Gastric mill ossicles record chronological age in the Caribbean spiny lobster (*Panulirus argus*)

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ABSTRACT

Information on individual age and size relationships is crucial for sustainable management of fished populations. Previous research has indicated it may be possible to directly age crustaceans by counting annular bands deposited within gastric mill ossicles. The method has been applied to multiple crustacean species; however, its applicability has been controversial because studies have shown gastric mill ossicles are reworked and/or replaced during ecdysis. Over the last 8 years, we have refined and improved upon methods of processing gastric mill ossicles and investigated the validity of this method for ageing Caribbean spiny lobster (*Panulirus argus*) using captive-raised animals of known age from the Florida Keys (USA). Although the biological process that creates annular bands within gastric mill ossicles remains unclear, validation of the method for *P. argus* based on known-age lobsters from 10 months–5.5 years old ($n = 33$) confirms that the deposition of bands correlates with chronological age. We compared precision and accuracy of age estimates for ossicles processed using two imaging techniques (transmitted vs. reflected light) and found that transmitted light provided the best images for ageing *P. argus*. The mean coefficient of variation (CV) of age estimates between two readers was 20.2 %. The consensus age estimates provided by the readers were, on average, within 0.5 years of the known age and overall, 94 % of consensus age estimates from readers were within 1 year of the known age. We also compared lobsters of the same size but different age as well as those of the same age but different size to address whether the number of endocuticular bands in the ossicle is a measure of true chronological age or simply related to the size of the animal. Our results suggest that the gastric mill ossicles record chronological age in *P. argus*, providing a reliable ageing method with which to inform age-based management of the Caribbean spiny lobster population. Future research is needed to understand the mechanism that controls the deposition of endocuticular bands within the crustacean gastric mill ossicles and to determine the applicability of the method for other crustaceans.

1. Introduction

Information on age and size relationships is crucial for sustainable fishery management of exploited populations (Campana, 2001). Accurately estimating age is essential for determining life history characteristics such as growth, lifespan, age at recruitment to the fishery, and age at sexual maturity. These data form the foundation of age-based stock assessments. For many marine animals, age is estimated by counting annual rings or bands deposited in hard structures like those found in fishes (otoliths, scales, fin rays, opercula, and vertebrae), bivalves (shells), and squids (statoliths) (Arkhipkin, 1997; Campana, 2001; Hollyman et al., 2018; Khan and Khan, 2009). However, for crustaceans, ageing studies have relied on indirect methods such as modal analysis of size frequency distributions or lipofuscin accumulation, mainly because of the assumption that all calcified structures capable of indicating chronological age were lost at ecdysis (i.e., molting) (Bluhm and Brey, 2001; Maxwell et al., 2007; Sheehy et al., 1998). These methods are useful in the absence of a direct age estimation method but are greatly affected by environmental and metabolic factors, making them unreliable and indicative of physiological age as opposed to chronological age (Tully et al., 2000; Vogt, 2012; Wahle et al., 1996).

A new method to directly age crustaceans using gastric mill ossicles was first published by Leland et al. (2011) after they found sequential endocuticular bands in the zygocardiac, mesocardiac, and pterocardiac

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ossicles from a range of species, including the redclaw crayfish (*Cherax quadricarinatus*), powerful crayfish (*Euastacus valentulus*), spanner crab (*Ranina ranina*), mud crab (*Scylla serrata*), and Moreton Bay bug (*Thenus orientalis*). These bands, which consisted of a broad translucent zone bordered by a narrow opaque zone, were hypothesized to record chronological age (Leland et al., 2011). Following that initial study, gastric mill ossicles have been used to age a variety of crustacean species (Bluhm et al., 2019; Gnanalingam et al., 2019; Kilada and Acuña, 2015; Kilada and Ibrahim, 2016; Kilada et al., 2012, 2015; Leland et al., 2015; Mouser et al., 2020). In some studies, the number of observed endocuticular bands corresponded to size-based age estimates, corroborating that the bands signified chronological age (Kilada and Ibrahim, 2016; Leland and Bucher, 2017; Mouser et al., 2020). Additionally, calcein (a calcium-binding fluorescent dye) has been used to stain gastric mill ossicles; the dye was injected before ecdysis and was detected in the ossicle endocuticle after ecdysis, supporting the hypothesis that gastric mill ossicles are retained through the molt and could record chronological age (Kilada et al., 2012; Leland et al., 2015).

Although several studies reported successful age determination using gastric mill ossicles, other studies determined that ossicles are lost during ecdysis and therefore do not record chronological age (Becker et al., 2018; Crook et al., 2018; Sheridan and O'Connor, 2018; Sheridan et al., 2016; Vatcher et al., 2015). Specifically, Vatcher et al. (2015) found that the ossicles of the blue crab (*Callinectes sapidus*) are partially resorbed and then shed at the molt. Further, loose gastric ossicles have been found in the stomachs of Norway lobster (*Nephrops norvegicus*), European lobster (*Homarus gammarus*), and velvet crab (*Necora puber*) after molting, indicating that they were lost during ecdysis (Becker et al., 2018). Sheridan et al. (2016) marked ossicles of *N. norvegicus* with calcein prior to ecdysis and still detected a calcein mark on the gastric ossicles of post-molt lobsters despite the old ossicles being shed within the stomach upon molting. They attributed the presence of the calcein mark on ossicles of post-molt lobsters to a process by which marked ossicles shed in the stomach were digested after molting and the calcein then redistributed in the new ossicles. Gnanalingam et al. (2019) examined the stomachs of post-molt Caribbean spiny lobsters (*Panulirus argus*) immediately after ecdysis and observed intact but decalcified ossicles in the foregut, with ossicles becoming fully calcified by 1-week post-ecdysis. They did not find loose ossicles in the stomach of post-molt lobsters but did find ossicle remnants within the cardiac sac of the shed exuviae. Further complicating the issue, Sheridan and O'Connor (2018) found that ossicles are lost internally and subsequently digested in some European green crabs (*Carcinus maenas*) while other crabs of the same species shed them in their foregut exuvia, suggesting that the fate of calcified gastric ossicles can vary within species. Despite these inconsistencies, the discovery that ossicles are lost at ecdysis has led to widespread skepticism about the validity of using ossicles to age crustaceans.

The physiological process underlying band formation in gastric ossicles is poorly understood and remains a subject of debate. Researchers who have dismissed gastric mill ossicles as a direct ageing method for crustaceans have provided an alternative hypothesis for what causes the endocuticular bands. They suggest that the number of bands correlates to the length of time it requires to complete the deposition of the cuticle following the molt (Becker et al., 2018; Sheridan et al., 2016). Older animals typically are larger and have a thicker carapace, so the hypothesis is that it would require a longer time for larger crustaceans to harden after the molt, resulting in more endocuticular bands. If so, then banding in ossicles should correlate with size but not necessarily chronological age. However, few studies of crustacean ageing have used gastric mill ossicle samples from known-age animals of the same age but sufficiently different sizes to directly examine this relationship (Gnanalingam et al., 2019; Huntsberger et al., 2020).

