Determination of Growth Bands in *Panulirus interruptus* (California Spiny Lobster)
Protocol constructed by Lauren Zaragoza for the CDFW in June-August 2019

Description:

This paper identifies the methodology for analyzing growth bands in *Panulirus interruptus*, the California Spiny Lobster. It was developed after an extensive review on aging alternative crustacean species. The methodology for this study was based off of Kilada et al. 2015, Gnanalingham et al. 2018, and Los Alamitos DFG Otolith Thin Sectioning Protocol by Kelley Voss.

Background:

The CDFW manages *P. interruptus* as it poses an important role in commercial and recreational fisheries (CDFW, 2019). To carefully manage and prevent overexploitation of the *P. interruptus* fishery, an understanding of population dynamics, stock assessments, and life history traits is necessary. These traits include longevity, mortality number, growth rate, and age-at-maturity. However, these traits fundamentally depend on an accurate determination of age which historically has been difficult due to periodic and seasonal ecdysis of crustaceans which limits the possibility of age determination. Age is usually achieved via band counts on hard calcium structures of wild animals such as bones, scales, otoliths in fishes, and shells of bivalves (Kilada et al. 2017).

Crustaceans, including *P. interruptus*, grow discontinuously through a series of molts separated by intermolts. During crustacean intermolt, growth is restricted due to its hard, inflexible exoskeleton, or integument. During the molting phase, the integument is shed and rapid growth occurs over a short period of time. The molting period has led to the assumption of the loss of all calcium structures and therefore, no way to directly determine the age of crustaceans (Kilada et al. 2017). With the lack of information on accurate age determination, current management practices are left to be decided without a full understanding of the life history of *P. interruptus*. Current models for the *P. interruptus* fishery rely on size modal analysis which involves the use of carapace length and width frequency to separate lobsters into size and age classes. This method leads to uncertainties between distinguishing slow-growing, old individuals and fast-growing, young individuals and therefore becomes an unreliable management tool. In a review on age determination of crustaceans done by Kilada et al. (2017), the size modal analysis was used in 83% of the 231 papers found in the literature, further pressing the needs for a more accurate method for age determination (Kilada et al. 2012, 2017).

In Kilada et al. (2012), the use of the fluorescent marker lipofuscin, which is a brain pigment correlated with animal senescence was observed after three molting events of *Homarus americanus*, the American lobster. Lipofuscin persisted in the eyestalks and the gastric mill ossicles. This led to the proposal that growth bands are also retained in these structures and age determination may be possible via the highest band counts. In a more recent study and the same genus as *P. interruptus*, age determination was performed by counting bands of the gastric mill ossicles for *Panulirus argus*, the Caribbean spiny lobster by Gnanalingham et al. (2019).

In this study, *Panulirus interruptus* was the focus species for the determination of band counts in the gastric mill ossicles and eyestalks.

Methodology:

I. **Sampling:** Allow for 4 days from the time of dissection to begin processing

Materials: Dissecting tray, dissecting scissors, scalpel, forceps, bucket, small vials

Sampling: Lobsters for study were primarily caught off the coast of Point Loma, San Diego -CA and Los Alamitos, Los Angeles –CA by divers in July 2019. The carapace length (CL; measured by calipers to the nearest mm), and sex were recorded. Existing tags, disease, or injuries were also noted. Lobsters were kept in a recirculating system tank until euthanized via rapid cold exposure (salt water ice slurry) where they were then dissected for their eyestalks and gastric mills. The eyestalks were detached with scalpel or dissecting scissors. The carapace was cut off and the gastric mill was dissected (Figure 1). The gastric mill was kept together via connective tissue and all exterior soft tissue was removed (Figure 2). The structures were stored in labelled vials that contained a solution of 70% ethanol, 4% glycerol, and 26% water (Gnanalingham et al. 2019). SDSU grad and post-doc students took measurements and removed the egg sacs of 3 females for an alternative study.

