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Investigation of the Definitive Age of Freshwater Decapod Crustaceans, *Orconectes propinquus* and *Procambarus clarkii*, via Growth Bands in the Calcified Regions Of the Eyestalk and Gastric Mill

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Investigation of the Definitive Age of Freshwater Decapod Crustaceans, *Orconectes propinquus* and *Procambarus clarkii*, via Growth Bands in the Calcified Regions Of the Eyestalk and Gastric Mill

Jayson L. Clore

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In

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ABSTRACT

A preliminary study that utilized the calcified regions of the eyestalks and gastric mills of decapod crustaceans for chronological age determination. The procedure is based on the traditional method for growth analysis of concentric growth bands in otoliths, shells, and genital plates. Transverse cross sections of the eyestalks and prepyloric, pyloric, and zygocardiac ossicles of the gastric mills of *Orconectes propinquus* and *Procambarus clarkii* crayfish showed the presence of alternating dark and light growth bands. These growth bands were recorded as a finer secondary series of growth bands found within the broader primary growth bands. Size modal comparisons showed that the secondary growth bands accumulated and became broader with increasingly mature crayfish specimens. Secondary growth bands potentially identified the molt history of the crayfish and post-molting growth of the calcified structures.

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INTRODUCTION

Worldwide there are over 550 freshwater crayfish species, with ~390 species in North America and 338 of these species in the United States. In the United States, 65 of these crayfish species are endangered and thus accurate assessment of these crayfish populations is critical in protecting them from extinction (Helfrich et al., 2009). Crayfish are also crucial invertebrates in many different freshwater aquatic ecosystems. They are omnivorous and consume other aquatic organisms as well as organic matter. Moreover, they also serve as an important food source for many higher topic levels, including humans. The Louisiana red-swamp crayfish species, *Procambarus clarkii*, is one such important decapod crustacean in commercial food sales. The United States produces over 90% of crayfish harvested worldwide at a commercial value of nearly 200 million dollars annually (Helfrich et al., 2009; LSU, 2013).

The accurate age determination of freshwater crayfish is important not only for regulation of commercial harvesting, but also for many research applications. Age determination of decapod crustaceans, and for that matter most invertebrates, is severely limited by the molting process where their entire exoskeletons are shed (Holdich, 2002; Kuballa & Elizur, 2007). This molting process creates a lack of permanent structures for age assessment. Hence, without a direct method for aging decapod crustaceans, indirect methods are utilized. Furthermore, determining the age of freshwater decapod crustaceans is often a formidable task due to the variations in species, gender, environmental conditions, and available nutrition.

Indirect age estimation methods such as size modal analysis are limited in that they are only applicable to the populations and years under examination. These size models also do not provide the age of the specific specimens, but rather an age estimate for animals of roughly the

same size (Campana, 2001; Farmer, 1973; France et al., 1991). This is important, because this method becomes increasingly unreliable for longer-lived species. Older, slower growing specimens may be grouped in the same size-age group as younger, faster growing specimens (Vogt, 2012).

Direct age determination has been done using lipofuscin accumulation in neural tissues as a result of oxidative processes. This permanent oxidative protein and lipid formation occurs throughout the life of the specimen and accumulates with age (Kodama et al., 2006; Vogt, 2012). The continuous accumulation of lipofuscin has been identified in the medulla terminalis of the eyestalk and optic lobe (Belchier et al., 1998; Maxwell et al., 2007; Sheehy, 1990; Sheehy et al., 1994). This model of biological age determination is limited due to the fact that lipofuscin is directly influenced by the environment and specific specimen (Vogt, 2012).

We propose a more precise, consistent and extrapolative method for ascertaining the actual chronological age of decapod crustaceans. In the past, tallying band deposits on calcified structures has been used for age determination of many aquatic animals. Previous studies have shown these aging patterns in the otoliths of fishes (Admassu & Casselman, 2000; Campana, 2001; Campana, 1984; Campana et al., 1985; Ewing et al., 2003; Lee & Byun, 1996; Neilson & Geen, 1982; Pannella, 1971; Smith & Deguara, 2003), shells of bivalves (Kilada et al., 2009; Thompson et al., 1980), genital plates of sea urchins (Flores et al., 2010), and more recently in eyestalks and gastric mills of saltwater crustaceans (Kilada et al., 2012) (Figure 1).

Age determination for fish has been somewhat straightforward and well documented in that they retain calcified otoliths for the extent of their lifetime. The banding patterns for these structures appear to occur annually (Ewing et al., 2003; Smith & Deguara, 2003). However, for freshwater crustaceans growth bands have been recognized with a primary and secondary set of

growth bands. The secondary growth bands appear as fine, microstructural growth between the broader, more noticeable, primary bands (Campana, 1984; Lee & Byun, 1996). This pattern has been identified for calcified structures of various animals (Admassu & Casselman, 2000; Nolan & Clarke, 1993), including freshwater crayfish (Leland et al., 2011).