There have been only a few age validation studies of crustacean gastric ossicles that used known-age animals with known molt histories; those that did had few specimens and/or only young animals (Kilada et al., 2012, 2015; Leland and Bucher, 2017). We previously investigated annual band formation in the gastric ossicles of *P. argus* using known-age animals up to 10 years old and found that the number of bands corresponded to chronological age (Gnanalingam et al., 2019). However, the number of known-age lobsters available was limited ($n = 11$) and lacked known-age lobsters in the 2- to 6-year age range. Additionally, our processing techniques and image quality have greatly improved since that original study. Previously, gastric mill ossicles from *P. argus* were imaged using reflected light (Gnanalingam et al., 2019); however, further study indicated that transmitted light may provide clearer images of the ossicles. Therefore, the objective of this study was to reassess the validity of using ossicles for age determination of *P. argus* by: 1) examining banding in the gastric ossicles over a wide range of known-age lobsters, 2) comparing the precision and accuracy of reader age estimates from known-age *P. argus* ossicles imaged using reflected and transmitted light, and 3) addressing the hypothesis that the number of endocuticular bands within the gastric ossicles relates to animal size as opposed to chronological age.

2. Methods

2.1. Known-age lobsters

Known-age *P. argus* were raised between January 2018 and July 2023 at the Florida Fish and Wildlife Research Institute's South Florida Regional Laboratory in Marathon, Florida (USA). Groups of recently settled pueruli and first-stage juvenile lobsters were collected from floating artificial habitats located 100 m offshore of Big Munson Island (24°37'N, 81°23'W) and Long Key (24°48'N, 80°50'W) in the Florida Keys. Caribbean spiny lobsters have a lengthy planktonic larval phase, usually estimated at 5–7 months, after which they metamorphose into the non-feeding post-larval (puerulus) phase and follow cues to migrate and settle in nearshore macroalgal habitats where they metamorphose into juveniles (Butler and Herrnkind, 1991; Goldstein et al., 2008; Herrnkind and Butler, 1986; Sims and Ingle, 1966). The spiny lobster larval foregut lacks a gastric mill and therefore contains no ossicles. The gastric mill is not formed until the molt to the puerulus stage and is not fully formed until the juvenile stage (Wolfe and Felegenhauer, 1991). For this study, lobsters were assigned age 0 at settlement when the gastric mill is formed, and they were approximately 6 mm in carapace length (CL). Ossicle-based age estimates are thus approximately 5–7 months younger than the true age of lobsters and instead document their age since settlement because of the timing of formation of the gastric mill.

Lobsters were collected as postlarvae from the plankton in January, May, and November of 2018 and November of 2021. Multiple collections were conducted because the survival and growth of postlarvae can vary. Each group of lobsters collected in 2018 was raised communally for the first 2–3 months, then raised individually until they were large enough to tag with a uniquely coded antenna tag. Lobsters collected in November 2021 were raised communally because they were intended to provide younger age-class animals (10 mo–1 yr) for analysis.

Lobsters were fed daily to satiation a diet of frozen shrimp, krill, squid, clams, scallops, and mussels and supplemented with fresh oysters, snails, or mussels once a week. Although raising animals in captivity for age validation introduces potential biases resulting from growth in an artificial environment (Campana, 2001), lobsters in this study were raised in a flow-through seawater system at ambient water temperatures and photoperiod conditions designed to mimic their natural environment to minimize those biases. A constant source of food and limited movement may have altered their growth but should not have affected annuli formation on the ossicles. The growth and molting history of each lobster was recorded starting from the time they were raised individually. Animals from each collection event were sacrificed at specific dates to provide a range of known ages. Lobsters were euthanized for this experiment humanely by immersion in 14°C seawater for 5 min until movement and reactivity to stimuli ceased and then cooling at −20°C for 30 min.

2.2. Processing ossicles

Gastric mills were removed from freshly euthanized lobsters, and the pterocardiac and zygocardiac ossicles were dissected (Fig. 1). Mesocardiac ossicles were not processed because the previous *P. argus* ageing study demonstrated the difficulty in identifying annular bands on those structures (Gnanalingam et al., 2019). Both pterocardiac and zygocardiac ossicles contained paired opaque and translucent zones within the endocuticle; however, only counts from the pterocardiac ossicles were conducted for this study because of their superior readability and greater ease of processing.

Ossicles were either frozen or preserved in a solution of 70 % ethanol, 26 % distilled water, and 4 % glycerol for at least 4 days (Kilada et al., 2015). Ossicles were then cleaned of any excess tissue, air dried (~2 hr), and embedded in epoxy resin (Araldite 502 resin and Aradur HY 956 EN hardener) using silicone molds. A thin (1 mm) layer of epoxy was placed in each mold as a base-layer and dried at 60°C for 3 hours. A final layer of epoxy (2–3 mm) was used to completely submerge each sample, positioning it at the center of the epoxy block. All air bubbles were removed using a metal probe and the epoxy was dried at 60°C for 3 hours or longer to ensure the epoxy was completely hardened. A low-speed IsoMet saw (11–1280, Buehler, Illinois, USA) with two diamond blades was used to make a 300–350 µm-thick transverse cross-section of each pterocardiac ossicle (Fig. 1). The cross sections were polished using 0.3–1.0 micron grit lapping film, wetted with distilled water, and viewed with reflected light on a 3000-LED Accu-Scope compound microscope (10x magnification). Images were then taken with a Jenoptik Gryphax Arktur 8-megapixel microscope camera and digitally enhanced using Adobe Photoshop Elements to increase contrast between the light and dark zones in the endocuticle. To compare imaging techniques between reflected and transmitted light, cross sections were then thinned to approximately 50–100 µm-thick sections using 800 grit wet sandpaper, polished with lapping film, and then imaged with transmitted light. Sections required further sanding because the thickness necessary to produce clear images with reflected light (300–350 µm) was too thick for the transmitted light to penetrate the ossicle. This resulted in two images produced for age estimation from each known-age lobster (Fig. 2).

