Abstract—The blue crab (*Callinectes sapidus*) plays an important economic and ecological role in estuaries and coastal habitats from the Gulf of Mexico to the east coast of North America, but demographic assessments are limited by length-based methods. We applied an alternative aging method using biochemical measures of metabolic byproducts (lipofuscins) sequestered in the neural tissue of eyestalks to examine population age structure. From Chesapeake Bay, subsamples of animals collected from the 1998–99 (n=769) and 1999–2000 (n=367) winter dredge surveys were collected and lipofuscin was measured. Modal analysis of the lipofuscin index provided separation into three modes, whereas carapace-width data collected among the same individuals showed two broad modes. Lipofuscin modal analysis indicated that most adults (carapace width >120 mm) were <2 years old. The results indicate that use of extractable lipofuscin can provide a more accurate and better resolved estimation of demographic structure of blue crab populations in the field than size alone.

Demographic assessment of the blue crab (*Callinectes sapidus*) in Chesapeake Bay using extractable lipofuscins as age markers*

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In fisheries management, age-structured models are the most common method for estimating optimal yields and determining the effect of fishing on population dynamics (Gulland, 1983). For some species of commercial marine fish, age can be determined by using otoliths or other hard parts (Secor et al., 1995). This is not possible for crustaceans, including the blue crab (*Callinectus sapidus*), which periodically molts its calcareous exoskeleton to accommodate future growth and thereby abandons any external evidence of age or previous size. As a consequence, modal analysis of length-frequency data has often been used as an alternative to direct aging methods (e.g. Rothschild et al., 1992). Unfortunately, this approach has proven difficult to validate because 1) growth is characterized by strong interannual and seasonal variability and 2) the spawning season is protracted, which leads to a wide, and sometimes multimodal distribution of sizes per year class (Prager et al., 1990; Ju et al., 2001). The critical need for understanding the demographic structure of crab populations led us to investigate an alternative method for age determination. Age pigments known as lipofuscins accumulate as stable mixtures in postmitotic tissue as a consequence of peroxidation reactions during normal metabolism (see review by Gutteridge, 1987). These biochemical measures have been applied to determine age in crustaceans (Ettershank and George, 1984; Belchier et al., 1994; Sheehy et al., 1995; Sheehy et al., 1996;

Wahle et al., 1996). Recently, a modified approach that relies on the concentration of these products normalized to tissue protein has been developed and validated for the blue crab (Ju et al., 1999).

We hypothesize that lipofuscin can provide a more robust measure of age than current size-based measures. For example, variance in size among individuals within the same year class, and declining growth rate with increasing age, can result in significant overlap of sizes among adjacent age classes and can reduce the accuracy of age determinations with length-frequency analysis. Additionally, the blue crab's natural longevity is an important factor and one still disputed by scientists because of the lack of reliable long-term tag returns and absence of age information. If blue crabs reach maturity quickly and live to age three to four as has been suggested (Ju et al., 1999; Van Engel et al., 1958, 1999; Helser and Kahn, 1999), then higher fishing rates may be sustained than those for crabs that mature more slowly and live to age six or older (Rugolo et al., 1998; Miller, 2001). In the present study, we conducted a large-scale application of the lipofuscin aging method using subsamples from fishery-independent winter dredge surveys (1998–99 and 1999–2000). The goal of this study was

to assess the age structure of the blue crab population in the Chesapeake Bay through measurement of lipofuscin and to compare the demographics with those determined by the more traditional size-based approach.

Materials and methods

Sample collections

Winter bottom-dredge surveys (WDS) are conducted annually in the Chesapeake Bay system to monitor blue crab recruitment and size. Surveys were conducted according to a stratified random design, and sampling intensity was apportioned according to depth and site area (for details, see Volstad et al., 2000). Although collections target all sizes of crabs, juvenile crabs (<15 mm carapace width [CW]) are not fully susceptible to the dredge gear and are underestimated in abundance (Rothschild et al., 1992). Further, lipofuscin (LF) index in small crabs (<40 mm CW) cannot be determined because of analytical limitations (Ju et al., 1999). Only juvenile crabs >40 mm CW were selected for our study. The elimination of crabs smaller than 40 mm CW in our lipofuscin sample should result in under-representation by juvenile crabs spawned late during the previous spawning season (i.e. August–September) and by other slow growing juveniles. Subsamples were collected from three different regions of Chesapeake Bay: the eastern shore (Fishing Bay and Honga River), western shore (Potomac River), and lower bay (James River) from December through February in 1998–99 and 1999–2000. A portion of juveniles <70 mm CW (*n*=58) from the 1998–99 survey was excluded for use in a growth and calibration study as recently reported in Ju et al. (2001).