Freshwater crayfish size can depend on species, gender, habitat, and nutrition availability. These factors often make it immensely more difficult to accurately determine the age of a specimen. This study will apply a method based on counting growth bands deposited on the calcified structures of theeyestalks and gastric mill of *Orconectes propinquus* and *Procambarus clarkii*. Our expectation is that our age determination method will allow for a more reliable and universal age determination for freshwater crayfish independent of size and nutrition.

The crayfish eyestalk wraps around the optic nerves and is located between the eye and cerebral ganglion (Figure 2). The foregut can be divided into three major regions, the esophagus, anterior cardiac stomach, and posterior, pyloric stomach (Chisaka & Kozawa, 2003). The gastric mill is housed within the cardiac stomach of the foregut (Figure 2). This structure is used for mastication of food particles in order to allow passage through the rest of the digestive system (McGaw, 2006; MaGaw et al., 2013). The gastric mill is composed of the central prepyloric and pyloric ossicles, with opposing zygocardiac ossicles (Chisaka & Kozawa, 2003) (Figure 3).

The banding patterns of the eyestalk and gastric mill could vary on whether the banding is caused by changing of the seasons, molting, or continuous growth. The presence of primary and secondary growth band series might be caused by different events as well. If the primary banding patterns are evenly distributed bands, then each year could correlate to a separate band. This could be explained by the warmer and cooler seasons that are encountered by *Orconectes propinquus*. However, the seasonal differences are much milder for *Procambarus clarkii* due to

their presence in humid subtropic regions, such as Louisiana. This would cause less prevalent primary banding patterns.

Secondary growth bands are potentially records of the molt history. This banding could be possible after molting with the initial strengthening of the gastric mill from the calcium stored as gastroliths. Gastroliths are amorphous calcium carbonate deposits found during each premolt on the anterior lateral walls of the cardiac stomach (Luquet et al., 2013). If the secondary banding patterns are due to molting, then there would be many close secondary bands that spread out as the gastric mill grows. This would likely be caused by increased molting frequency as juveniles and less frequent bands in the adult crayfish that molt less often. However, either banding pattern also has the potential to be independent of either hypothesis and could be due to normal, continuous growth.

MATERIALS AND METHODS

Animals used in this study were obtained from local aquatic ecosystems and ordered from another region of the United States. The *Orconectes propinquus* were harvested from streams and rivers in west Michigan. *Orconectes propinquus* was selected due to its availability and prevalence in Michigan. These northern North American crayfish allow for the study of the growth bands for temperate climate species in which there are prevalent seasonal changes. The *Procambarus clarkii* crayfish were ordered from the Louisiana agriculture industry. *Procambarus clarkii* was chosen because of its importance as a commercial harvested food source and the fact that it is indigenous to humid subtropic regions.

Orconectes propinquus and *Procambarus clarkii* crayfish were anesthetized using an ice water bath. After about 15 minutes, each crayfish was removed from the bath to be measured and gender identified before dissection. Measurements were recorded from the anterior end of the rostrum to the posterior edge of the cephalothorax (Figure 4). The eyestalks were removed and collected. The carapace was then removed to expose the gastric mill. The entire gastric mill was dissected from the specimen to allow for detachment of the prepyloric, pyloric and zygocardiac ossicles under stereoscope magnification. The eyestalk was bisected along the midline into left and right halves. This allowed for improved sectioning quality. The prepyloric (PP), pyloric (PY) and zygocardiac (ZC) ossicles were separated as well to allow for improved sectioning (Figure 3). All samples were cleaned of all remaining organic materials.

Isolated regions of the eyestalk and gastric mill were then placed in fresh 4% paraformaldehyde (PFA) for ≥48 hours at 4°C to allow for perfusion. This PFA fixative was created using a 7.4 pH phosphate buffer solution (10X PBS) to better preserve the tissue.

Samples were then removed from the 4% PFA and washed three times with 1X PBS.

Dehydration was also done by placing the samples in 70% ethanol for 5 minutes, followed by 95% ethanol for 5 minutes (Table 2). This procedure was adjusted from the original (Table 1) to allow for a less brittle sample during sectioning. The samples were treated with xylene for 20 minutes in order to clean up the tissue. Further adjustment of the procedure by only storing the isolated samples in exclusively 70% ethanol (Leland et al., 2011) for >24 hours permitted further conservation of the calcified structures after sectioning. Lastly, the samples were placed in a heated paraffin bath (61°C) for 20 minutes prior to embedding, allowing for permeation of paraffin into the samples. A Micron® paraffin embedding station was used to embed the tissues for later sectioning. Samples were orientated in the paraffin in a manner to allow for proper orientation of cross sections. Embedded samples were allowed to solidify within tissue cassettes for 24 hours at 4°C.

