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Direct determination of age in shrimps, crabs, and lobsters

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Abstract: The detection and measurement of annual growth bands preserved in calcified structures underlies the assessment and management of exploited fish populations around the world. However, the estimation of growth, mortality, and other age-structured processes in crustaceans has been severely limited by the apparent absence of permanent growth structures. Here, we report the detection of growth bands in calcified regions of the eyestalk or gastric mill in shrimps, crabs, and lobsters. Comparison of growth band counts with reliable, independent estimates of age strongly suggests that the bands form annually, thus providing a direct and accurate method of age determination in all of the species examined. Chemical tags in the lobster cuticle were retained through one or two molts that occurred over the duration of an experiment, as apparently was the mesocardiac ossicle containing the growth bands in the gastric mill. Growth bands are not the previously documented lamellae of the endocuticle, and their formation was not associated with molting. Sex-specific growth curves were readily developed from growth band examination in multiple species, suggesting that routine measurement of growth and mortality in decapod crustaceans may now be possible.

Résumé : La détection et la mesure des bandes de croissance annuelles conservées dans des structures calcifiées sous-tendent l'évaluation et la gestion des populations exploitées de poissons partout dans le monde. Cependant, chez les crustacés, l'estimation de la croissance, de la mortalité et d'autres processus qui sont dépendants de l'âge a été très limitée par l'apparente absence de structures permanentes liées à la croissance. Nous faisons ici état de la détection de bandes de croissance dans des régions calcifiées du pédoncule oculaire ou du moulin gastrique chez des crevettes, crabes et homards. La comparaison des décomptes de bandes de croissance avec des estimations fiables et indépendantes de l'âge suggère fortement que les bandes se forment annuellement, fournissant ainsi une méthode directe et précise pour la détermination de l'âge chez toutes les espèces examinées. Des marques chimiques dans la cuticule du homard ont été conservées durant une ou deux mues effectuées sur la durée d'une expérience, comme l'a apparemment été l'ossicule mésocardiaque qui contient les bandes de croissance du moulin gastrique. Les bandes de croissance ne sont pas les lamelles de l'endocuticule décrites précédemment et leur formation n'était pas associée avec la mue. Des courbes de croissance par sexe ont été facilement développées à partir de l'examen des bandes de croissance chez plusieurs espèces, ce qui suggère que la mesure de la croissance et de la mortalité chez les crustacés décapodes pourrait maintenant se faire couramment.

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Introduction

Most aquatic animals can be aged by counting annual growth bands deposited in hard structures, such as the otoliths of fishes (Campana 2001) and shells of bivalves (Kilada et al. 2009). However, no equivalent structure has yet been found in crustaceans, nor would such a structure be expected to exist, given that crustaceans grow by molting. Although molting

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frequency varies considerably among species, sex, and size classes of crustaceans, molting individuals are assumed to lose and replace all calcified structures, including cuticle (skeleton) that might record annual growth layers (Vogt 2012). In the absence of a direct method for ageing crustaceans, a number of indirect approaches have been developed to infer age, such as size modal analysis. Although these approaches have proven useful, they have two main limitations (Vogt 2012). First, the size–age relations they establish are applicable only to the populations and years under examination, because of environmental controls on growth rate. Secondly, these size–age relations do not provide individual ages but rather a probabilistic and aggregated evaluation of age for animals of the same size. This can lead to substantial errors in age estimates for individuals, as younger, fast-growing animals may be impossible to discriminate from older, slow-growing animals of the same size. More recently, the use of lipofuscin, which is accumulated in neural tissues as a result of routine cellular oxidative processes, has provided promising results for ageing crustaceans (Sheehy et al. 1999). However, this technique also generates results that are context-specific, as the accumulation of lipofuscin is directly influenced by the environment and individual circumstances (Wahle et al. 1996). Therefore, a technique to directly and accurately age individual crustaceans does not currently exist.