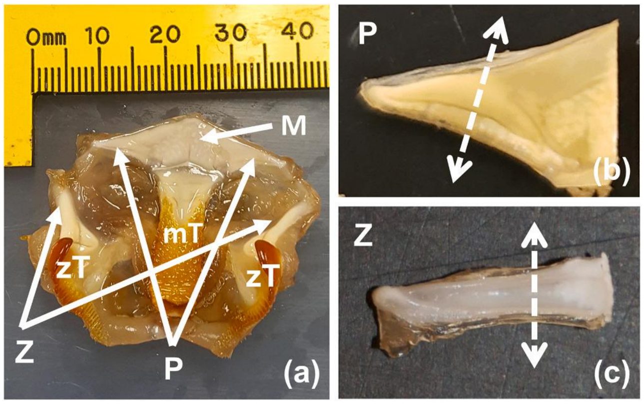

Fig. 1. (a) The gastric mill from a 126.4 mm carapace length Caribbean spiny lobster (*Panulirus argus*) showing the mesocardiac (M), paired pterocardiac (P), and paired zygocardiac (Z) ossicles. The mesocardiac and zygocardiac ossicles also have tooth plates used for grinding food (mT = mesocardiac tooth, zT = zygocardiac teeth). The disarticulated and cleaned ossicles were sliced transversely as indicated by the white arrows for the (b) pterocardiac and (c) zygocardiac ossicles after being embedded in epoxy.

2.3. Age determination

Two readers, each with 5 years of experience ageing ossicles, conducted counts independently, without prior knowledge of the lobsters' CL, sex, or known age. Annular bands were identified as paired opaque and translucent zones within the endocuticle. Age estimates were produced by counting the opaque, typically narrow bands. If there was any material past the last narrow band, readers would indicate that as a "+". For analysis purposes, the "+" was denoted as a half year. As documented previously for other species, the ossicles of *P. argus* contained both a series of more prominent primary bands and less prominent secondary bands located between the primary bands (Kilada et al., 2012; Leland et al., 2011). The more prominent, evenly spaced primary bands were counted as annular bands. In processing the ossicles from the known-age lobsters, we discovered that for *P. argus* there is a false annulus or check before the first annular band (Fig. 2). Consequently, when producing age estimates, the readers used this false annulus to establish the starting point for subsequent counts but did not count this first band in the age estimate. For any image the two readers did not agree on age, they re-examined it together and provided a consensus age.

2.4. Reader precision and accuracy

The precision of counts among the two readers was assessed using the mean coefficient of variation (CV; Chang, 1982). We used age-bias plots to evaluate systematic differences in age estimates among readers and between known-age and consensus-age estimates (Campana et al., 1995). The accuracy of counts was defined as how closely age estimates aligned with the known age, measured as the difference between chronological age and ossicle estimated age. We calculated the percentage of age estimates that agreed exactly (PA-0), were within a half year (PA-0.5), and were within 1 year (PA-1) of the known age. Additionally, we calculated the mean accuracy for the consensus-age estimates as the average difference between known-age and consensus-age.

2.5. Size and age

The differential growth of lobsters in this study allowed for the analysis of endocuticular banding as it relates to lobster size and cuticle deposition time following the molt. The batches of *P. argus* postlarvae grew at different rates, even though they were subjected to the same conditions and feeding schedules. We examined lobsters of the same age but different sizes, as well as those of the same size but different ages. In addition to our known-age lobsters, we also included one partially known-age lobster to increase the age range in the comparison. This lobster was an 83.5 mm CL, egg-bearing female when brought to the laboratory in 2016, estimated to be at least 2 years old at that time based on previous growth and maturity data for lobsters in Florida, and retained for an additional 7.25 years in the laboratory after capture. This lobster was assigned an estimated age of at least 9.25 years old and was used only in the comparison of size and age.

3. Results

3.1. Known-age lobsters

The 33 known-age lobsters in this study ranged in age from 10 months to 5.5 years, and in size from 31 to 169 mm CL (Fig. 3a). Overall, the number of observed paired transparent and opaque primary bands corresponded well to the chronological age of the known-age lobsters (Fig. 4). Post-settlement molting history was recorded for 24 of the 33 known-age lobsters in this study. Molts were not tracked until lobsters were 2–3 months post-settlement after transfer to individual containers; consequently, the total number of molts was slightly fewer than the true

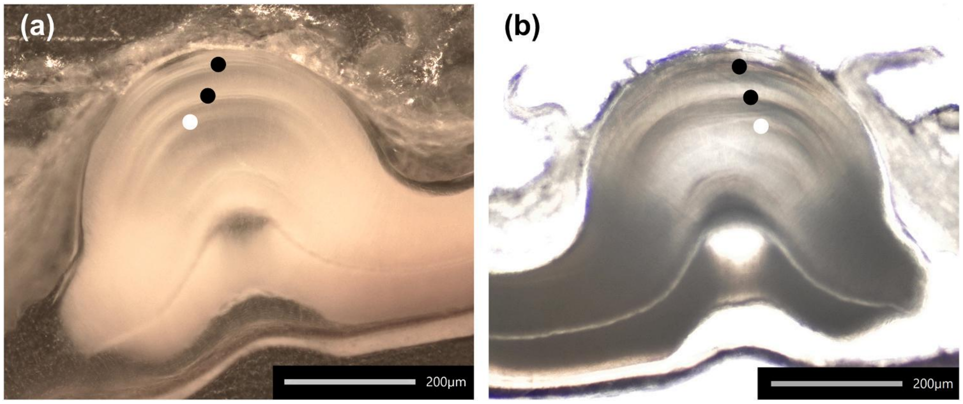

Fig. 2. Images of a sliced pterocardiac ossicle from a 2.6-year-old female known-age lobster (106.5-mm carapace length) with (a) reflected and (b) transmitted light. Annual bands are indicated by the black dots in each image. The first band is a false annulus, is not counted in the age, and is indicated by the white dot in each image.

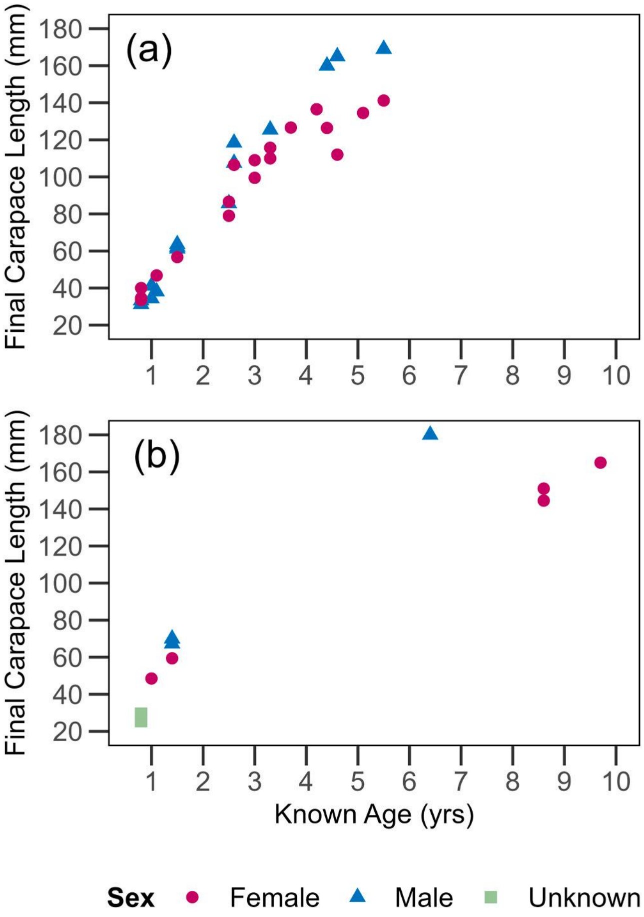

Fig. 3. Age, sex, and final carapace length for all known-age lobsters (a) in this study ($n = 33$) and (b) in Gnanalingam et al. (2019) ($n = 11$; one 11-month-old female lobster not shown due to unknown carapace length).