Analysis of lipofuscin index

Crabs were anesthetized on ice prior to being sacrificed. Carapace width (mm) was measured and eyestalk tissues were carefully dissected. Each collected tissue was transferred to a 4-mL amber vial for extraction of lipofuscins with a solvent. Measurement of fluorescence intensity was modified slightly from Ju et al. (1999) to improve sensitivity and accommodate larger sample numbers by switching from individual sample detection to a scanning fluorescence spectrophotometer (Waters 474) equipped with a flow cell. Volumes of 10 μL from each extract were injected by an auto-sampler with methanol (MeOH) as the carrier solvent (1 mL/min). Fluorescence intensity was measured at a maximum emission wavelength of 405 nm by using a maximum excitation at 340 nm at constant temperature (10°C).

To provide a quantitative measure of lipofuscin, fluorescence intensities of extracted lipofuscin were calibrated by using quinine sulfate (in 0.1N H_2SO_4) and normalized to protein content of extracted tissue measured by the modified bicinchoninic acid assay (Nguyen and Harvey, 1994). Although fluorescence intensity can be accurately calibrated against external standards, the lipofuscin amount per unit tissue volume (wet or dry weight) has often been measured

either before or after extraction (e.g. Ettershank and George, 1984). Such measures are inherently variable and subject to differences among tissue types and processing methods. The use of cellular protein as a basis for measurement of extractable lipofuscin concentrations eliminates many previously encountered difficulties. Protein-normalized lipofuscin content is expressed as the lipofuscin index or normalized-lipofuscin (μg-LF/mg-protein).
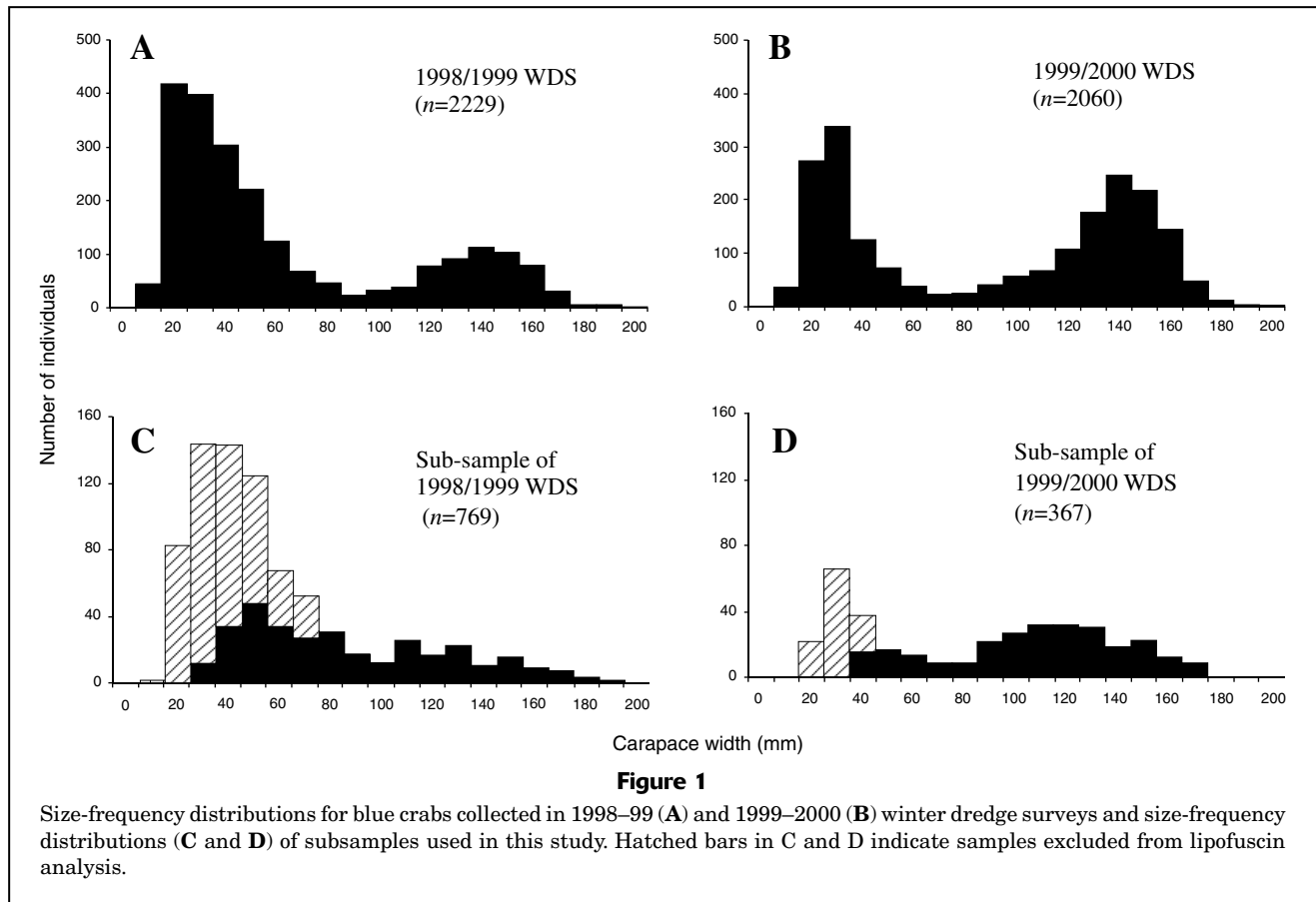
Statistical analysis

