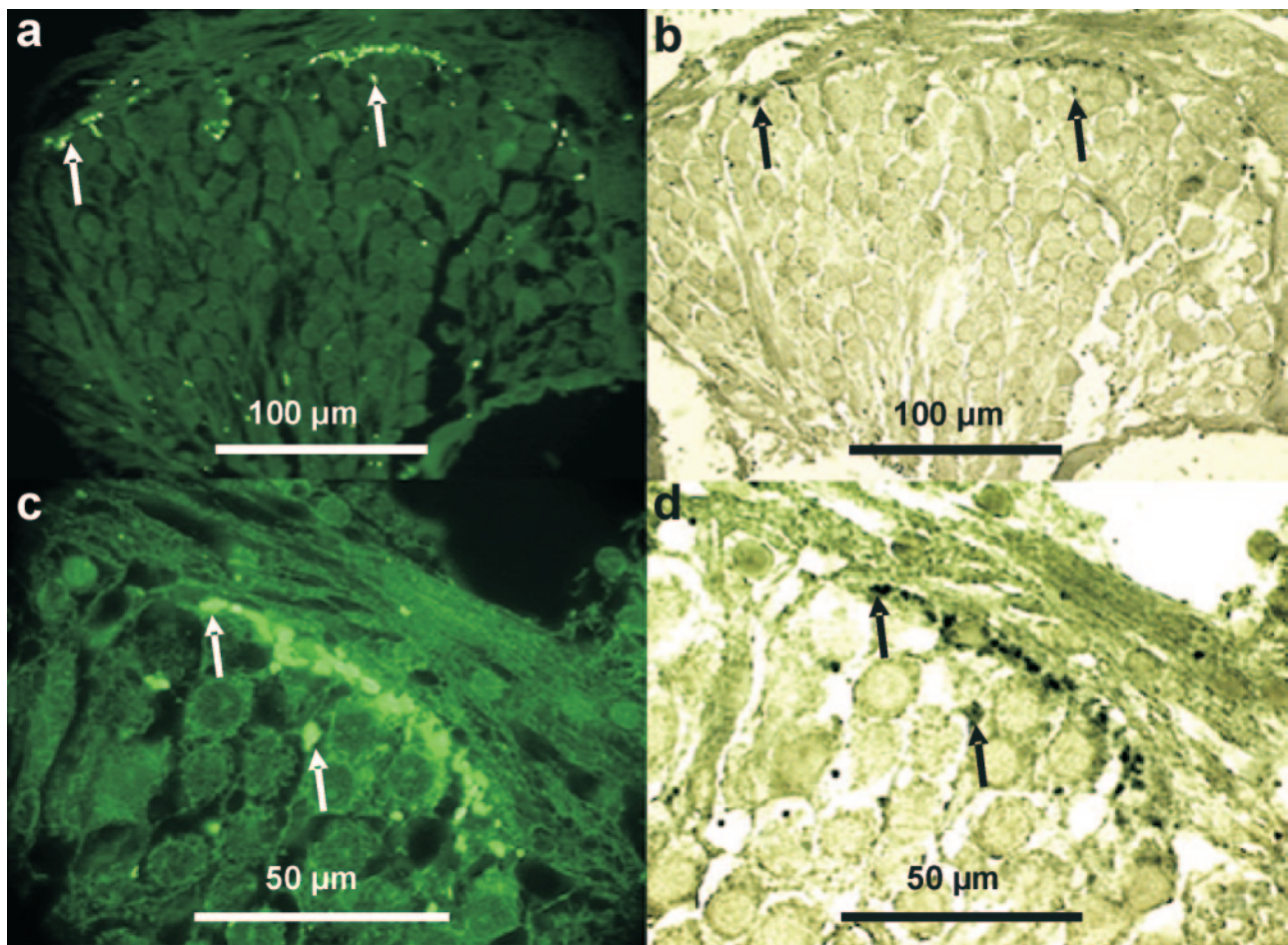


Figure 2. Cross-sectional images of the proximal ganglion of the eyestalk. Autofluorescence (a and c) and Sudan black staining (b and d) of a single section of cell cluster A of the eyestalk hemiellipsoid body in *Panulirus argus*. Arrows indicate selected granules exhibiting both autofluorescence and Sudan black staining, confirming their identity as neurolipofuscin.

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 unevenly distributed throughout cluster A—it 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).

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 having a percent volume fraction (%VF) greater than 1.0 (*i.e.*, in older animals), although such values were rare in our population. Three of the oldest animals (all male) with ages greater than 35 months had highly variable concentrations of neurolipofuscin between eyestalks and

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 *Homarus gammarus* (coefficient of variation = 61.29 for *P. argus* and 69.06 for *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*.