Fig 1.

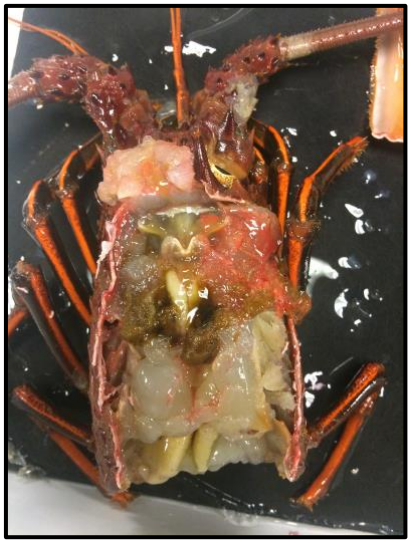

Figure 1. Carapace cut off, Gastric mill exposed.

Fig 2.

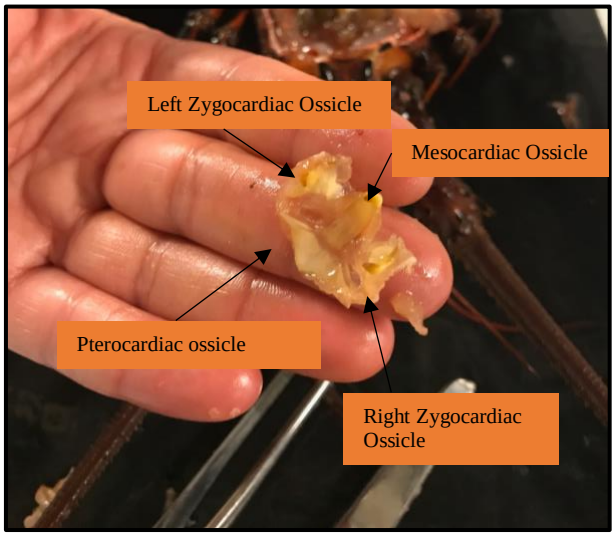

Figure 2. Dissected gastric mill, consisting of the left zygocardiac ossicle, mesocardiac and pterocardiac ossicle (one piece), and the right zygocardiac ossicle.

II. **Preparation for Sectioning:** Panulirus interruptus was preserved in a solution of 70% ethanol, 4% glycerol, and 26% water for four days before preparation. Allow for 3 days to complete the entire processing stage.

Materials: paper tags, pencil, Devcon 2-ton clear epoxy, disposable container, wooden manicure stick, soft touch forceps

Methods:

1. With a pencil, draw two perpendicular, intersecting lines that divide each tag into equal quarters. This will allow for proper placement of ossicles and to guide sectioning. Mark identification information in the back of the tag.
2. Separate the gastric mill ossicles into three parts total (the left and right zygocardiac ossicles and the mesocratic connected to the pterocardiac ossicles). See Figure 2. Clean all of the ossicles and eyestalks from remaining muscle and connective tissue and place back in respective vials for further storage until ready for placement on tags.
3. Paste each zygocardiac ossicle (L and R) and eyestalks (L and R) on separate tags using Devcon 2-ton clear epoxy and let dry overnight. To do so, mix epoxy in a disposable container according to the manufacturer's instructions. Use the pointed end of the manicure stick to place epoxy on the intersection of the two lines on the paper tag. Using forceps, press the section into the epoxy and add a thin layer of epoxy over the top of the section to protect during sectioning.
    a. Devcon 2-ton epoxy consists of two parts. Each part should be equal in amount when beginning to mix.
    b. For best results and consistency, paste the part of the zygocardiac ossicle where it has the most surface area to paper contact. Additionally, orient the ossicle so multiple transverse sections can be achieved. Avoid getting epoxy on the edges of the tag or else the tag won't slide into the chuck. For orientation reference, see Gnanalingham et al. (2018).
    c. For eyestalks, orient the eyestalk so longitudinal sections can be cut with the dorsal side facing up. For orientation reference, see Kilada et al. (2015).
4. Manufacturer's instructions indicate a 30-minute drying period. To ensure complete solidification, let the tags dry overnight.