number that had occurred post-settlement. However, we began recording molts at a similar time for all lobsters in this study, which allowed us to compare the total recorded molts among lobsters. The number of molts increased linearly with age. Lobsters molted approximately 15 times by age 1; by age 2 lobsters molted only 2–3 times per year and continued molting at this rate through age 6. The total molts, however, far exceeded the consensus age estimates provided by the readers, which were based on counts of the primary bands on the ossicles, indicating that the primary bands were not a result of the number of molts (Fig. 5).

3.2. Precision

Age estimates from pterocardiac ossicles were precise regardless of the microscopy method. Mean CV values were 19.6 % and 20.2 % for ossicles imaged with reflected light and transmitted light, respectively. Age-bias plots indicate some bias among readers based on the imaging technique (Fig. 6). With reflected light, Reader 2 often had lower age estimates when compared to Reader 1, whereas with transmitted light Reader 2's estimates were higher for younger lobsters and lower for older lobsters.

3.3. Accuracy

Consensus age estimates for images using transmitted light were approximately twice as accurate for exact percent agreement (PA-0) when compared with reflected light images (15 % and 27 %, respectively) (Table 1). However, the accuracy of consensus age estimates for transmitted and reflected light images was similar for PA-0.5 (70 % and 76 %) and PA-1 (94 % and 97 %). On average, the consensus age estimates were within 0.56 years of the known age for images with reflected light and 0.55 years for images with transmitted light. Overall, consensus age estimates from the readers were slightly higher than the known age, particularly for younger lobsters (Fig. 7).

3.4. Size and age

The accuracy of reader counts of annual bands and the ossicle images for the known-age lobsters in this study demonstrate that the number of primary bands is not dictated by the size of the lobster but instead its chronological age (Fig. 7, Fig. 8). The band counts from the readers aligned closely with the known chronological age of the lobsters (Fig. 7) despite the large variance in lobster size (see Fig. 3a). The batch of lobsters collected in November 2018 grew more slowly compared to lobsters collected in January 2018; this provided a wide range of lobster sizes for ages in which lobsters from both batches were sacrificed. This was particularly evident for known-age 2.5- and 4.5-year-old lobsters, where the range in sizes was 79–118.4 ($n = 6$) and 112–165 ($n = 5$) mm CL, respectively. However, despite the large variance in size the consensus reader estimates for ossicle images with transmitted light were still accurate at 2.75 ± 0.52 (SD) and 4 ± 0.61 (SD) years, respectively.

Images of the ossicles from the known-age lobsters in this study visually demonstrate that the number of primary bands represents the

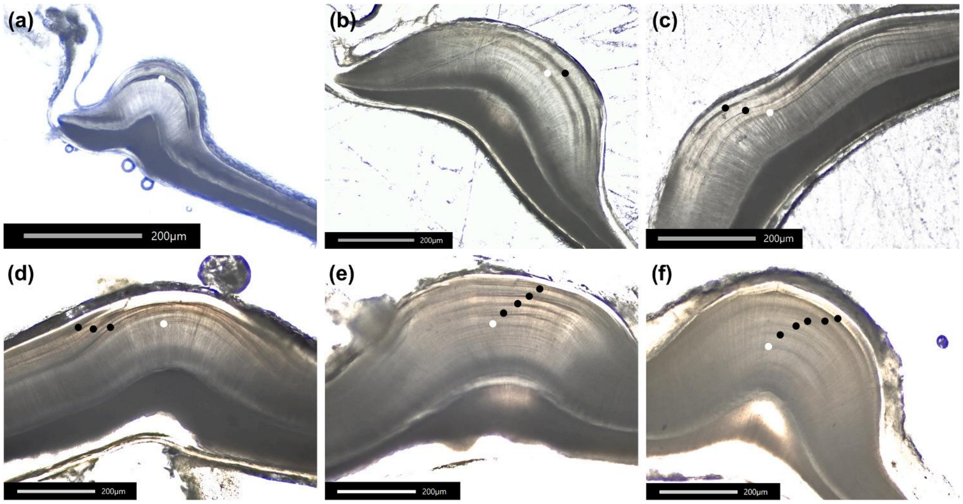

Fig. 4. Annual bands in sections of the pterocardiac ossicle from six known-age lobsters imaged with transmitted light. Annual bands are indicated by the black dots and the first band is a false annulus, not counted, and indicated by the white dot in each image. Lobster information: (a) male, 31.3 mm carapace length (CL), age 10 months; (b) male, 62.2 mm CL, age 1.5 years; (c) female, 86.6 mm CL, age 2.5 years; (d) female, 115.7 mm CL, age 3.5 years; (e) female, 126.4 mm CL, age 4.4 years; (f) male, 169 mm CL, age 5.5 years.

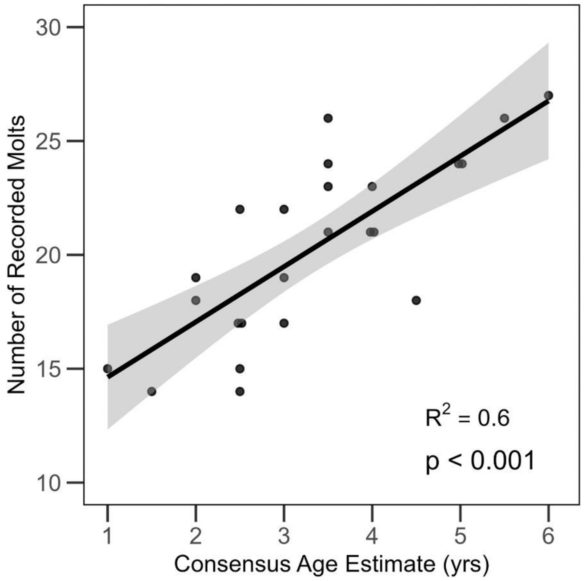

Fig. 5. The number of recorded molts corresponding to the consensus age estimate provided by the two readers (from pterocardiac ossicles imaged with transmitted light) for all known-age lobsters with molting history ($n = 24$). The trend line indicates the linear regression with 95 % confidence intervals. Overlapping values are offset for visualization.