Sectioning of the samples embedded in paraffin was done using a Micron® microtome. 20μm sections were prepared, allowing for appropriate tissue transparency while maintaining continuity. Serial transverse cross sections were created using the microtome. Sections were unfurled and adhered to glass slides using a warm water bath (37°C). Slide samples were examined and documented using an Olympus BX51 digital microscope. Slide images were digitally processed using Adobe Photoshop. Growth bands were documented and easily recognizable as dark and light alterations in the endocuticle.

RESULTS AND DISCUSSION

Both *Orconectes propinquus* and *Procambarus clarkii* crayfish species were identified as having growth bands in the eyestalk and gastric mill. Representative cross sections were acquired from the prepyloric ossicles of the gastric mills. Most samples were documented within 24 hours to avoid any further dehydration or crumbling of the sectioned samples. However, unsectioned paraffin embedded samples appeared to retain their composition and clarity for weeks if necessary.

Comparison of the different sectioned regions demonstrated that growth band numbers often differed, but could be explained as a result of positioning and depth of the section within the sample. Similar discrepancies in growth count precision were found in other studies, however, the ossicles found with the highest number of growth bands were deemed necessary. (Beamish & McFarlane, 1983; Campana, 2001; Horn, 2002; Leland et al., 2011).

Cross sections of the prepyloric, pyloric and zygocardiac ossicles showed the presence of two types of growth bands. The growth bands were identified as dark and light alternating regions of the endocuticles. These growth bands were recorded as a finer secondary series of growth bands found within the broader primary growth bands (Figure 5 & Figure 6). More juvenile crayfish contained 0 – 1 primary growth bands, while more mature crayfish contained 2 – 3 primary growth bands. The presence of additional secondary growth bands appeared to increase with size (Figure 8 & Table 3) and age. This was similar to the finding of Leland, Coughran, and Bucher (2011).

The finer, secondary growth bands appear to widen progressively across the endocuticle (Figure 7). This pattern could be explained by the molt history of the crayfish and post-molting

growth of the ossicles. However, this is only speculation, as it could be merely a record of growth over time, independent of molt cycles.

The broader, primary growth bands could be identified as an annual period growth (Figures 5 & 6). However, without specific ages for the crayfish samples, it is difficult to determine the exact nature of the primary bands. Cross sections of the eyestalks revealed banding patterns similar to that of the gastric mill.

CONCLUSION

Cross sections of the eyestalks and prepyloric, pyloric and zygocardiac ossicles showed the presence of two types of growth bands. The growth bands were identified as dark and light alternating regions of the endocuticles (Figures 5-7). These growth bands were recorded as a finer secondary series of growth bands found within the broader primary growth bands. The secondary growth band counts were compared with size modal analyses and showed that the size of the crayfish was proportional to the number of secondary growth bands (Figure 8).

Although this study found a clear presence of growth record within the ossicles, determining the exact period of time between both the primary and secondary series is of great importance. More research is needed in order to determine exactly what each band represents. This might involve the rearing and periodic recordings of crayfish growth bands over an extensive period of time, including the time lapse between molts. More research could also be done in order to compare the aging results of lipofuscin assays with those of calcified banding.

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Figure 6: Primary Growth Bands

Figure 7: Secondary Growth Bands

Figure 8: Graph Comparing Size and Number of Growth Bands

Figure 1: Seen here are growth bands in the gastric mill, a tooth-like digestive structure, from an American lobster, *Homarus americanus* (Kilada et al., 2012).