III. Sectioning

Materials: Isomet low-speed saw, custom chuck (to hold tags), weights, water, two diamond saw blades, paper or plastic spacers, forceps, Kimwipes, caliper, lab tape

Methods:

1. Place blades on arbor (stationary arm) of the saw in the following order: metal spacer, blade, paper/ plastic spacers, blade, metal spacer, cap, screw. Spacer thickness required varies on size of substrate being cut. For the zygocardiac ossicles, 180-360 micrometers were attempted based on Kilada et al. 2015 and Gnanalingham et al. 2018. To achieve different sizes, I traced the shape of the metal spacer and cut out sections of rite-in-the-rain paper and Exceed notebook paper and measured the thickness with a caliper. We found 180 micrometers to be most efficient for the zygocardiac ossicles which would be 2 Exceed notebook paper spacers.
    a. Careful handling the blades to prevent distortion or breakage
    b. Depending on the paper type, the thickness will be different. Measuring thickness with calipers is recommended. When making spacers, be sure to cut them to a size where the edge is far enough away from the edge of the blade to allow for the ossicle to be fully cut.
    c. For eyestalks we used a 360 micrometer spacer at first based off of previous studies. They appeared to be too thick. Thinner sections are recommended.

2. Fill the water bath in the saw with water for lubrication. The water should barely touch the bottom of the blade when the saw in turned on. The blade speed can be adjusted. A speed of 4 or 5 was consistently used.
3. Slide the first tag for sectioning all the way into the chuck with the hole end of the tag sticking out. Before turning the blade on, use the dial attached to the moving arm to orient the sample left or right depending where you want the two blades to cut it. Then adjust the angle of the chuck so the saw will cut the whole sample, and not be inhibited by slicing the chuck.
4. With the sample oriented on the blade, turn the saw on, make sure the blade is barely touching the water (too much water inundates the tag and the tag will be torn apart). After several seconds the saw will have sliced through the ossicle and the epoxy and metallic grinding nose may be heard. By observation, attempt to prevent the blade cutting into the chuck, as most times it leads to a lost section. Place the section on a labelled glass slide to prepare for mounting.
    a. Many times the section isn't with the rest of the tag and it is either lost or in between the blades. To check for the section, loosen the screw and separate the blades just enough to see the inside of the blades. At a low speed, turn the saw on and off to check for the section while using forceps to remove any clumps of paper that may have gotten stuck. Only use the forceps when the saw is off.
    b. For eyestalks, more weight was needed along with manipulation of the chuck back and forth.
5. When finished sectioning, rinse all the components of the blade with water (no hard water!) and let everything dry flat on a paper towel and store flat to prevent distortion.

IV. Mounting with Cytoseal

Materials: Cytoseal, glass slides, non-plastic disposable container, fume exhaust snorkel/ fume hood, protective gloves, wooden manicure stick, tray with raised edges

Methods:

1. Pour a small amount of cytoseal into a non-plastic disposable container (we used a ceramic thimble with aluminum tin inside.
    a. Warning: Cytoseal is a hazardous material. Perform all steps involving the adhesive in a fume hood or fume exhaust snorkel and with protective eye gear and gloves on.
    b. Work efficiently when using the cytoseal. It dries rapidly and becomes hard to work with.
2. The sections should already be placed on glass slides labelled accordingly post sectioning. Remove one section at a time and place in a watch glass. Spread a small drop of cytoseal using the pointed end of the wooden manicure stick in the center of the glass slide. Make sure it is large enough to encase the section. Using forceps, transfer the section onto the glass slide and use the blunt end of the manicure stick to press the section into cytoseal. This ensures no air pockets remain underneath the section.
    a. Not thoroughly checking and removing air pockets will obscure bands on the section.

    b. In between each mount, clean off the manicure stick to prevent sticking of the section on the stick.
3. Allow the cytoseal to dry flat overnight under the fume exhaust snorkel.