The lipofuscin index (μg-LF-content/mg-protein) of samples were natural-log–transformed before statistical analyses to satisfy assumptions of homogeneity of variances and normality of residuals. In order to determine whether the lipofuscin index varied between sexes in each sampling year, analysis of covariance (ANCOVA) with CW as a covariate was performed. Regression analysis also was performed to compare the relationship between size (CW) and lipofuscin index for each sex in each sampling year. Statistical analysis was done with SAS (SAS, Inc., 1996).

The frequency distribution of CW and lipofuscin index frequencies were analyzed for modal separation. We specified that modal means should be separated by more than two standard deviations (SD) (Gulland and Rosenberg, 1992). Lipofuscin index frequency distributions, which showed more than two modes, were analyzed by using ENORMSEP (FiSAT; Gayanilo et al., 1996), which is a maximum likelihood method for identifying modes. A chi-squared analysis was used to test the assumption that frequencies were normally distributed for each mode. Class interval was specified at 10 mm and 0.04 ug lipofuscin-content/mg-protein for CW and lipofuscin index, respectively. Although this exceeded the precision of the lipofuscin measurement, it provided class size ranges between 5 and 25 individuals to facilitate the analysis of distribution modes. The age class of each mode was then assigned based on the lipofuscin index accumulation rate determined through rearing experiments (Ju et al., 2001). The formula for this age assignment is

$$Age\,(\mathrm{yr}) = 0.824 \times \exp^{(1.133 \times LF\,index)}. \qquad (1)$$

Age classes were defined as 0, 1, and 2 and consisted of individuals 0 to <1, 1 to <2, and ≥2 yr old, respectively. The chronological "age" in this study is related to hatching dates of animals, i.e. the release of the first stage zoea. The age class of each mode identified from modal analysis was assigned according to the lipofuscin index accumulation rate (Eq. 1). Size (CW)-based age classification of 0, 1, 2+ age individuals corresponded to crabs of CW < 60, 60 ≤ CW < 120, and CW ≥ 120 mm, respectively, according to a recent Chesapeake Bay blue crab demographic assessments (Rugolo et al., 1998). In our study, age 2+ references all crabs ≥2 years of age. All crabs of CW > 60 mm (*n*=217 and 208 for 1998–99 and 1999–2000 WDS, respectively) were included to estimate the relative abundance of each age class determined by either lipofuscin or CW. Although some juveniles (<80 mm CW) were not measured, they were assumed to be <1 yr old (age-0 class).