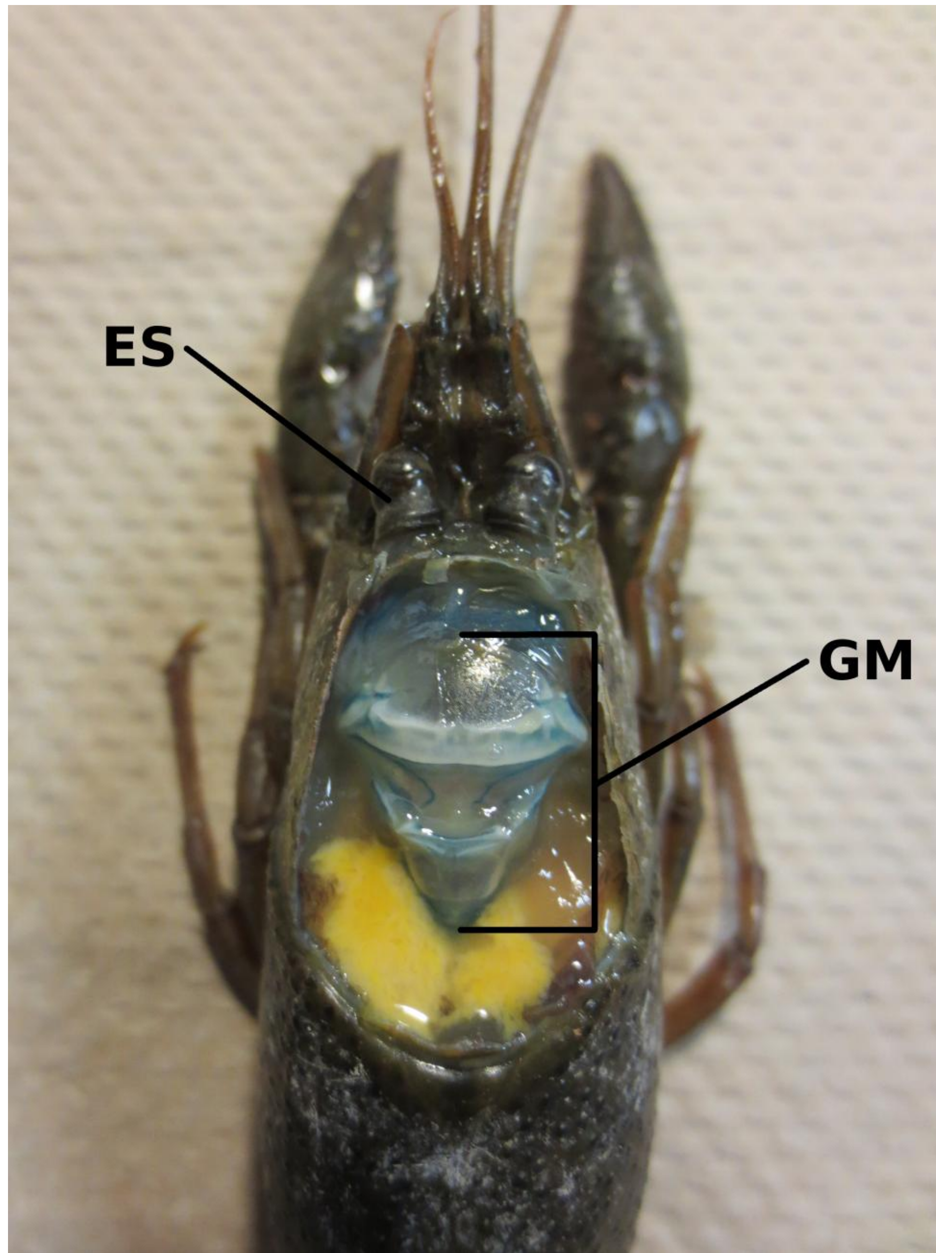

Figure 2: Internal anatomy of the crayfish showing the location and position of the eyestalk (ES) and gastric mill (GM).