In this paper, we describe a direct method to determine the age of decapod crustaceans. The method is based on counting growth bands deposited in calcified body structures: the eyestalk and gastric mill. The technique is illustrated in four species of decapod crustaceans: American lobster (*Homarus americanus*), snow crab (*Chionoecetes opilio*), sculptured shrimp (*Sclerocrangon boreas*), and northern shrimp (*Pandalus borealis*). The implications of this method for future stock assessments and biological studies of crustaceans around the world are likely to be substantial.

Materials and methods

Animals used in this study were obtained from scientific surveys, commercial fisheries, or rearing facilities in various localities from 2008 to 2011 (Supplementary Table S1[1]). Individuals were sexed and their body size measured (±0.1 mm) as carapace width (CW) for snow crab or carapace length (CL) for the other species (Supplementary Table S2[1]). Where the actual age was unknown, individuals were assigned an estimated age and instar number (the number of molts after larval settlement) based on their body size using species- and location-specific growth models that were developed from size modal analyses (e.g., Supplementary Fig. S1[1]), laboratory, and (or) field observations of molt increment and intermolt period or a combination thereof (details in Supplementary Table S1[1]).

Eyestalks and gastric mills were obtained by dissection after anesthesia. All structures were cleaned and embedded in Cold Cure epoxy resin before preparing serial transverse or longitudinal sections (120 μm thickness) with a diamond-bladed Isomet saw (Supplementary Figs. S2 and S3*a*–S3*b*[1]). Sections were epoxy-mounted on a standard microscope slide, polished by hand using dry 0.3 μm grit lapping film, and viewed with transmitted light under 90% ethyl alcohol with a CX41 Olympus compound microscope at 10×–40× magnification. Digital images were taken with a DP72 Olympus video camera attached to the microscope. Images were digitally enhanced using Adobe Photoshop 12.0.4 ×32 to increase the contrast between adjacent bands. Growth bands were recognized as paired light and dark zones in the endocuticle. They were counted from the basal (adjacent to the membranous layer and hypodermis) to the distal region of the endocuticle (Fig. 1) by observers without knowledge of the animals' actual age or size-based estimate of age. We used "Image J" software to measure the width of each growth band. Precision (reproducibility) of band counts between left and right eyestalks and between two independent readers of the same structure was measured using the mean coefficient of variation (CV) of paired records of band counts (Campana 2001).

Immature crabs of 16–63 mm CW were maintained in the laboratory in 2009 and 2010 and allowed to molt once to determine if growth band formation was more closely related to time or to molting. After ablation of a single eyestalk (under anesthesia), the crabs were monitored daily and then killed up to 6 months after molting, at which point the remaining eyestalk was removed. Sections of premolt and postmolt eyestalks of crabs were prepared, examined, and interpreted as above.

To determine whether any mineralized features of the cuticle are conserved through molting, 13 juvenile lobster (8.5–36.5 mm CL) were immersed in seawater containing the fluorescent marker calcein, before or during molting, and then returned to regular holding conditions where they were allowed to molt 0, 1, or 2 times before euthanasia (details in Supplementary Table S3[1]). Sections of the eyestalk and gastric mill were prepared as described above and then viewed with transmitted light at 10×–40× magnification under a Leica DM IRE2 Confocal Laser Scanning Microscope to detect calcein. Digital images were recorded with a Leica DC 500 camera using two different excitation wavelengths to distinguish the calcein mark (520–530 nm: green) from natural autofluorescence (600–790 nm: red). Background-corrected images highlighting the calcein mark (if present) were prepared using Adobe Photoshop 12 by subtracting the red image channel from the green channel, then converting to grayscale.