Figure 1

Size-frequency distributions for blue crabs collected in 1998–99 (**A**) and 1999–2000 (**B**) winter dredge surveys and size-frequency distributions (**C** and **D**) of subsamples used in this study. Hatched bars in C and D indicate samples excluded from lipofuscin analysis.
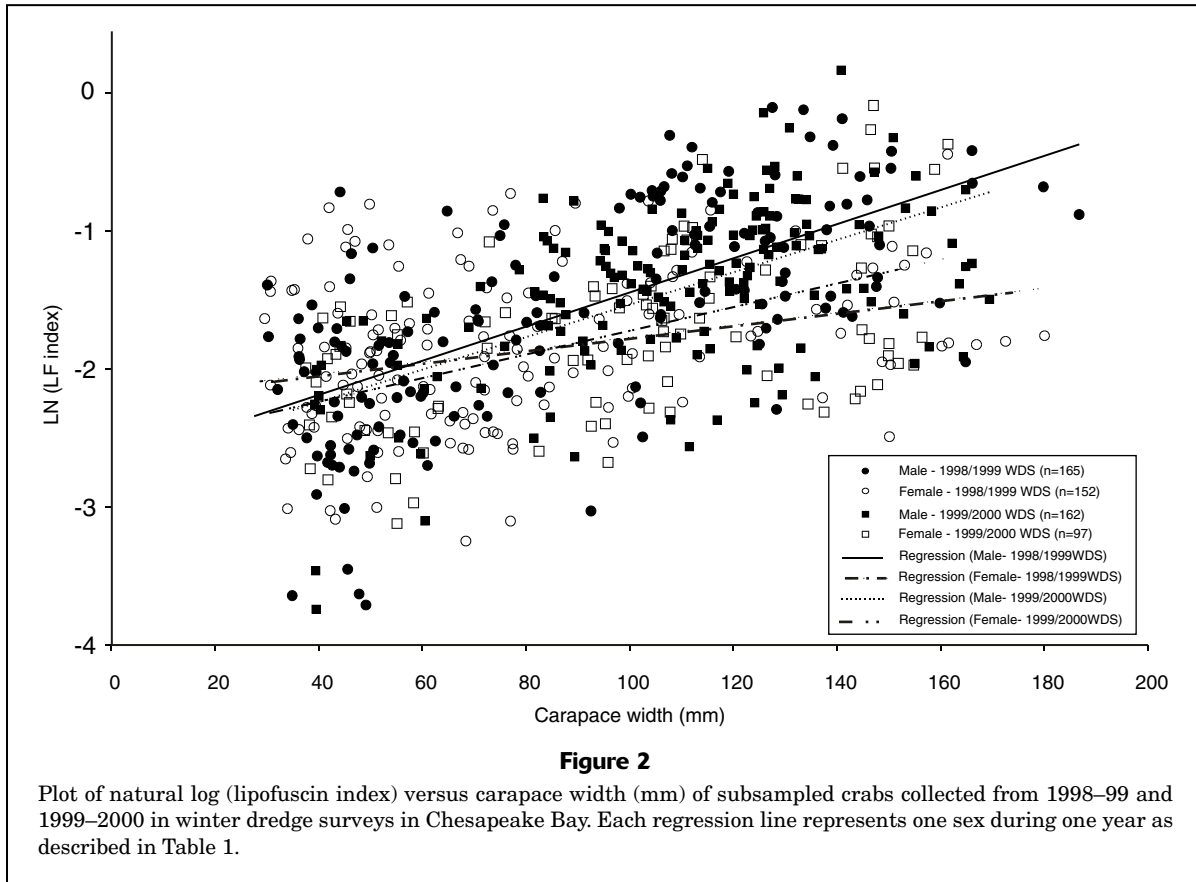
Results

Size frequencies of blue crabs collected in the winter dredge survey differed significantly between the two sampling years (chi-square test; α=0.05; Fig. 1, A and B). Although the total number of crabs collected from 1998–99 and 1999–2000 winter surveys were equivalent, a greater proportion of crabs >80 mm CW occurred in the second year. For both years, frequency distributions of CW data supported the fit of two modes (Fig. 1) and juveniles were clearly distinguishable from adult crabs as individuals <80 mm CW (or 90 mm CW for 1998–99). For 1998–99 WDS, males and females subsets of crabs had mean CW of 56.4 ±2.0 mm (mean ±SD; n=165) and 52.2 ±2.0 mm (n=152), respectively. For 1999–2000 WDS, CW for males and females was 91.5 ±2.7 mm (n=162) and 72.0 ±3.0 mm (n=97), respectively. The number of crabs sampled for lipofuscin analysis was halved (from n=769 to 367) in the second year because of a much more restricted analysis of juveniles <80 mm CW that were assumed to be <1 year of age. In both years, the subsamples examined for lipofuscin were skewed towards crabs >60 mm CW (Fig. 1, C and D), reflecting our analytical criteria and objective to include all adults in the analysis of age structure.

Lipofuscin index varied positively with CW (Table 1, Fig. 2) but there was high variability in lipofuscin index for a given size. All subcategories (sex and year) supported

significant regressions of lipofuscin on CW, but coefficients of determination were quite low, ranging as low as 0.04 for females in the first sampling year. ANCOVA analysis showed that lipofuscin adjusted for CW effects was significantly different only between sexes for the first sampling year (Table 2). We had expected that the lipofuscin index in mature females (CW >120 mm) would be higher at a given CW than in males because females discontinue molting following their first mating (Millikin and Williams, 1984). As a result, females living beyond their final molt would be predicted to accumulate lipofuscin independently of size. Results were scattered, however, and there was no statistical evidence for higher lipofuscin index in mature females. The lack of compelling evidence for differences in overall lipofuscin levels among sexes allowed males and females to be combined in the subsequent age structure analysis.