The analysis evaluating whether fewer paired eyes or more single eyes improved precision of neurolipofuscin measurements for a hypothetical population found that larger samples of single eyes were less variable than smaller paired eye samples. Although the mean %VF of neurolipofuscin was lower in the population generated from paired eyes because those with high variability were discarded and the majority of the highly variable eyes had high concentrations of neurolipofuscin (Fig. 3), the difference in mean %VF of neurolipofuscin between the two strategies amounted to less than 2 months of neurolipofuscin accumulation. However, the standard deviation decreased as the sample size increased and was lower in the population generated from single eyes (Table 1).

The analysis evaluating the number of sections necessary to achieve precise measurements of neurolipofuscin demonstrated that a random subsample 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 average neurolipofuscin concentration in *P. argus* eyestalks increased linearly with age across years, with indications of seasonal variation in neurolipofuscin accumulation rate within years. The neurolipofuscin value for each animal is an average value of the two eyes, except for cases in which only one eye was available. There was no difference in neurolipofuscin concentration (ANCOVA with age as the covariate, $P > 0.05$) related to the sex of these lobsters, so the sexes were pooled in subsequent analyses. For lobsters raised in the laboratory under thermal conditions similar to those in the Florida Keys, the annual average neurolipofuscin accumulation rate was 0.29%VF as indicated by 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. Examination of the seasonal

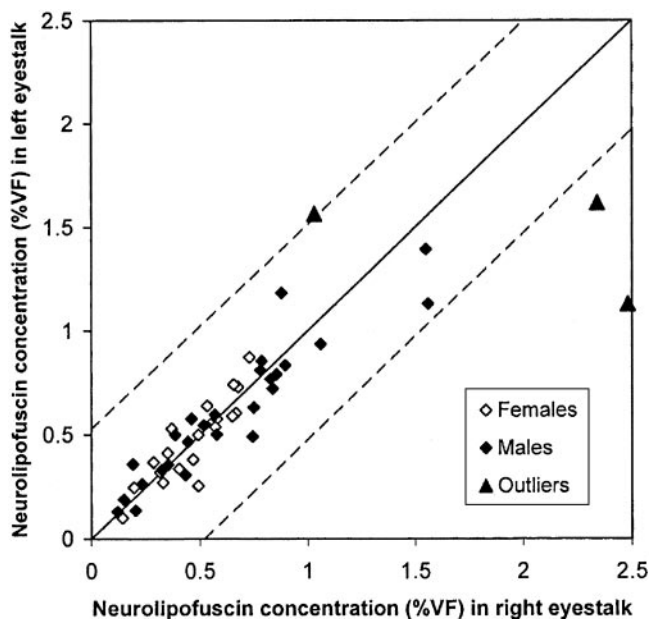


Figure 3. Neurolipofuscin concentrations in left and right eyestalks were strongly correlated (Pearson correlation: $n = 49$, $r = 0.85$, $P < 0.0001$). There was no significant bilateral difference in the neurolipofuscin concentrations (Wilcoxon signed rank test: $n = 49$; $P = 0.434$). Solid reference line has a slope of 1. Dashed lines define the 95% prediction interval. Outliers (triangles) defined as points deviating by 2 standard errors from the expected slope of 1; these were not included in further analyses.

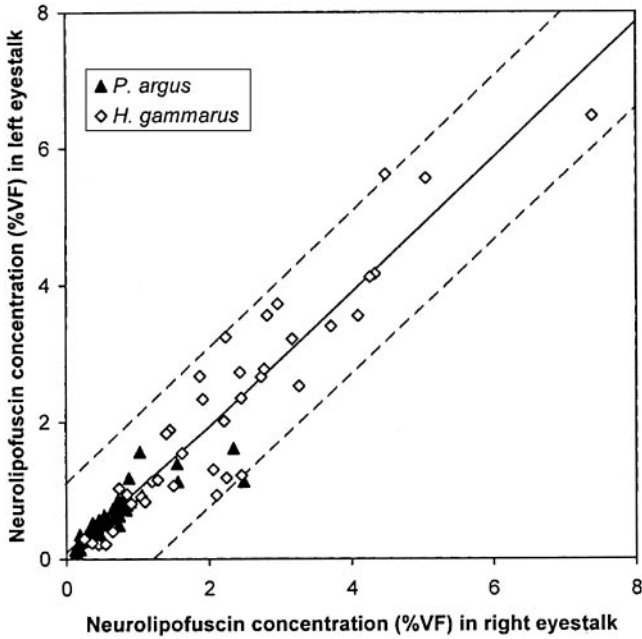


Figure 4. Bilateral comparison of neurolipofuscin concentrations in paired eyestalks in *Panulirus argus* (this study) and in *Homarus gammarus* (from Sheehy, 2002a). Bilateral variability is similar for the two species, but the range of neurolipofuscin concentrations (physiological ages) in the *P.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

patterns of neurolipofuscin accumulation was limited to lobsters collected in September of 2001 so as to avoid effects from the different collection dates of the various batches of lobster. This eliminated all lobsters over age 35 months and several younger lobsters from the seasonal