The spatial distribution of calcium and phosphorus in the cuticle of the eyestalk and gastric mill of lobster was determined with X-ray elemental maps collected on a JEOL-6400 scanning electron microscope equipped with an EDAX Genesis X-ray microanalyser. Operating conditions consisted of a probe current of 10 nA, an accelerating voltage of 15 kV, and a dwell time of 200 ms per pixel. Map resolution was 512 × 512 pixels and based on 46 sequential frames. Trace element scans were made with a Thermo Finnigan Element 2 high-resolution inductively coupled mass spectrometer (ICP-MS) coupled with a Merchantek LUV213 UV laser ablation microprobe.

Results and discussion

Features consistent with growth bands were observed in the endocuticle in transverse and longitudinal sections of the base of the eyestalk or in the mesocardiac ossicle of the gastric mill of the four study species (Figs. 1*a*–*c*; Supplementary

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjfas-2012-0254.

Fig. 1. Growth bands (indicated by dots) in (*a*) transverse section of the eyestalk of a 41 mm carapace width snow crab; (*b*) longitudinal section of the eyestalk of an 18 mm carapace length (CL) northern shrimp; (*c*) longitudinal section of the mesocardiac ossicle of the gastric mill of a 116 mm CL American lobster. Narrow and nonprominent laminae are clear in the images and are distinguishable from the growth bands. Panels (*d*)–(*f*) show the relation between band count and mean ± 95% confidence interval of estimated age (solid symbol) and instar number (open symbol) in the same species. For lobster, band count is shown in relation to age for individuals from the Bay of Fundy (BOF) and Magdalen Islands (MGI) and to instar number for MGI. A 1:1 equivalence line between age and band count or instar number is shown on all panels. For lobster and northern shrimp, band count was divided by 1.2 and 5, respectively, for graphical purposes; in these two cases, the mismatch between instar number and band count is thus even greater than suggested by the distance separating instar values from the 1:1 equivalence line. The age data from Magdalen Islands in the case of lobster (*f*) and the instar number data of the snow crab (*d*) were offset by +0.2 on the *x* axis to improve clarity by eliminating overlap with other age data in each panel. Similar data and results are shown for sculptured shrimp in Supplementary Fig. S4.[1]