chronological age of the lobster, irrespective of CL (Fig. 8). In Fig. 8a and b the lobsters are the same age (2.5 and 2.6 years old) but have different carapace lengths (85.7 mm and 118.4 mm) and both have two endocuticular bands. The ossicle in Fig. 8c is from a 4.6-year-old lobster with a 112 mm CL (similar in size to the 2.6-year-old animal in Fig. 8b) but shows four bands in its ossicle. The ossicle in Fig. 8d is from a lobster of similar age (4.5 years old) to Fig. 8c, however with a much larger CL (160 mm) and also has four bands in its ossicle. Further, Fig. 8e and f contains images of ossicles from lobsters with similar carapace lengths (141.2 and 145.6 mm); however, one is a known-age 5.5-year-old lobster, and the other is the partially known-age lobster estimated to be at least 9.25 years old, and they show the expected number of bands per their chronological age.

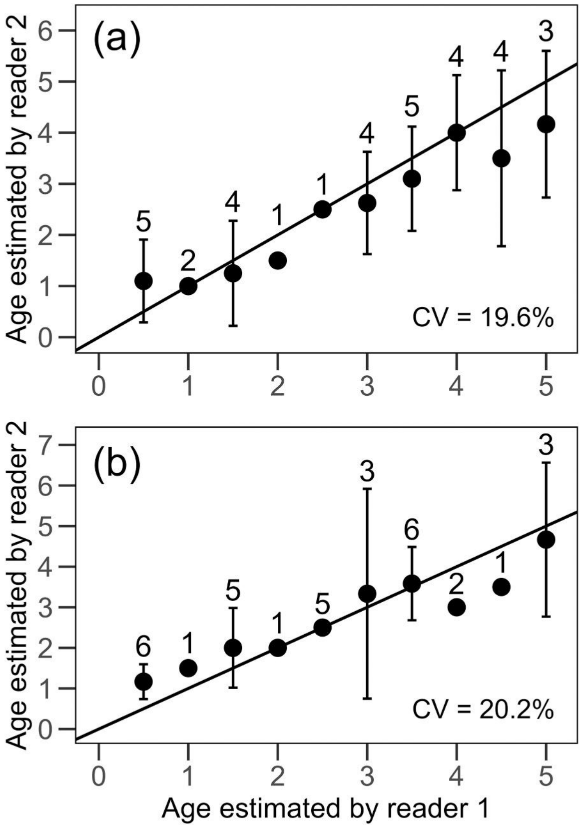

Fig. 6. Age-bias plots for pair-wise comparisons of age estimates of the known-age lobsters from two independent readers for images with (a) reflected and (b) transmitted light. The solid lines represent exact agreement. Error bars represent the 95 % confidence intervals for the mean age assigned by reader 2 to all lobster assigned a given age by reader 1. The mean coefficient of variation (CV) for each image type is provided. The numbers above the points indicate the sample size for each age estimated by reader 1.

Table 1
Percent agreement between reader age estimates and known ages for pterocardiac ossicles imaged with transmitted and reflected light for the 33 known-age lobsters. PA-0 is the percent of age estimates that matched exactly with the known age, PA-0.5 is the percent that were within a half year, and PA-1 is the percent that were within 1 year.

	Reflected Light			Transmitted Light		
	Reader 1	Reader 2	Consensus	Reader 1	Reader 2	Consensus
PA-0	30	30	15	36	24	27
PA-0.5	70	67	76	70	49	70
PA-1	85	91	97	97	88	94

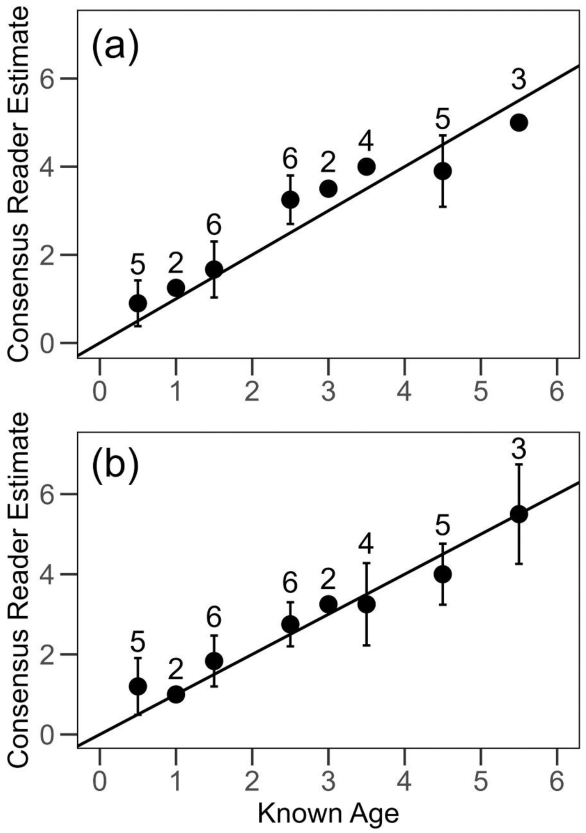

Fig. 7. Age-bias plots comparing the known-age to the consensus-age determined by the two readers for images of pterocardiac ossicles with (a) reflected and (b) transmitted light. The solid lines represent exact agreement and error bars represent 95 % confidence intervals. The numbers above the points represent the sample size for each known age.

3.5. Ossicles during ecdysis

While raising and processing lobsters for this study, we observed that the old gastric mill ossicles of *P. argus* are lost at ecdysis. We found two sets of ossicles in the gastric mill of pre-molt animals; the old ossicle and tooth were present with a larger, newer ossicle and tooth forming (Fig. 9a,b). In a post-molt animal, sacrificed within 12 hours of molting, the gastric mill contained the new larger ossicles that were not fully calcified (Fig. 9c). The gastric mill in the shed exuviae from that same animal contained the old, smaller ossicles, which were decalcified (Fig. 9d).

4. Discussion

The primary goal of this study was to evaluate the use of ossicles for estimating chronological age in the Caribbean spiny lobster. We have increased the sample size and age range of known-age lobsters (Gnanalingam et al., 2019, see Fig. 3) and compared lobsters with different growth and molt history to explore whether gastric mill ossicles in the Caribbean spiny lobster can be used to determine their chronological age. Our results show that the age of *P. argus* can be reliably determined within a half-year accuracy using the pterocardiac ossicles.