Table 1

Monte Carlo simulation of the effects of sample size and replication on means and standard deviations of lobster neurolipofuscin concentration estimates from single eyes and paired eyes: standard deviation decreases as the sample size increases and is lower in the samples generated from single eyes

Sample type	Number of samples	Mean %VF	SD %VF
Single eyes	40	0.5256	0.0507
	60	0.5263	0.0401
	80	0.5247	0.0320
	100	0.5288	0.0292
Paired eyes*	20	0.5002	0.0614
	30	0.5019	0.0529
	40	0.5009	0.0430
	50	0.5064	0.0404

* Values differing by more than 2 standard deviations removed.

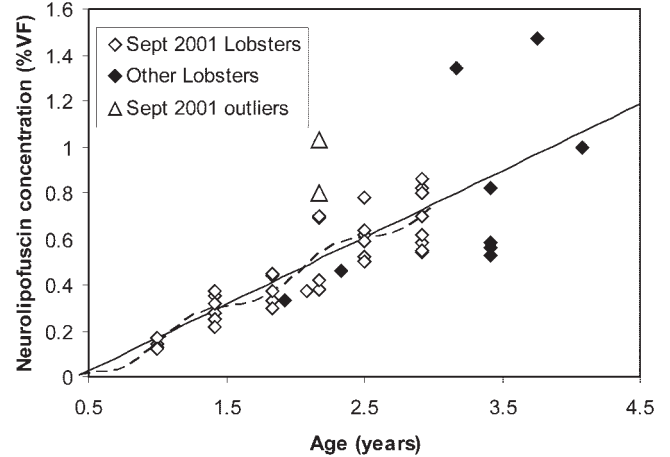


Figure 5. Relationship between age and neurolipofuscin concentration (average of both eyestalks) in *Panulirus argus* reared in the laboratory at ambient sea temperatures. The linear regression (solid line) of neurolipofuscin concentration on age is $N = 0.290t - 0.119$, where t = time in years, and N = percent volume fraction (%VF) of neurolipofuscin ($r^2 = 0.61$). Seasonal oscillation in neurolipofuscin concentration was detected in lobsters collected in September of 2001 (open diamonds) by fitting Sheehy's (2002b) seasonalized linear model (dashed line). Other animals (solid diamonds) were excluded from the seasonalized 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 triangles).

analysis. We used the seasonalized linear model of neurolipofuscin concentration on age developed by Sheehy (2002b):

$$N = b(t - t_0) + bc(2\pi)^{-1}\sin 2\pi(t - t_s) + bc(2\pi)^{-1}\sin 2\pi(t_0 - t_s)$$

where N = neurolipofuscin concentration (%VF) at age t (years), $b = 0.297$ (the average neurolipofuscin accumulation rate), $c = 0.796$ (the amplitude of seasonal oscillation), $t_s = 1.036$ (the fraction of the year that has elapsed at the point when neurolipofuscin accumulation rate is the highest), and $t_0 = 0.511$ (the age, in years, at onset of resolvable neurolipofuscin accumulation) ($n = 37$, $r^2 = 0.81$, $P < 0.0001$) (Fig. 5). The overall slope of this model (b) is consistent with the linear model applied to the larger database, which included older lobsters. Two statistical outliers were excluded from the model fit because they deviated from the model by more than 2 standard errors.

Growth and age

Spiny lobsters in our study grew rapidly and averaged 63 mm in carapace length (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 appeared to begin to approach an asymptote (Fig. 6),