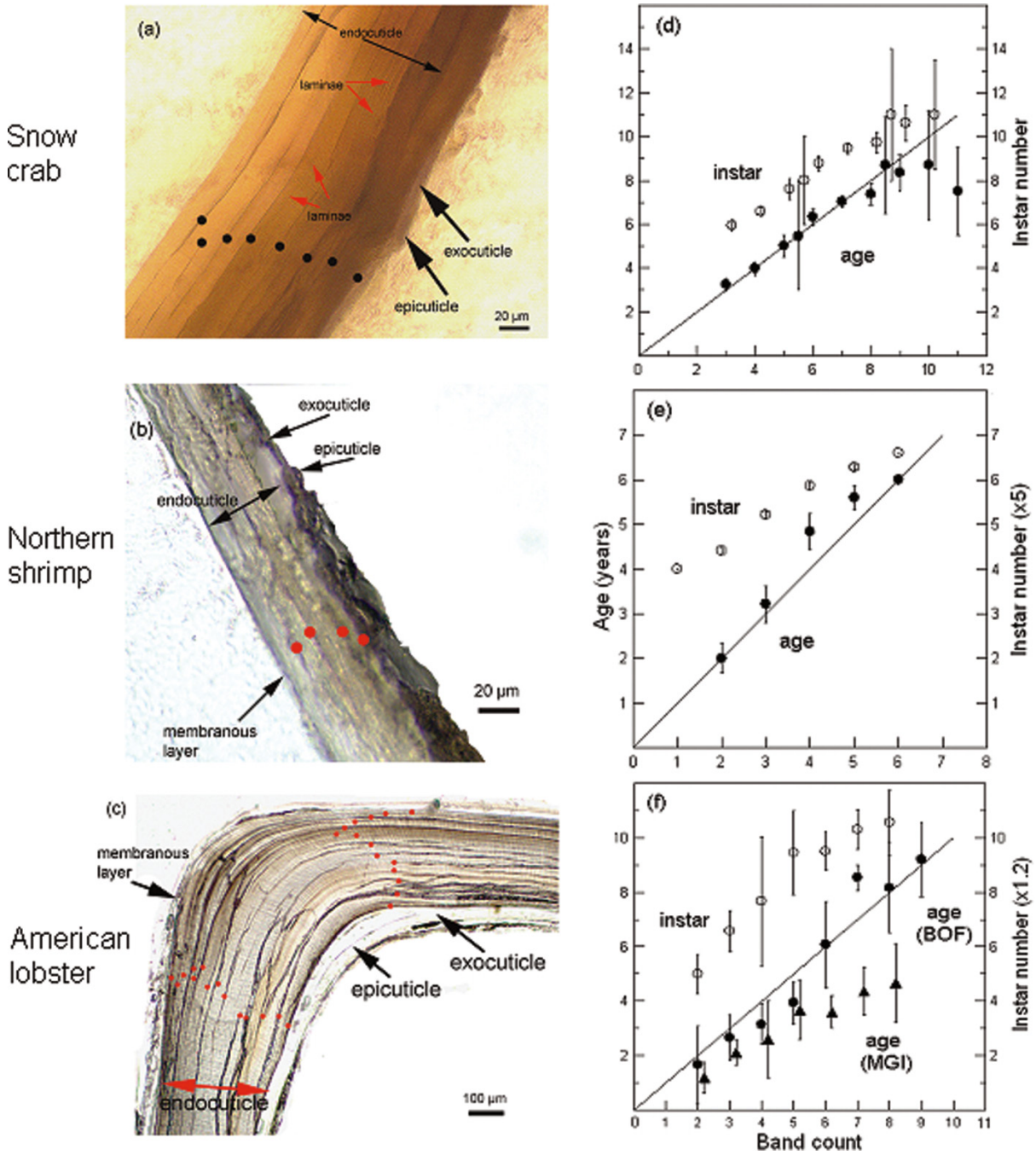


Fig. S4*a*[1]). All four layers of the crustacean cuticle — the epicuticle, exocuticle, endocuticle, and membranous layer (as ordered from the external surface) — were visible in these sections (Supplementary Fig. S3*c*[1]) and are underlain by the hypodermis. In crab, the growth bands were most easily interpreted in transverse sections of the eyestalk (Fig. 1*a*), whereas in the two shrimp species, longitudinal sections of the eyestalk provided the best visibility (Fig. 1*c*; Supplementary Fig. S4*a*[1]). In lobster, the growth bands were most clearly seen in longitudinal sections of the mesocardiac ossicle, on either side of the ossicle's prominent arch (Fig. 1*c*), and less easily seen in the eyestalk (Supplementary Fig. S5[1]). The bands seen in the lobster's mesocardiac ossicle appear to be the same as those described recently from the gastric mill of five species of decapod crustaceans (Leland et al. 2011). In all species and both structures, the bipartite growth bands consisted of a broad translucent zone bordered by a narrower opaque band (Fig. 1). The growth bands (5–22 μm width) were easily distinguished from the narrower microlamellae (1–4 μm width) (Fig. 1) previously described in the cuticle of various crustacean species (Amato et al. 2008). Up to six growth bands were visible in each of the two shrimp species and up to 10 or 15 in the crab and lobster, respectively, agreeing with longevity expectations. Growth band counts were consistently and substantially less than instar number in all species, indicating that growth bands were not merely a record of molting (Figs. 1*d*–1*f*; Supplementary Fig. S4*b*[1]). In contrast, band count was very similar to estimated age in crab (Fig. 1*d*) and the two shrimp species (Fig. 1*e*; Supplementary Fig. S4*b*[1]), where age was strongly inferred from known periodicity of molting and distinct size frequency modes, respectively. For lobster, mean band count matched known age (up to 4 years for juveniles obtained from rearing experiments) and estimated age from modal analysis or tag–recapture information for juveniles and subadults in the Bay of Fundy (Fig. 1*f*). However, the match between band count and estimated age was not as good for the Magdalen Islands, especially for larger lobsters (Fig. 1*f*). We are currently unable to explain the poorer fit for Magdalen Islands lobsters, but similar challenges have been encountered in the application of well-established, otolith-based ageing techniques to certain fish species (Treble et al. 2008).