Frequency distributions for the lipofuscin index were fitted by multiple modes in both years (Fig. 3, A and B). Intervals between modal means were greater than two standard deviations for the first three modes in each sample (Table 3) and lipofuscin index-based modal distributions were not significantly different from expected normal distributions (chi-square test; α=0.05). Modes were consistent with the presence of 0, 1, and 2 age classes in each collection. Ages derived from lipofuscin index values (Eq. 1) for the first mode in each year were higher (ages 0.8–1.0 yr) than those expected for juveniles produced during the previous spawn-

Figure 2

Plot of natural log (lipofuscin index) versus carapace width (mm) of subsampled crabs collected from 1998–99 and 1999–2000 in winter dredge surveys in Chesapeake Bay. Each regression line represents one sex during one year as described in Table 1.
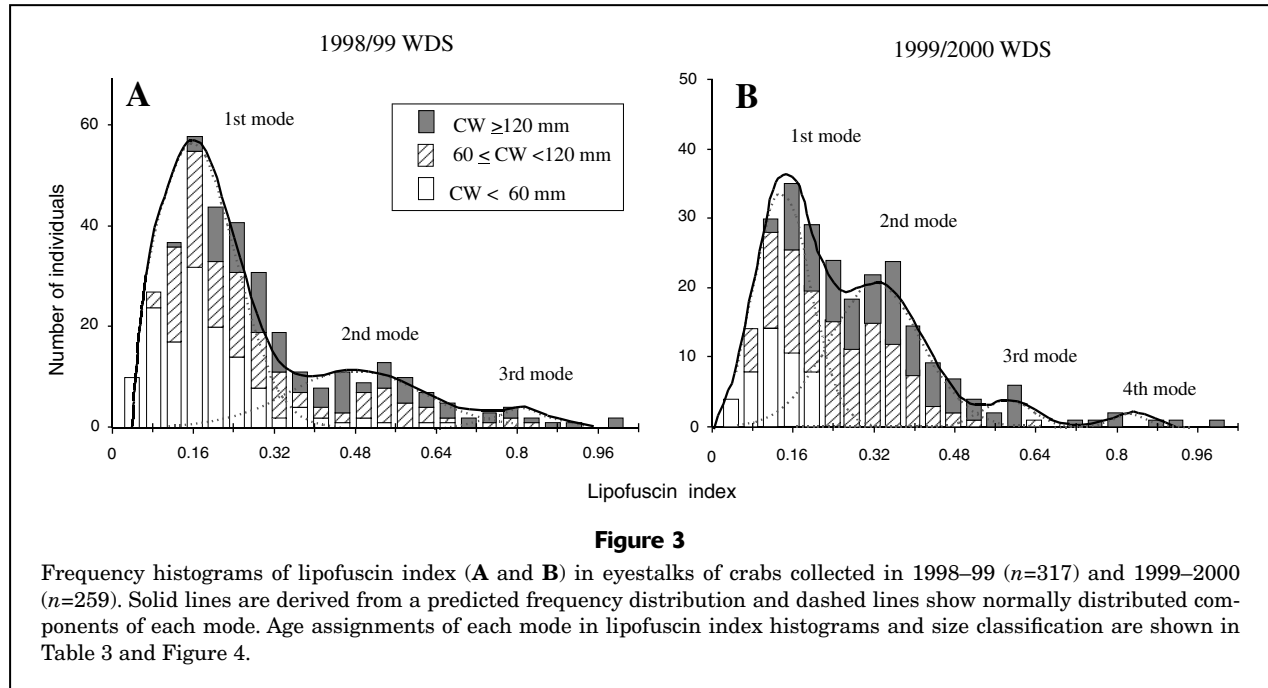
Table 1

Lipofuscin index ($\log_e$-transformed) and size (CW) relationship for each sex in each sampling year as determined in the regression analysis. The slope (±95% CL), intercept (±95% CL), adjusted coefficient of determination (adj. r^2), and probability of type-I error (P) are shown. WDS = winter bottom-dredge survey.