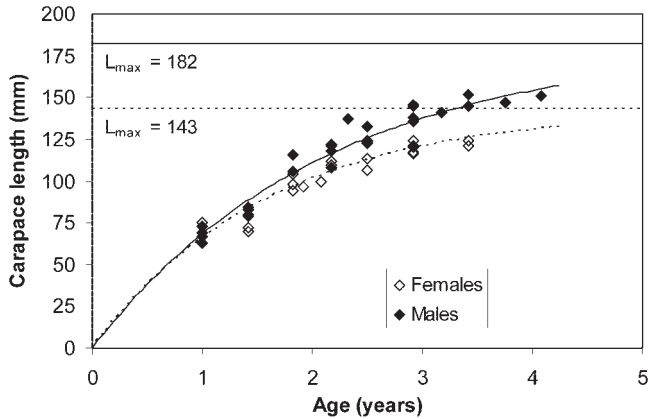


Figure 6. The length-at-age data for tank-reared *Panulirus argus* were well described by a von Bertalanffy growth model $L(t) = L_{\max} * (1 - \exp(-K * (t - t_0)))$ with $L_{\max} = 182$ mm CL, $K = 0.47$ years⁻¹, $t_0 = 0$, and $r^2 = 0.95$ for males; and $L_{\max} = 143$ mm CL, $K = 0.62$, $t_0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

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 $L(t) = L_{\max}(1 - \exp(-K(t - t_0)))$, where asymptotic length $L_{\max} = 182$ mm CL, K (growth constant) = 0.47 years⁻¹, and $t_0 = 0$ for males ($r^2 = 0.95$, $n = 27$, $P < 0.05$), and $L_{\max} = 143$ mm CL, $K = 0.62$, and $t_0 = 0$ for females ($r^2 = 0.84$, $n = 21$, $P < 0.05$) (FiSAT II for growth models, Table Curve 2D for calculation of r^2 and P). These L_{\max} 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) (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_{\max} .

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. 5) that are similar to those 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 in lobsters with higher quantities of neurolipofuscin. The clustering of neurolipofuscin is mentioned in previous studies of crustaceans, including a freshwater crayfish, *Cherax cuspidatus* (Sheehy, 1989); *Homarus americanus* (Wahle et al., 1996); two Antarctic amphipods, *Waldeckia obesa* and *Eurythenes gryllus* (Bluhm et al., 2001b); and the Norway lobster, *Nephrops norvegicus* (Fonseca et al., 2005a); as well as in a species of 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.

Because these marginal neurolipofuscin deposits were often the densest aggregations in a section, they were included in our measurements of neurolipofuscin according to our methods. Although including the margins caused some histological sections to contain less tissue, a comparison of our results with those obtained previously for *Homarus 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, after evaluating the internal variability within cluster A, we realized 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 per individual (Sheehy, 1989, 1990a, b; Wahle et al., 1996; O'Donovan and Tully, 1996; Sheehy et al., 1998; Vila et al., 2000; Lomovasky et al., 2002; Kodama et al., 2005). Most of these studies did not specifically evaluate the sample sizes needed to optimize precision, but de Kerros 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 consistent with Sheehy's (2002a) recommendation of 20 to 30 sections. It is, however, noteworthy that Kodama et al. (2006) recently tracked well-resolved cohorts of *Oratosquilla oratoria* in a seasonal series of frequency distributions of neurolipofuscin concentration by using as few as 5 equidistant sections of the protocerebral bridge cell mass per individual, thereby increasing the achievable sample size several-fold.

Considering the 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 than by collecting replicate samples to identify those lobsters with bilateral differences for the purpose of defining outliers. The erroneous samples, 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 having a percent volume fraction (%VF) greater than 1.0. 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 whether the bilateral variability observed in our oldest animals is a common feature.

Our results on neurolipofuscin accumulation and the growth of laboratory-reared individuals of *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 °C, the approximate mean rate of neurolipofuscin accumulation in *P. argus* was 0.29%VF per year, whereas in *P. cygnus*, the accumulation rate was a little slower, 0.21%VF per year, at a lower average temperature of 20.6 °C (Sheehy *et al.*, 1998). The observed age-specific variability in neurolipofuscin concentration, as indicated by r^2 , was 0.61 when obtained by using a linear regression and was 0.81 when obtained by using a seasonalized model for *P. argus* (outliers removed); the variability was 0.80 when a linear regression was used for *P. cygnus* (all data used) 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, unpubl. data). These results appear contrary to those of 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 using either size or neurolipofuscin concentration to predict ages of individuals 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 of carapace length (CL) per year for both sexes, and for the second year was 46 mm/y in males and 37 mm/y in females (Fig. 6). Previous studies using tag-recapture data from Florida es-

timated yearly growth in the first year at 22–66 mm/y, with the vast majority being 22–40 mm/y (Witham *et al.*, 1968; Eldred *et al.*, 1972; Davis and Dodrill, 1980; Davis, 1981; Hunt and Lyons, 1986; Forcucci *et al.*, 1994; Sharp *et al.*, 2000). Growth estimates from tag-recapture studies, however, may underestimate growth because of the growth inhibition caused by tagging injuries and the possible interference of tags 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 to food and did not experience any injuries.