Growth band counts should not differ systematically across structures within an animal if they are valid age indicators. There was no significant bias in growth band counts between right and left eyestalks within individuals, indicating bilateral symmetry in the deposition of the growth bands (Supplementary Fig. S6[1]). Nor was there bias in band counts between the eyestalk and mesocardiac ossicle of lobster (Supplementary Fig. S7[1]). Precision (CV) in band counts between left and right eyestalks ranged from 8% in northern shrimp to 11% in sculptured shrimp and 15% in crab; counting precision between the eyestalk and mesocardiac ossicle of lobsters was 7%. Replicate growth band counts of crab eyestalks and lobster mesocardiac ossicles by two independent observers resulted in CVs of 7% and 10%, respectively. These values of precision are similar to those reported in ageing studies for most fishes (5%–12%: Campana 2001) and bivalves (5%–7%: Kilada et al. 2009).

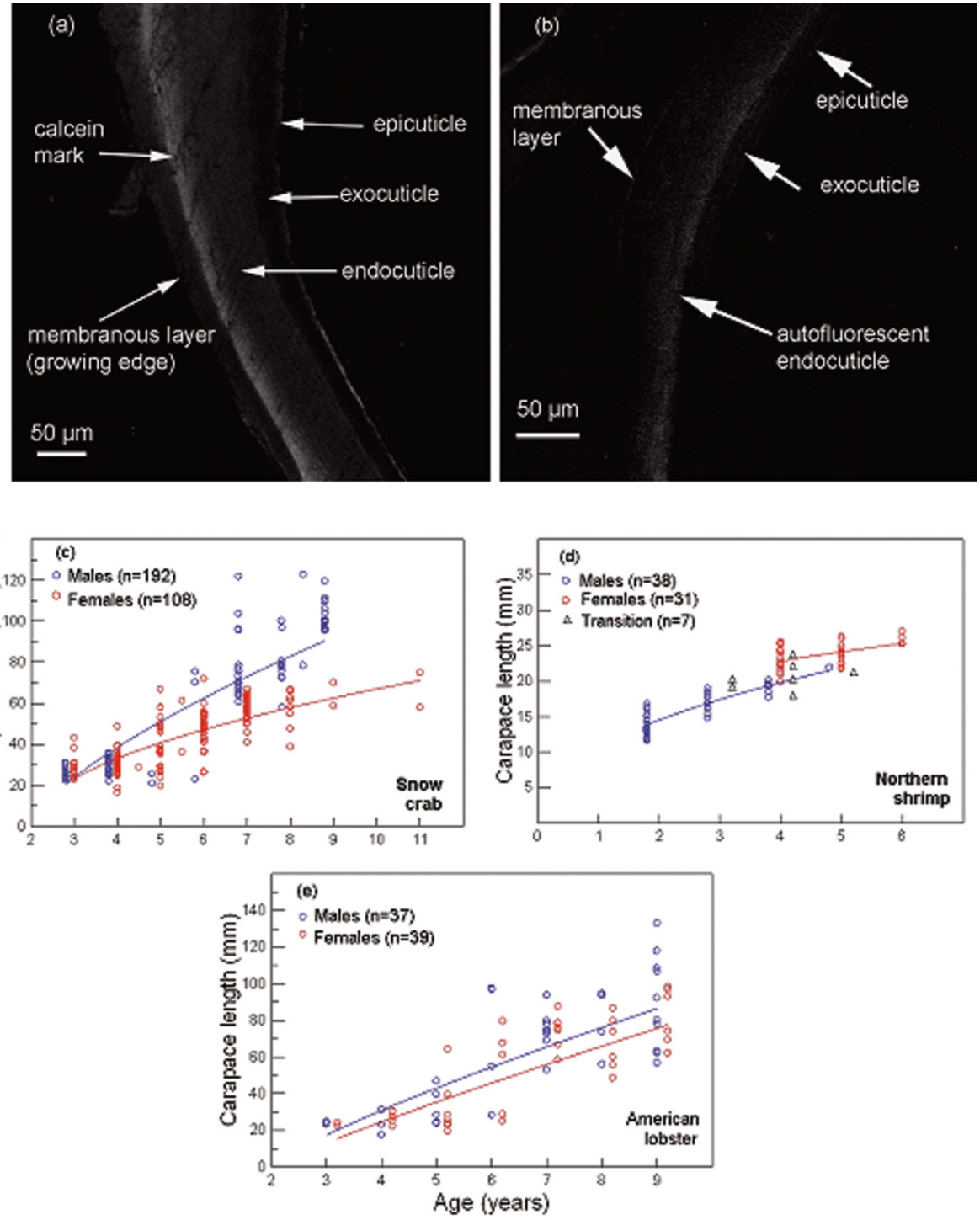
Molt-independent band formation was experimentally demonstrated in crab. The mean number of bands added to the nonablated eyestalk after molting increased progressively and did not differ significantly from 0 ($P < 0.05$) until 6 months postmolt, when it reached 0.75 ± 0.25 bands (Supplementary Fig. S8[1]), demonstrating that growth bands were added over time rather than shortly after each molt.