	Slope (±95% CL)	Intercept (±95% CL)	Adj. r^2	P
1998–99 WDS				
Male	0.012 ±0.002	−2.684 ±0.218	0.41	<0.001
Female	0.004 ±0.003	−2.118 ±0.218	0.04	<0.005
1999–2000 WDS				
Male	0.012 ±0.003	−2.709 ± 0.283	0.34	<0.001
Female	0.009 ±0.003	−2.581 ±0.290	0.28	<0.001

ing seasons (ages 0.4–0.6 yr) (Fig. 4). It seems likely that juvenile modes were skewed towards older juvenile ages because small crabs (40 mm CW) were excluded from analysis. In the 1999–2000 WDS sample, modal analysis supported a second subannual mode for age-1 crabs, which infers a bimodal recruitment pattern during the summer and fall of 1998 and may not have occurred in 1997 (i.e. 1998–99 WDS samples). Several lipofuscin values actually exceeded the third dominant mode (the fourth mode for 1999–2000 WDS samples), which suggests that several crabs attained ages ≥3 years. Nevertheless, these individuals could not be statistically distinguished from the last dominant mode.

There was a large discrepancy between the individuals assigned age classes by lipofuscin index and those assigned by CW criteria. Lipofuscin measures suggested that most crabs could attain 120 mm CW in less than two complete years of life (Table 4). Conversely, the index predicted that some individuals could grow quite slowly, remaining <60 mm CW into their second year of life (Fig. 3A). Although the samples analyzed did not include every animal from the WDS, the age structure suggested a pattern of declining

Figure 3

Frequency histograms of lipofuscin index (**A** and **B**) in eyestalks of crabs collected in 1998–99 (*n*=317) and 1999–2000 (*n*=259). Solid lines are derived from a predicted frequency distribution and dashed lines show normally distributed components of each mode. Age assignments of each mode in lipofuscin index histograms and size classification are shown in Table 3 and Figure 4.

abundance with the lipofuscin index, consistent with a predominate pattern of mortality among age classes. Based on lipofuscin analysis, the relative abundance between age classes (age 1 and 2+ classes) was not significantly different between two sampling years (*t*-test; *P*>0.05).

Discussion

Size-specific patterns of crab abundance in the Chesapeake Bay exhibit significant interannual variation; a significant decline in juveniles was seen during the second year (1999–2000) of the study (Fig. 1, A and B). Such variations have been observed previously in Chesapeake Bay (Abbe, 1983; Hines et al., 1987; Lipcius and Van Engel, 1990), and may result from interannual variation in recruitment of larvae, postlarvae, and juveniles (related to the spawning stock), physicochemical conditions, and food availability (Holland et al., 1987; McConaugha, 1988). Density-dependent processes such as cannibalism and predation may also regulate juvenile abundance and dampen recruitment variation, particularly when stock levels are high (Lipcius et al., 1995; Kahn et al., 1998).

Age estimates based upon lipofuscin index were more highly resolved than those based on CW, and there were at least three age modes (ages 0, 1, and 2) apparent in both sampling years. Perhaps more importantly, individuals were classified differently with lipofuscin than with CW, which indicated that age estimates based upon CW could lead to substantial errors in determination of growth, mortality, and fishery yield estimates. Recent growth-rate measures have indicated that Chesapeake Bay blue crabs may grow substantially faster than previously recognized, but also that some crabs may grow at very slow rates under

Table 2
The results of analysis of covariance with CW (carapace width) as a covariate to determine effects between sex and the lipofuscin index (log$_e$-transformed) of crabs collected from winter dredge surveys. NS = not significant at *P*=0.05. WDS = winter bottom-dredge survey.

Source	df	F	P
1998–99 WDS			
CW	1	85.12	<0.001
sex	1	13.23	<0.001
sex (CW)	1	25.86	<0.001
1999–2000 WDS			
CW	1	114.71	<0.001
sex	1	0.39	0.532[NS]
sex (CW)	1	2.74	0.100[NS]