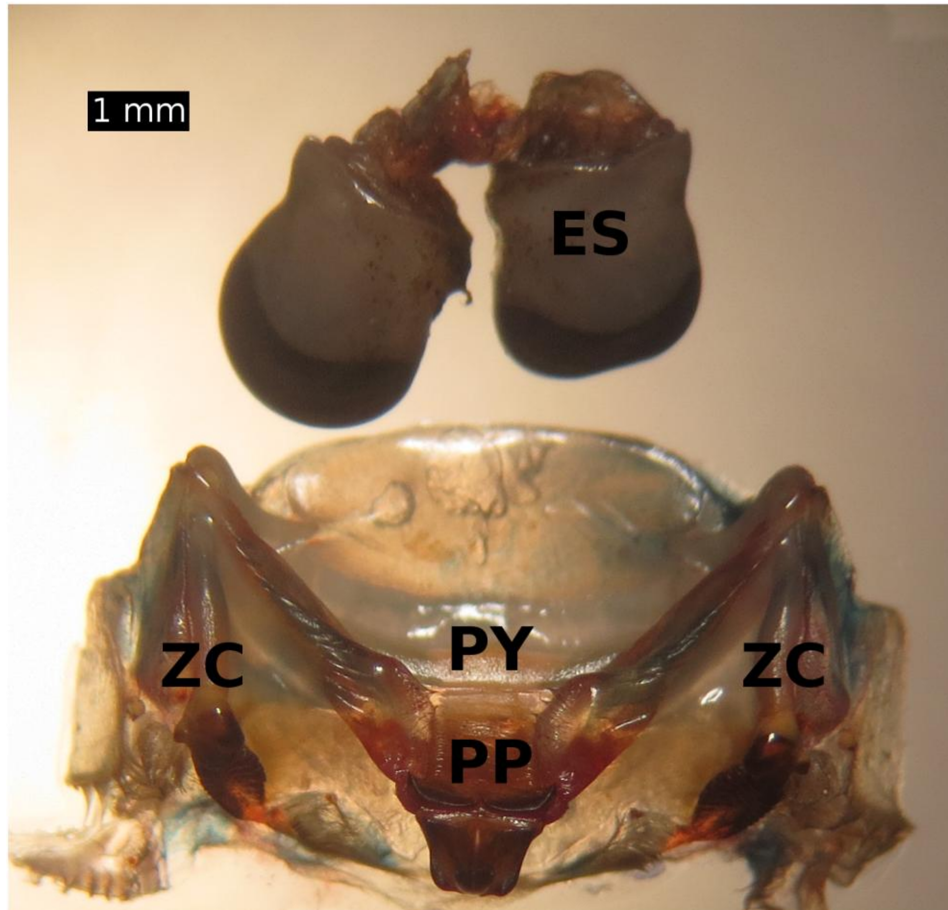

Figure 3: Eyestalk (ES) and gastric mill isolated from the crayfish, *Orconectes propinquus*. The prepyloric ossicle (PP), pyloric ossicle (PY), and zygocardiac ossicles (ZC) of the gastric mill are labeled.

Figure 4: Position of caliper for size modal analysis shown on a *Procambarus clarkii*. The measurement is taken from the anterior end of the rostrum to the posterior edge of the cephalothorax.