Juvenile lobsters exposed to calcein (a calcium-binding compound) and examined several days after exposure displayed a clear fluorescent mark near the basal region of the endocuticle (i.e., close to the membranous layer) of both the eyestalk and mesocardiac ossicle (Fig. 2; Supplementary Fig. S9[1]). The bright green fluorescent mark was easily distinguished from the less intense, natural autofluorescence visible in the outer region of the endocuticle (Fig. 2; Supplementary Figs. S10–S11[1]). The calcein mark was observed in the cuticle of three lobsters sacrificed 21–42 days after molting in a calcein bath, and importantly, the mark was retained in the cuticle of tagged lobsters that molted once ($n = 9$) or twice ($n = 1$) after the calcein treatment (Fig. 2; Supplementary Table S3; Figs. S10–S11[1]).

To our knowledge, the chemical tagging experiment represents the first evidence that any mineral features of the cuticle are perpetuated through molting, which provides a possible mechanism for growth bands to accumulate and record age in the endocuticle throughout the life of the crustacean. It is well known that calcium carbonate is transported from the cuticle to specialized storage structures (e.g., gastroliths on stomach wall) during premolt, to later be remobilized and incorporated into the newly forming cuticle (Shechter et al. 2008). If calcein-labeled minerals had been remobilized rather than retained, a diffuse distribution of fluorescence throughout the new endocuticle would have been expected, not the distinct bands that were observed (Supplementary Figs. S10–S11[1]). Nor does it seem likely that calcein was only remobilized into regions of the endocuticle composed of calcium carbonate, given that elemental maps of calcium and phosphorus revealed a matrix likely made of hydroxyapatite (Supplementary Table S3[1]) that was uniformly distributed throughout the lobster endocuticle (Supplementary Table S4; Fig. S12[1]). Nevertheless, mechanisms explaining the persistence of calcein (and growth bands) through molting may differ between structures. Careful dissections revealed that the mesocardiac ossicle was not shed externally with the exuvia during molting in lobster (Supplementary Figs. S13*a*–S13*b*[1]) and in a crayfish (Supplementary Fig. S13*c*[1]). Furthermore, the fact that the ossicle was found in a relatively hard state just 1 hour after molting suggests it was not shed at all. We were, however, unable to identify any cuticular structure missing from the exuvia of the more structurally simple eyestalk. However, the lack of calcein fluorescence in the exuvial eyestalk of tagged lobsters (Supplementary Fig. S10*e*[1]) is consistent with the interpretation above, that mineral features of the cuticle may be locally retained.