unfavorable environmental conditions (Ju et al., 2001). In other words, lipofuscin-based age determinations indicate highly variable individual growth rates. We speculate that such variability is the result of the discontinuous nature of crab growth combined with the protracted spawning season and strong seasonal pattern in growth (driven by temperature), which together amplify relatively small differences in hatching dates and early growth rates into large differences in carapace sizes. Spatial heterogeneity in habitat would also be a contributor to high variability in crab growth rates. Alternatively, the degree with which lipofuscin reflects true chronological age may have contributed to the high variation seen in CW-based ages versus
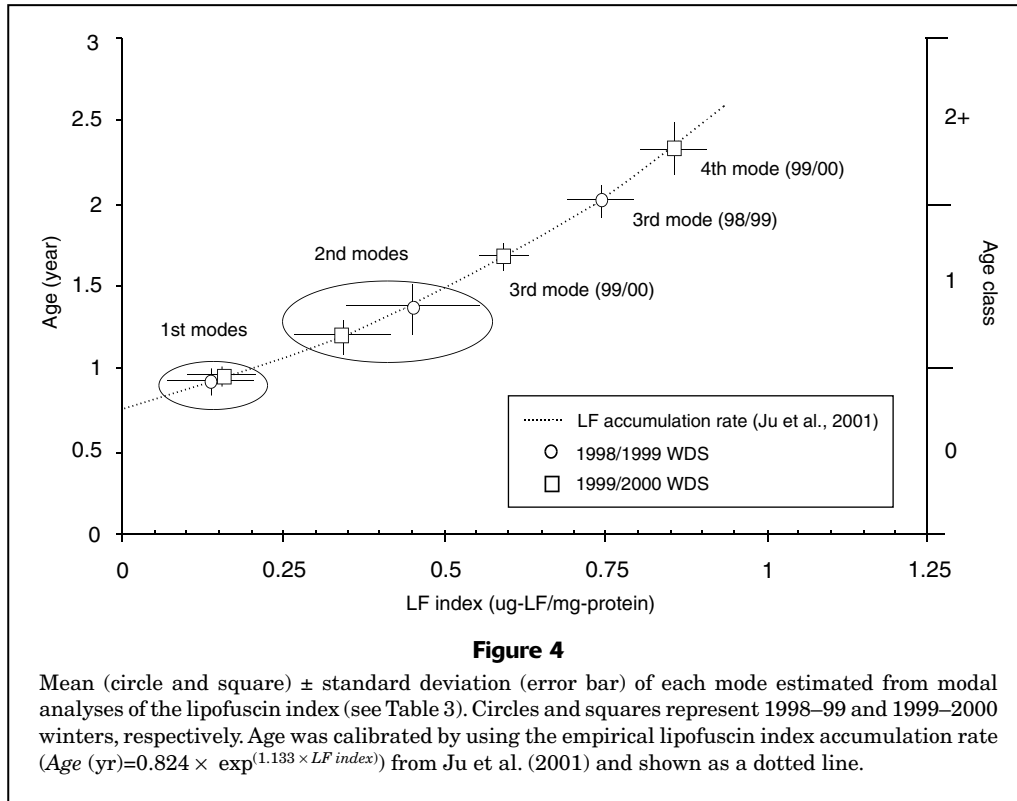
Figure 4

Mean (circle and square) ± standard deviation (error bar) of each mode estimated from modal analyses of the lipofuscin index (see Table 3). Circles and squares represent 1998–99 and 1999–2000 winters, respectively. Age was calibrated by using the empirical lipofuscin index accumulation rate (Age (yr)=0.824 × $\exp^{(1.133 \times LF\,index)}$) from Ju et al. (2001) and shown as a dotted line.

Table 3

Modal frequency analysis of lipofuscin (LF) index for crabs from 1998–99 and 1999–2000 winter bottom-dredge surveys (WDS). χ^2, chi-square test; df, degree of freedom.

Group	1998–99 WDS		1999–2000 WDS	
	Mean LF ±SD	No. of ind./mode	Mean LF ±SD	No. of ind./mode
1st mode	0.146 ±0.076	219	0.150 ±0.053	119
2nd mode	0.440 ±0.120	86	0.330 ±0.088	119
3rd mode	0.734 ±0.060	12	0.590 ±0.046	12
4th mode			0.860 ±0.066	9
Total		317		259
χ^2	21.04		18.83	
df	19		22	

lipofuscin-based ages. In past studies on crustaceans (e.g. O'Donovan and Tully, 1996), lipofuscin has been shown to be significantly influenced by metabolism, leading some authors to conclude that lipofuscin is a closer measure of physiological than chronological age. Recent work on laboratory- and pond-reared crabs, however, has shown that LF accumulation is relatively constant and that average winter and summer rates differ by 27% (Ju et al., 2001). During winter months LF continued to accumulate in the eyestalks of pond-reared crabs, even though growth in CW ceased on account of winter temperatures. Further, the

lipofuscin-index versus age relationship in these rearing studies (coefficient of determination: r^2=78%) was substantially less variable than the CW versus age relationship (r^2=59%). Nevertheless, variance in the lipofuscin index versus age relationship is sufficient to cause misclassification of individuals, and it is important to obtain sufficient samples to permit age modes among cohorts to be resolved.