Very few studies on crustacean ageing have benefited, as did ours, from a range of known-age animals with which to validate annulus formation. For an ageing method to be fully validated, the annuli formation in each age group must be confirmed (Campana, 2001). Although we did not have known-age animals available to us at the extreme end of their size range (~ 200 mm CL for males; ~160 mm CL for females; SEDAR, 2010), we processed lobsters up to 85 % of their known maximum size and those individuals were 5.5 years of age (Fig. 3a). In the previous *P. argus* ageing study, known-age lobsters reaching 180 mm CL (male, 90 % of maximum size) and 160 mm CL (female, 100 % of maximum size) were 6.5 and 9.7 years of age (Fig. 3b; Gnanalingam et al., 2019). These are size and age estimates well beyond most lobsters landed in the Florida fishery where the mean size of *P. argus* is ~80 mm CL at an estimated age of 18–24 months (Forcucci et al., 1994; Matthews et al., 2009; Sharp et al., 2000). Given the size structure of lobsters within the Florida fishery (Bertelsen and Matthews, 2001) and our age-at-size estimates, it is unlikely that many harvested lobsters grow beyond 3 years of age (Matthews et al., 2009), and therefore the age range validated in this study includes all ages likely to be caught in the Florida fishery.

Although raising captive animals is often not considered the most robust method for age validation, other methods of age validation in use, such as release of known-age and marked animals, length frequency analysis, bomb radiocarbon dating, and mark-recapture of chemically tagged animals all present problems for crustacean species (Campana, 2001). Molting precludes the long-term retention of an external physical tag and the capture of a large percentage of harvestable lobsters each year by the fishery in Florida prevents multiyear periods at large. Size is a potentially biased measure of age for crustaceans because it is highly dependent on temperature, injury, health, or other environmental factors. This was demonstrated with the animals in this study, as they were fed the same diet and subjected to the same conditions, but the size-at-age was considerably different between the animals that settled as postlarvae different times of the year. The reworking and/or loss of the ossicle at the molt inhibits bomb radiocarbon analysis and chemical tagging methods (e.g., calcein). We have tried marking the ossicles of *P. argus* with a barium stable isotope (^{137}Ba) using laser-ablation gas chromatography, a method that has been used for marking fish otoliths (Thorrold et al., 2006); however, like calcein tagging that had been attempted previously (Gnanalingam et al., 2019), barium was unsuccessful due to the reworking of the ossicles at ecdysis. Banding patterns on the ossicles and size-at-age may differ between captive-raised and wild animals and warrants further research; however, results from this study with captive-raised known-age animals demonstrates the potential for ossicles to record chronological age. An interesting example of potential changes in banding in laboratory-raised vs. wild lobsters can be visualized in the ossicles from the partially known-age >9-year-old lobster analyzed in this study. The bands laid down during her time in the wild were darker at the base of the endocuticle in both of her paired pterocardiac ossicles (Fig. 10). Within that region there were three endocuticular bands, which would correspond to her estimated time in the wild. Thus, even though the color of ossicle matrix differed between the time she spent in the wild as compared to the years she was in captivity, annual bands were still evident.

The CV of band counts between readers in this study were similar for pterocardiac ossicle images using reflected (19.6 %) and transmitted

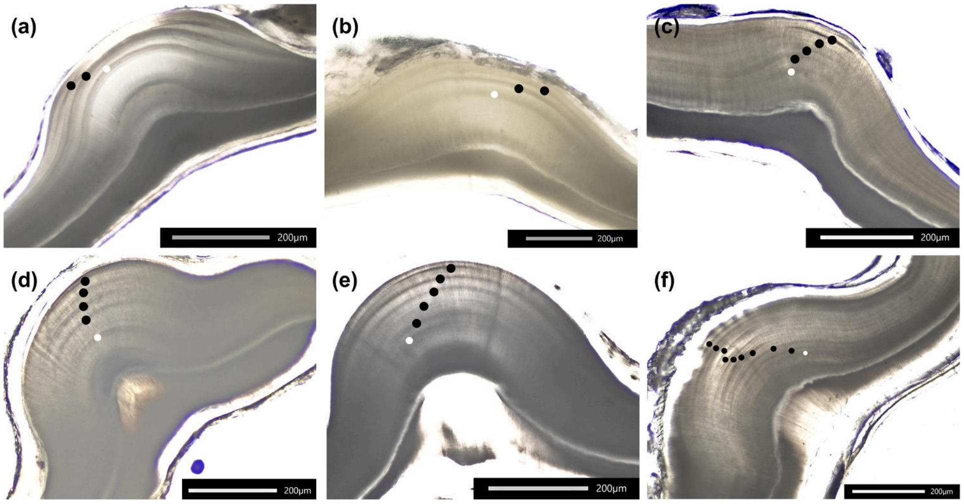

Fig. 8. Examples of pterocardiac ossicles from lobsters with a range in size (carapace length, CL) and known age, demonstrating the independence of annuli counts and lobster size. Annual bands are indicated by the black dots and the first band is a false annulus, not counted, and indicated by the white dot in each image. Lobster information: (a) 2.5-year-old male, 85.7 mm CL; (b) 2.6-year-old male, 118.4 mm CL; (c) 4.6-year-old female, 112 mm CL; (d) 4.5-year-old male, 160.0 mm CL; (e) 5.5-year-old female, 141.2 mm CL; (f) estimated >9-year-old female, 145.6 mm CL. The estimated >9-year-old animal was raised in the lab for 7.25 years and was an 83.5 mm CL egg-bearing female when brought into the laboratory.