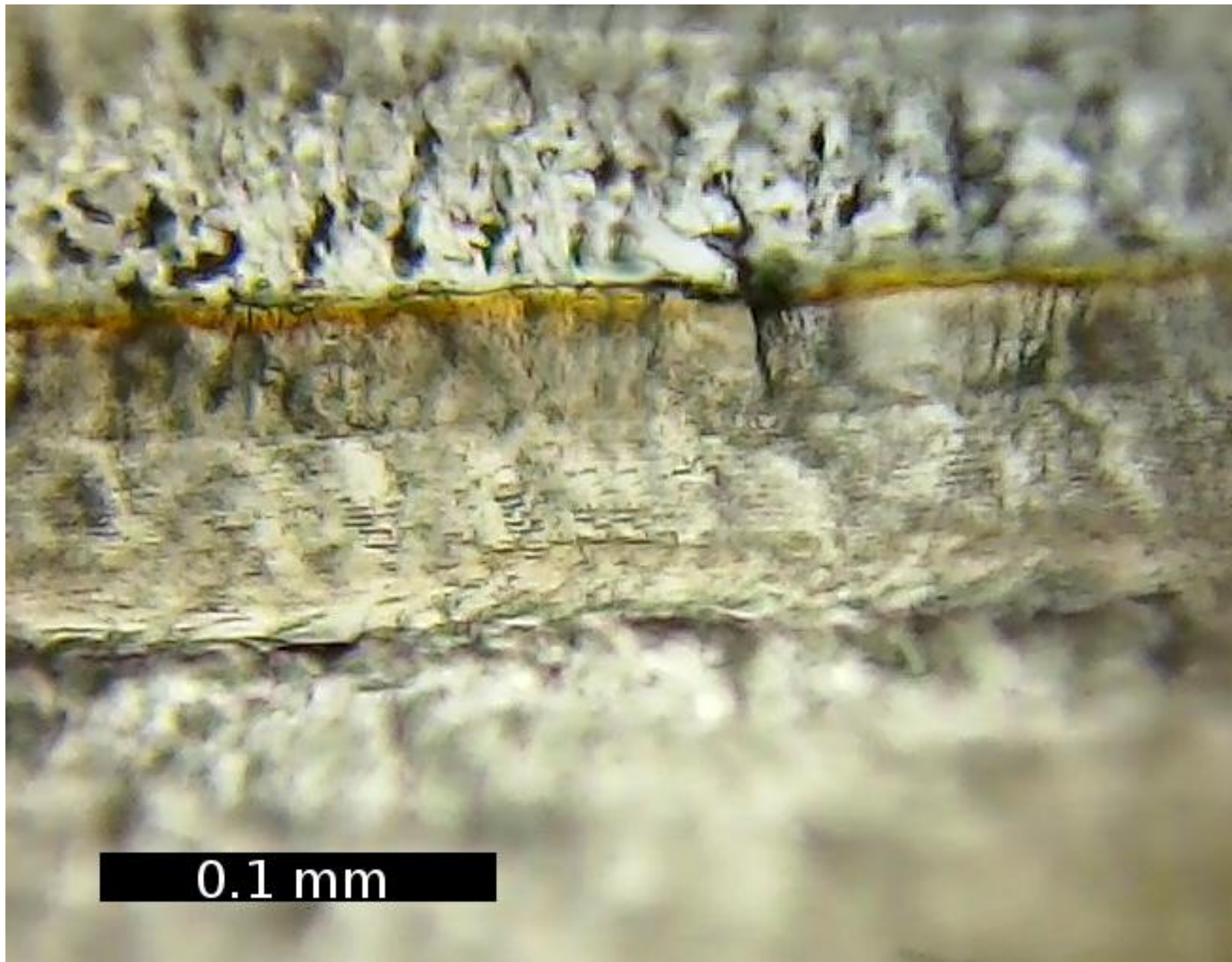

Figure 5: Microscopic image (40X) taken of the prepyloric ossicle from an *Orconectes propinquus*. Image shows the presence of both primary and secondary bands.

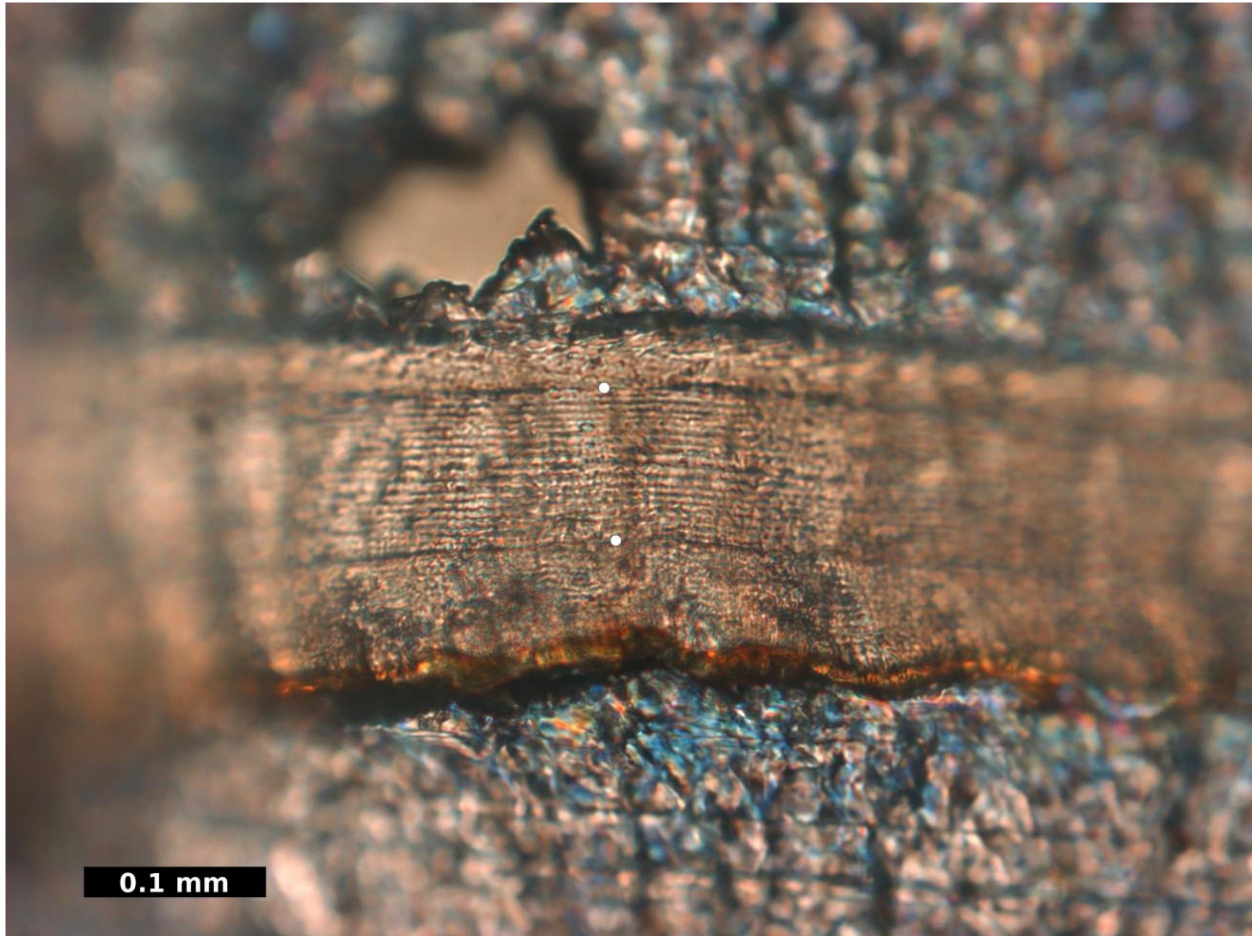

Figure 6: Microscopic image (20X) taken of the prepyloric ossicle from a *Procambarus clarkii*. Image shows the presence of two primary bands (white circles) and numerous secondary bands between.