The protracted spawning season for Chesapeake Bay blue crabs (mid-May through mid-September; Van Engel, 1999) has important implications for subsequent size and

Table 4

Lipofuscin (LF)-based age composition and relative abundance (in parentheses) of each age group in subadult (60 ≤ carapace width <120 mm) and adult (≥120 mm) size classes for crabs from 1998–99 and 1999–2000 winter bottom-dredge surveys (WDS). Crabs <60 mm were excluded due to gear selectivity and our exclusion of crabs ≤40 mm (see "Materials and methods" section).

Age group (LF based)	1998–99 WDS Size class		1999–2000 WDS Size class	
	60 ≤ CW < 120 mm	CW ≥ 120 mm	60 ≤ CW < 120 mm	CW ≥ 120 mm
0	80 (0.63)	31 (0.34)	67 (0.57)	30 (0.33)
1	44 (0.35)	50 (0.56)	60 (0.51)	54 (0.59)
2+	3 (0.02)	9 (0.10)	0 (0.00)	7 (0.08)
Total number	127	90	117	91

age structure. Crabs that spawn early may grow rapidly and attain "subadult"size (>60 mm) by the end of the first growth season (Ju et al., 2001). If this is the case, then a significant fraction of subadults (defined as ≥60 mm CW) may have been misclassified on the basis of past CW criteria as being 1-yr-old subadults. We would argue that crabs spawned late in the season (late summer–early fall) may overwinter at small sizes but will emerge the next spring and experience rapid growth in warm temperatures to reach maturity within their first year of life. In support of this view, Ju et al. (2001) have shown in growth studies that pond-reared crabs from late-spawning cohorts overwinter and undergo extremely rapid growth during the following summer months. Most of these crabs attained 127 mm CW (size of entry into the hard crab fishery) before their second winter. Occasionally such a protracted period of spawning may result in multimodal patterns in recruitment. As reported by van Montfrans et al. (1990, 1995), we observed two subannual cohorts of age-1 blue crabs in 1999–2000; therefore it is likely that these crabs were recruited from a bimodal pattern of juvenile production during 1998. These subannual cohorts were statistically separated into modes (Fig. 3) based on known lipofuscin accumulation rate in blue crabs (Fig. 4). Modal analysis of lipofuscin index for 1998–99 did not show evidence of multimodal recruitment (Fig. 3A). The inconsistent appearance of "subannual" cohorts between sampling years may result from the interannual variation of settlement and spawning patterns, which in turn is related to yearly spawning stock conditions and physicochemical conditions in the Chesapeake Bay region (McConaugha et al., 1983; van Montfrans et al., 1990, 1995).

According to lipofuscin modal analysis, crabs <2 yr old are a significant fraction of the harvestable (>127 mm CW) stock in Chesapeake Bay. This finding argues that size (CW) criteria, on which past assumptions of greater ages in the Chesapeake Bay blue crab stocks are based, is insufficient for assessing blue crab demographics. It appears that size may be more reflective of interannual differences in growth rates than age structure alone.

Given that lipofuscin-based age distributions show that ≥2 year old crabs are a minor contributor to the harvestable stock, we speculate that the population dynamics of crabs

available for harvest is strongly influenced by numbers of juveniles produced in each year and that growth conditions experienced by these juvenile cohorts during their first full year is a dominant determinant of the reproductive potential of the blue crab. It also suggests that seasonal yield patterns in commercial fisheries are strongly influenced by seasonal patterns in time of spawning and subsequent juvenile growth. If relatively older (>2 yr) crabs are minor contributors to the adult stock, then the Chesapeake Bay fishery may essentially depend on an annual crop of crabs, produced over a protracted spawning season. The consequence of this pattern is that landings will be more tightly coupled with juvenile production levels (e.g. settlement rates of postlarvae) and environmental conditions (e.g. winter duration) that affect their growth into the adult stock, not unlike the dynamics observed in penaeid shrimp fisheries (e.g. Haas et al., 2001). Other attributes including sex ratio and size- and age-specific reproductive rates are other important considerations in assessing the reproductive condition of the Chesapeake Bay blue crab stock (Jivoff and Hines, 1998; Rugolo et al., 1998).

Uncertainties remain in applying these results to the assessment of the Chesapeake Bay. We note that samples used in our study did not cover the entire Chesapeake system and that the abundance and size distributions of blue crabs are expected to vary locally. Nevertheless, these results strongly suggest that for moderately large sample sizes, crabs can be assigned to annual or subannual cohorts on the basis of lipofuscin measures, thereby significantly improving our knowledge of population dynamics and life history. Natural longevity in Chesapeake Bay blue crabs remains an open question, particularly because crabs (≥3 yr old) are likely to be very rare because of the combined effects of high exploitation and natural mortality (Miller, 2001).

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