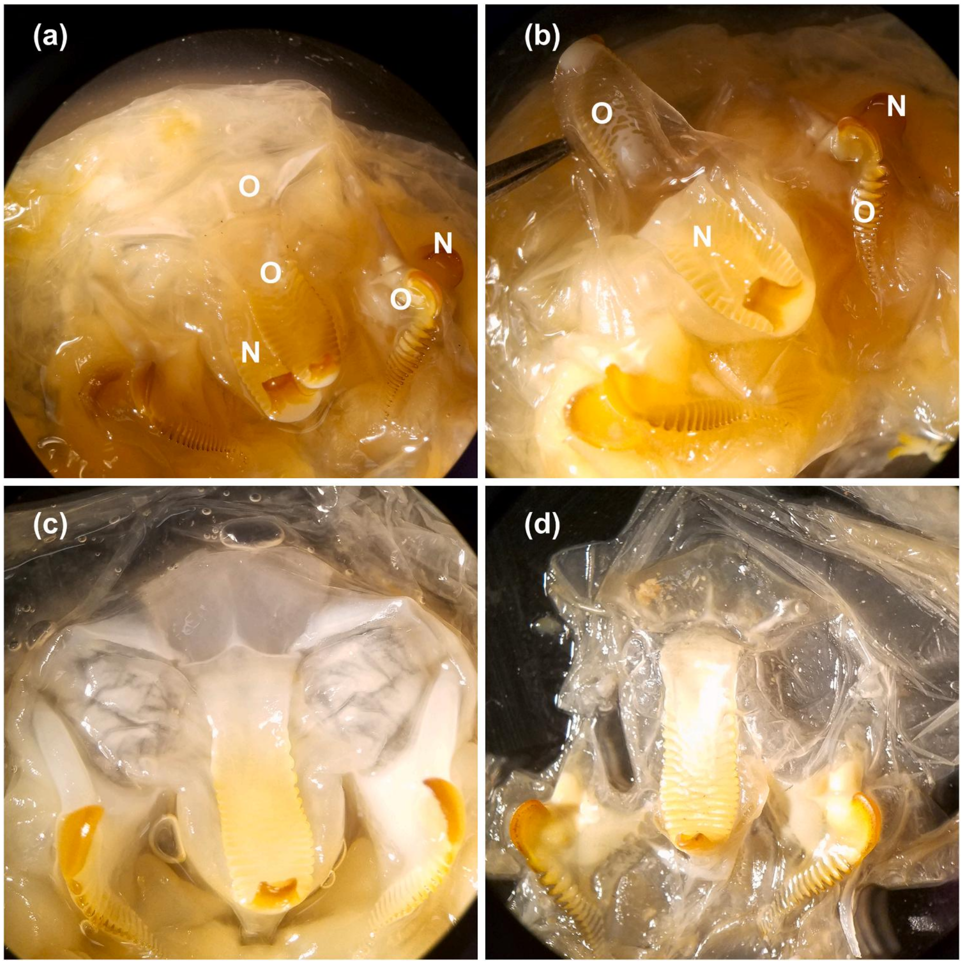

Fig. 9. Photographs of the gastric mill from a pre-molt and post-molt *Panulirus argus.* (a, b) The gastric mill from the pre-molt animal shows two sets of teeth and ossicles with the smaller old ossicles and teeth on top (O) and the larger new ossicles and teeth on bottom (N). (c) The gastric mill from a post-molt animal, sacrificed within 12 hours of molting, shows a fully formed gastric mill containing ossicles that are not yet fully calcified. (d) From that same post-molt animal, the shed gastric mill exuvia contains the old teeth and decalcified ossicles.

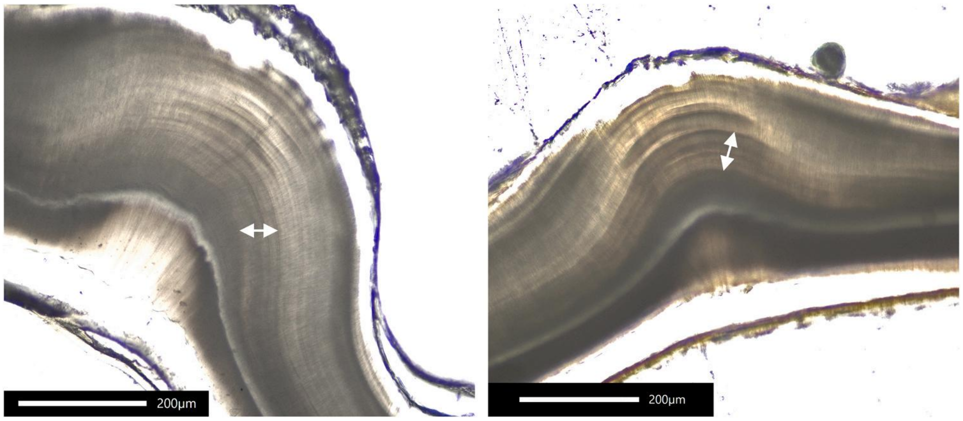

Fig. 10. Images from both pterocardiac ossicles for the partially known-age female lobster that was estimated to be at least 9.25 years old based on carapace length (83.5 mm) when collected from the wild, her egg-bearing status at that time, and the subsequent 7.25 years raised in the laboratory. The arrows indicate the darker region at the base of the endocuticle in both images that would align with years in the wild.

(20.2 %) light and indicate reproducibility of measurements among readers; however, they fall above the generally accepted values for fish otoliths (5–12 %) and bivalves (5–7 %) (Campana, 2001; Kilada et al., 2009). Mean CV values serve as a measure of precision of age estimates between readers, and although no *a priori* target for reader precision can be designated in ageing studies (Campana, 2001), our CV estimates are within the range observed for other crustacean species using this technique. Other studies have shown CV values of 10 % for mesocardiac ossicles of *Homarus americanus* (Kilada et al., 2012), between 15 % and 19 % for mesocardiac ossicles of *Portunus pelagicus* (Kilada and Ibrahim, 2016), 27.9 % across all gastric mill ossicles of *Faxonius neglectus* (Faxon) (Mouser et al., 2020), between 24 % and 33 % for zygocardiac ossicles of known-age *P. argus* (Gnanalingam et al., 2019), and 6 % for mesocardiac ossicles of *Pleuroncodes monodon* and *Cervimunida johni* (Kilada and Acuna, 2015). From this current study we now have a reference collection of known-age ossicles to train readers, which will aid in improving both our precision and accuracy in the future.

Accuracy of reader age estimates indicate transmitted light is the preferred imaging technique for ageing *P. argus* using pterocardiac ossicles. The exact percent agreement between consensus age estimates and known ages for images with transmitted light was approximately double that for images with reflected light. Additionally, 94 % of the consensus age estimates obtained from images with transmitted light were within 1-year of the known age, indicating ageing *P. argus* with pterocardiac ossicles imaged with transmitted light is highly accurate.

We found similar endocuticular banding in both zygocardiac and pterocardiac ossicles. Pterocardiac ossicles were chosen for age estimation due to superior clarity of bands and shorter processing time when compared with zygocardiac ossicles (1.5 hr vs 3 hr). Embedding ossicles at the correct angle was particularly difficult for zygocardiac ossicles because of their concave shape and often resulted in slices that were tilted. Tilted slices had extra material past the growing edge of the ossicle that was difficult to distinguish from true annular bands. Additionally, the groove of the zygocardiac ossicles created air bubbles in the epoxy that prevented proper polishing of the ossicle slices and resulted in prolonged processing times and poor-quality images. In other studies where multiple ossicle types have been examined, researchers have also found a preferred ossicle type. In the redclaw crayfish (*C. quadricarinatus*) (Leland et al., 2015) and the red king crab (*Paralithodes camtschaticus*) (Bluhm et al., 2019) the pterocardiac ossicles produced clearer bands, whereas in ringed crayfish (*F. n. neglectus*) (Mouser et al., 2020) and blue swimmer crabs (*P. pelagicus*) (Kilada and Ibrahim, 2016) zygocardiac ossicles produced more precise age estimates and clearer images. In the previous *P. argus* study it was suggested that the zygocardiac ossicles were the best structure to use when ageing based on the precision of reader estimates (Gnanalingam et al., 2019). However, the pterocardiac ossicles from the known-age animals in that study (Fig. 3b, $n = 11$) were not processed. Those 11 known-age animals were not included in this study, as there were no pterocardiac images to compare.