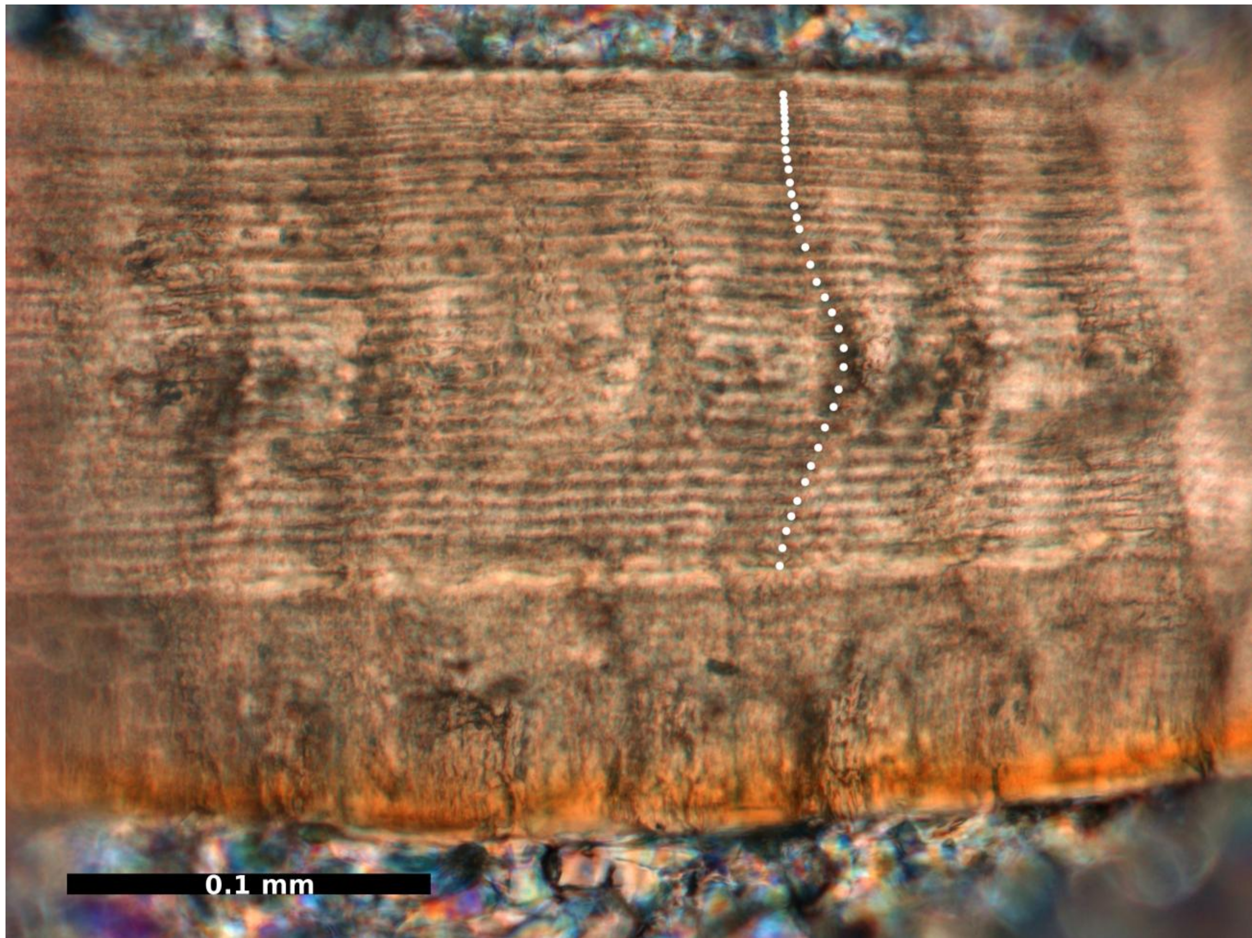

Figure 7: Microscopic image (40X) taken of the prepyloric ossicle from a *Procambarus clarkii*. Image shows the presence of secondary bands (white circles) within the endocuticle.