We found relatively little individual variability in the growth rates of our laboratory-reared lobsters and, consequently, found 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 have more variable growth rates owing to a variety of causes, and thus that size would be a poor indicator of age in the field. Less growth variability in laboratory populations than in 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* ($r^2 = 90.3\%$ and 46.8%, respectively) (O'Donovan and Tully, 1996) and *H. americanus* ($r^2 = 92.2\%$ and 82.8%, respectively) (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 to 9.6 years, from various fisheries in the United Kingdom 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 (Sheehy and Bannister, 2002). 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 2 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, $n = 877$; Uglem *et al.*, unpubl. data). Thus, the strong association between age and size in *H. gammarus* in this case appears to be the 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 about 85% of the maximum length (L_{\max}) estimates of 182 mm CL and 143

mm CL for male and female, respectively. The greatest neurolipofuscin concentration and age of our experimental lobsters was 1.9%VF and 4.1 years, respectively, which represent only about 25%–30% of the 6%–7% maximum possible neurolipofuscin values reported in other species (Sheehy *et al.*, 1998; Sheehy, 2002a) and the estimated maximum age of 20 years in *P. argus* (this study and Kanciruk, 1980). The point of these comparisons is to illustrate that in Figure 6 we are observing almost the entire size range, whereas Figure 5 includes only a 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 the higher correlations between neurolipofuscin concentration and age 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 composing that cohort. This relationship does not differ significantly across a wide range of decapod species (Sheehy and Bannister, 2002), and our data for *P. argus* are no exception. On the basis of this conserved relationship, it is possible to estimate the precision achievable for neurolipofuscin-based age estimates for individual *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 from frequency distributions of neurolipofuscin concentration, as has been achieved for *P. cygnus* (Sheehy *et al.*, 1998), *W. obesa* (Bluhm *et al.*, 2001a), *N. antarcticus* (Bluhm and Brey, 2001), and *O. oratoria* (Kodama *et al.*, 2005, 2006), remains to be tested for wild populations of *P. argus*.

The seasonal oscillation of neurolipofuscin accumulation (Fig. 5) appears to describe the majority of the variation associated with age in this study. The elimination of two statistical outliers at 2.3 years of age greatly reduced the amplitude of the seasonalized oscillation. Although these data points are bilaterally consistent and their variability is similar both to that observed for our lobsters older than 3 years and to that for other species (Sheehy and Bannister, 2002), we chose not to include the outliers in our seasonal oscillation analysis, because of their disproportionate influence on the model. Additional observations are required to better evaluate the true magnitude of seasonal neurolipofuscin accumulation and estimate more robust confidence limits.

In our laboratory study, the influence of seasonality, based principally on temperature, was initially obfuscated because of the inclusion of lobsters collected at different times of the year. Despite the more tropical distribution of most *P. argus* populations in the Caribbean, in Florida,

spiny lobsters are still subject to seasonal temperature variation and appear to have neurolipofuscin accumulation patterns similar to those of temperate species reared under ambient thermal conditions (Sheehy *et al.*, 1994; Vila *et al.*, 2000; Tully *et al.*, 2000; Sheehy, 2002b). Kodama *et al.* (2006) demonstrated that seasonal oscillation in neurolipofuscin accumulation rate also occurs in nature. Amplitudes of seasonal oscillation reported in these previous studies ranged from 0.71 to 1.3. Also, we might expect to see latitudinal gradients in the neurolipofuscin accumulation patterns as have been observed in *H. gammarus* (Tully *et al.*, 2000; Sheehy, 2002b; Sheehy and Bannister, 2002). Because the temperature of our tanks was maintained at levels found in waters of the Florida Keys, the neurolipofuscin deposition rates observed in this study should provide a guide to neurolipofuscin accumulation rates in wild lobsters in Florida. Confidence in the accuracy of age determinations of wild individuals of *P. argus* will depend heavily on future neurolipofuscin-concentration frequency analyses.

It is possible to estimate the maximum potential lifespan of *P. argus* from the present data. Recent findings for a range of species, including lobsters, suggest a strong inverse relationship ($r^2 = 0.92$, $P < 0.0001$) between mean rate of neurolipofuscin accumulation and longevity in arthropods (Sheehy, 2002b; Fonseca *et al.*, 2005b). On the basis of this relationship and our average neurolipofuscin accumulation rate (0.29%VF), the maximum potential lifespan of *P. argus* in Florida can be estimated at roughly 20 years. Perhaps fortuitously, this life expectancy is identical to a previous length-based estimate (Kanciruk, 1980).

Our 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 properly managing and creating regulations that will ensure a sustainable fishery for this ecologically important and commercially valuable species.

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