The prominent primary series of bands in the ossicle endocuticle are deposited annually, as shown by the results of this study; however, it is still unclear what the secondary bands represent. While the molting history of the lobsters in this study showed that the number of molts far exceeded the number of primary bands, it remains possible that the secondary bands are correlated with molting frequency. The number of molts for the known-age lobsters in this study was relatively consistent each year in the laboratory and increased linearly with age. The resolution of the ossicle images in this study, while adequate for counting primary bands, made it difficult to count the secondary bands, and thus we cannot say for certain if secondary bands correlated to molting frequency. Future study of what the secondary banding signifies would benefit from improvements in ossicle processing that could allow for better visualization of the secondary bands.

Using known-age animals in this study allowed us to confirm that *P. argus* deposits one false annulus prior to the first annual band. Identification of the first true annual increment is important for unbiased age validation (Campana, 2001). For example, multiple fish species have otoliths that contain false annuli, often referred to as checks, especially in early years of life (Anderson et al., 1992; Buckmeier and Smith, 2020; Rodriguez-Marin et al., 2019). Accounting for the first false annulus across the full range of ages in this study resulted in accurate age estimates. The previous *P. argus* ageing study did not account for this false annulus, explaining why readers consistently overestimated the age of animals in that study (Gnanalingam et al., 2019). It remains unclear what mechanism causes the false annulus in *P. argus*, but it may result from the highly complex early life history of spiny lobster. The gastric mill does not form until after the 5- to 7-month larval phase when the larvae molt into the puerulus stage and is not formed fully until the molt to the juvenile stage (Wolfe and Felegenhauer, 1991). Hence, it is possible that the false annulus is produced during the transformation from puerulus to juvenile or during the juvenile stage itself.

Ossicles from some animals exhibited what appeared to be a decalcified area adjacent to the membranous layer, or growing edge (Fig. 4d,f; Fig. 8a). This could have been an artifact of the preservation solution, as it was not seen in any of the ossicles that were frozen. Alternatively, it is possible that it was an effect of very warm water temperatures (>32°C) around the time those animals were sacrificed that caused degradation of the ossicles. These ossicles were included in the age estimation analysis, as readers were able to count bands on the peripheral edges of the ossicles.

Recent studies showing that gastric ossicles are lost during ecdysis have challenged the relationship between endocuticular bands and chronological age in crustaceans (Becker et al., 2018; Crook et al., 2018; Sheridan and O'Connor, 2018; Sheridan et al., 2016; Vatcher et al., 2015). In this study we also found that ossicles were shed at ecdysis. However, even though the old ossicles decalcified during ecdysis and were lost with the old exuvia at the molt, it did not preclude the retention of bands in the developing cuticle that corresponded to chronological age, as demonstrated by the results of this study. Leland et al. (2015) suggest a memory-like mechanism whereby previously recorded ossicle bands are duplicated at the molt and added to over time. Crustaceans can preserve information through the molting process, as has been shown with the retention of individual color patterns on crustacean shells that have been used to track individuals (Frisch and Hobbs, 2007; Gallardo-Escárate et al., 2007). For the Caribbean spiny lobster, a unique horn color pattern persists molt to molt over the life of the lobster and is a method we use to track individual lobsters in the laboratory. The exact mechanism as to how bands form on the ossicles is unknown; however, the known-age lobsters in this study indicate that primary bands are deposited annually and record chronological age even though the ossicles are shed at the molt.

Results from this study also provide no evidence that the number of endocuticular bands correlates with the length of time an animal requires to complete the deposition of the cuticle following the molt, a hypothesis suggested by researchers who found that gastric mill ossicles are shed at ecdysis and rejected them as a direct ageing method (Becker et al., 2018; Sheridan et al., 2016). Few studies have measured the thickness of the cuticle and how it changes with age and size in crustaceans. However, Vittori et al. (2020) compared body length and cuticle thickness for two species of terrestrial isopods and found that the thickness of the cuticle corresponded to body size, with cuticle thickness increasing with increasing body length. In the blue crab (*Callinectes sapidus*) it was also shown that the total thickness of the cuticle was positively correlated with carapace size (Waugh et al., 2009). With larger animals having a thicker cuticle, it was theorized they would take longer to harden post-molt and therefore have more endocuticular bands (Becker at al., 2018). Typically, older animals are larger, and thus there would be a secondary correlation to chronological age as opposed to a direct relationship between age and number of endocuticular bands. We used known-age crustaceans of the same age but different sizes and same size but different ages to examine this relationship. We were able to show with ossicle images and accuracy of reader counts of the number of endocuticular bands from our known-age *P. argus* that the number of primary bands was not related to the size of the animal but to its chronological age.

The controversy surrounding the species-specific fate of the ossicles through the molt and the validity of using band counts to age crustaceans warrants further investigation into the mechanism by which bands in the endocuticle of gastric mill ossicles are formed. At present, the mechanism driving the formation of endocuticular bands in the crustacean gastric mill ossicles is unclear. Although we have validated annual band deposition in ossicles for the Caribbean spiny lobster, uncertainty over the mechanism of deposition hinders the acceptance of this method and greatly impedes validation of the methodology for wild-caught lobsters across environmental gradients. Future research into the mechanism of annuli formation is needed for developing this technique further and understanding if it can be applied throughout the range of *P. argus* and to other crustacean species.

5. Conclusions

Although there remains uncertainty about the biophysical mechanism underlying the formation of bands on ossicles, we have demonstrated the presence of annular bands in known-age Caribbean spiny lobster that were read to within a half-year accuracy using the pterocardiac ossicles. The Caribbean spiny lobster fishery is one of the largest lobster fisheries in the world and one of Florida's most valuable fisheries with ex-vessel values ranging from US$20 to US$50 million annually (Florida Fish and Wildlife Conservation Commission, unpubl. data; FWC, 2021; Phillips et al., 2013; Wahle et al., 2020). However, the development of an accurate stock assessment has been hindered by the previous inability to directly determine the age of lobsters. Directly ageing *P. argus* using gastric mill ossicles will provide essential life-history measures and inform age-based management of the Caribbean spiny lobster. Further research will compare size, age, and sexual maturity between regions in Florida to improve our understanding of population structure, longevity, size, and reproductive maturity at age for *P. argus*.

CRediT authorship contribution statement

Erica Ross: Writing – review & editing, Methodology, Investigation, Formal analysis. **Mark J Butler IV:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Samantha Hagedorn:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Thomas R Matthews:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Emily Hutchinson:** Writing – original draft, Visualization, Supervision, Project administration, Methodology, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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