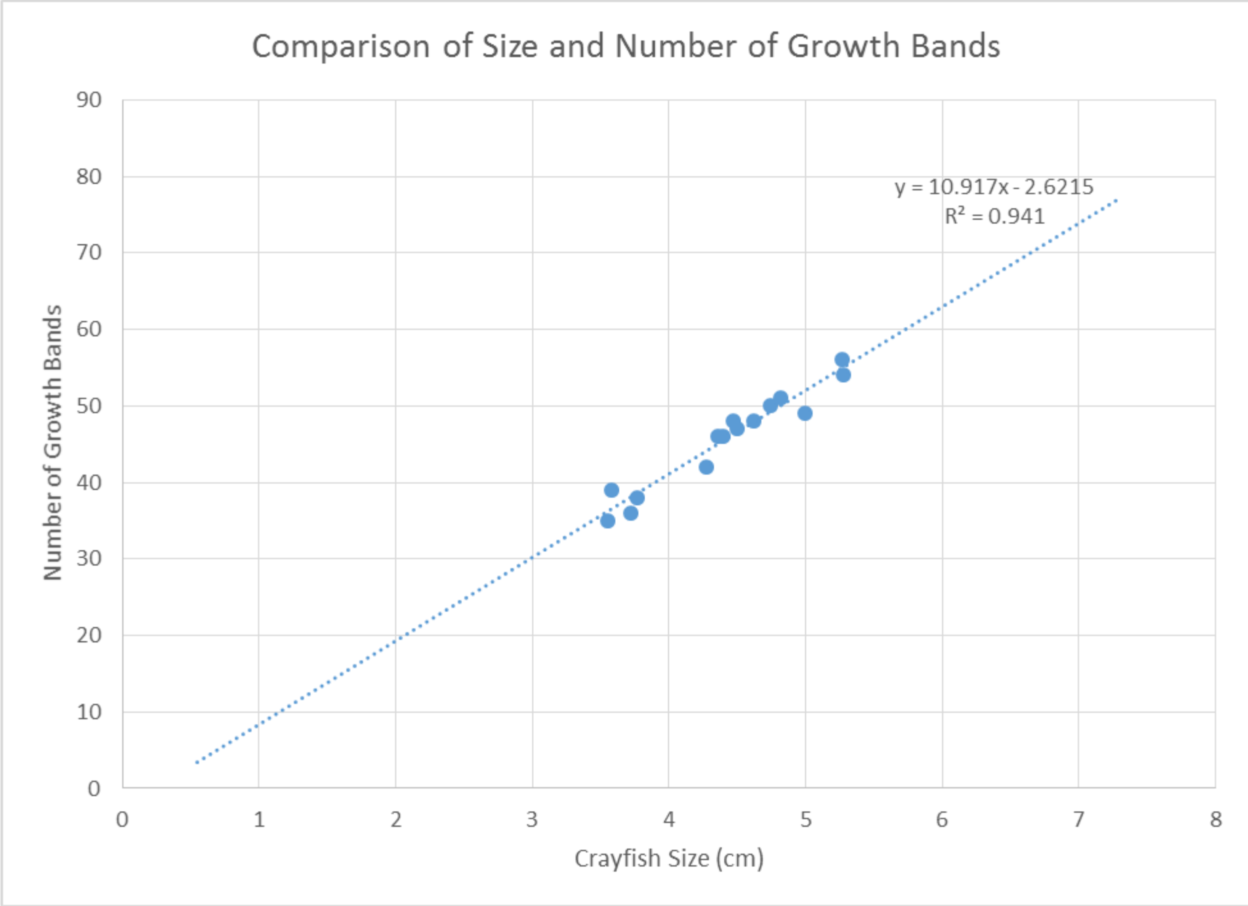

Figure 8: Comparison of the size of the *Procambarus clarkii* crayfish to the number of secondary growth bands within the endocuticle.

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Table 1: Initial Tissue Preparation Procedure

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Table 3: Comparison of Size and Number of Growth Bands

4% PFA (4°C)	≥ 48 hours
1X PBS	Wash (X3)
70% Ethanol	15 min
95% Ethanol	15 min (X2)
100% Ethanol	15 min
Xylene	15 min (X2)
Paraffin (61°C)	60 min

Table 1: Initial tissue preservation, dehydration, and pre-embedding procedure.

4% PFA (4°C)	≥ 48 hours
1X PBS	Wash
70% Ethanol	5 min
95% Ethanol	5 min
Xylene	20 min
Paraffin (61°C)	15 min

Table 2: Final tissue preservation, dehydration, and pre-embedding procedure. This procedure was implemented in order to allow for more quality cross-sections to be obtained.

P. clarkii	Size (cm)	Band Count
1	3.55	35
2	3.58	39
3	3.72	36
4	3.77	38
5	4.27	42
6	4.36	46
7	4.40	46
8	4.47	48
9	4.50	47
10	4.62	48
11	4.74	50
12	4.82	51
13	5.00	49
14	5.27	56
15	5.28	54

Table 3: Comparison of the size of the *Procambarus clarkii* crayfish to the number of secondary growth bands within the endocuticle.