Age information obtained from growth band counts allowed the development of preliminary, sex-specific size-at-age relationships for all four study species. In crab and lobster, there was a gradual divergence in size at age between the sexes, with males becoming progressively larger at age than females (Figs. 2*c* and 2*e*). This pattern is consistent with the view that females of both species grow more slowly and reach smaller maximum sizes than males (Waddy et al. 1995; B. Sainte-Marie, personal observation). Size-at-age patterns for the two shrimp species were also consistent with our understanding of

Fig. 2. (*a*) Fluorescent microscope image showing calcein retention in a longitudinal section of American lobster eyestalk (carapace length (CL) = 22 mm) after molting. The eyestalk was sectioned 63 days after tagging with the calcium-binding compound calcein, and despite a second molt 22 days after tagging, the fluorescent calcein mark remained clearly visible near the base of the endocuticle layer, indicating that it had been retained through molting. In contrast, (*b*) a control (untagged) individual (CL = 12 mm) only showed diffuse autofluorescence in the outer part of the endocuticle. Additional images from calcein tagging experiments involving the eyestalk and mesocardiac ossicle of the American lobster are shown in Supplementary Figs. S9, S10, and S11. Panels (*c*)–(*e*) show the size-at-age relationships for different decapod species. Symbols represent males (blue) and females (red), and fitted lines are power regressions. Age was determined by counting growth bands in thin sections of the eyestalk in (*c*) snow crab and (*d*) northern shrimp and of the mesocardiac ossicle of the gastric mill in (*e*) lobster. Male and sex-transitioning individuals were offset by −0.2 and +0.2 years, respectively, to improve clarity by eliminating overlap with the female data. Similar size-at-age relationships are shown for male and female sculptured shrimp in Supplementary Fig. S14.[1]



the biology of these shorter-lived species (Fig. 2*e*; Supplementary Fig. S14[1]). In northern shrimp, as expected of an obligate protandric hermaphrodite (Bergström 2000), we found no female younger than 4 years of age, a predominance of smaller younger males, and overlapping sizes for the two sexes only at intermediate ages of 4–5 years (Fig. 2*e*). Sex-transitioning northern shrimp, which can be recognized on the basis of morphology (Bergström 2000), overlapped predictably in size with the largest males and smallest females (Fig. 2*e*). The sculptured shrimp is not a protandric hermaphrodite, but females in this species live longer and attain larger body sizes than males (Sainte-Marie et al. 2006), as was evident in our results (Supplementary Fig. S14[1]). Small female sculptured shrimp were absent from our samples and thus could not be aged. More complete growth curves for all four species will require larger numbers and a broader size range of aged animals.

The inability to directly and accurately determine the age of decapod crustaceans has hitherto impeded the biological understanding and fisheries management of these commercially important species. Our method to determine age in decapod crustaceans based on cuticle growth bands was validated in young known-age lobster and corroborated in four species by independent size-based estimates of age (using well-established methods routine to fishes; Campana 2001). Furthermore, we have confirmed that the growth bands are not directly associated with molting and that mineralized features of certain parts of the cuticle can be retained through molting. Nevertheless, further age validation and associated research would be useful, including multiyear chemical tagging experiments demonstrating long-term annual deposition of growth bands, inquiry into the processes that determine accretion of cuticular growth bands, and exploration for growth bands in warm-water (shorter-lived) decapod crustaceans and in other crustacean orders. Through the use of absolute age determinations derived from counting endocuticle growth bands, it should soon be possible to consider age-based stock assessments in support of the management of socially and economically important crustacean fisheries worldwide.

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