V. Mounting with Crystal bond

Materials: glass slides, crystalbond, aluminum tin, hot plate, forceps, metal spatula

Methods:

1. Break off a small section of crystalbond and place into aluminum plate. Place the aluminum plate onto the hot plate before turning it on. Once situated, turn the hot plate onto level 4 and let the crystalbond liquefy for a few minutes.
    a. Warning: careful when using the hot plate as burning yourself can be a serious risk and may result in the loss of the ossicle section.
2. Remove the section from the labelled glass slide and place in a watch glass. Place the glass slide onto the hot plate with the labelled side sticking off of the hot plate like a "holding tab." This will allow for easy handling of the slide without it getting too hot.
    a. The crystalbond solidifies as soon as it begins to cool. Keeping the crystalbond on the hot plate allows for it to be manipulated until mounting is complete.
3. Place the pointed end of the metal spatula into the heated crystalbond and twist a few times to equally coat the bottom of the spatula. Place the same head onto the glass slide (on the hot plate) and twist again to achieve a small round dot of crystalbond on the glass slide. With forceps, transfer the ossicle section from the watch glass to the slide with crystalbond. Press the section into the crystalbond or using the spatula, drip a small amount of crystalbond onto the top of the section to completely encase the section.
    a. For the crystalbond to drip from the spatula onto the section, the spatula has to be extremely hot.
    b. Upon contact with the crystalbond, the section may start to bubble (Depending on the thickness of the section. We observed the thinner the better). To mitigate bubbling, manipulate the section in the heated crystalbond for a while by moving it around with the pointed end of the spatula. Once the bubbles die down, be sure to orient the section completely parallel to the glass slide or it will create complications during polishing.
    c. The longer the section is on the hot plate, the more likely it will burn and no longer be able to be read for bands.
4. Once, the section is oriented correctly, remove the glass slide from the hot plate by grabbing the tab situated off the hot plate (most likely the labelled edge of the glass slide) and place on a tray to cool. Crystal bond dries quite readily, however, to ensure solidification, allow it to dry for a few hours.

VI. Polishing/ Analyzation

Materials: Wet/dry lapping film (4 levels of grit), polishing paper, distilled water, rinse bottle, large beaker, small suction cup, slide box, Kimwipes, compound microscope, Photo software ISC

Methods:

1. Before sanding, analyze all of the samples under a compound microscope and take pictures using photo software ISC. Label and save accordingly to the desktop in a folder with sub folders specified to specimen.
2. Create a space near a compound microscope dedicated to the sanding/polishing station that consists of a clear plastic plate with 3 different grades of lapping film and 1 polishing section (Green: 30, Blue: 9, White: 0.3, Brown: Polishing). Apply distilled water to the three grades of lapping film.
3. One sample at a time, remove the label, apply the small suction cup to the back of the slide and begin sanding in a circular motion. Rinse the slide by holding it over a beaker and rinsing it with a bottle. Dry both sides of the slide thoroughly with a Kim wipe and examine under a microscope under 4x magnification.
    a. Each sample will be different and need different methods. Remember to start slowly starting with green, blue, white, and then emphasize polishing. Experiment with 5-20 circular motions on each while remembering to continuously check the sample under the microscope. More often than not this process will need to be repeated more than once.
    b. For thicker sections, use the separate higher grade lapping film included with the sanding set. Remember to use it carefully and slowly with a low number of strokes as the coarseness may result in the loss of the sample.
    c. Be sure to document the stages of sanding by saving images to the specimen specific folder. Over polishing can render the sample to be unreadable and may result in sectioning again but with proper documentation of the stages that may not be necessary.
4. On the microscope experiment with the light exposure and in photo software ISC adjust the white balance and experiment with gamma, contrast, and gain. Adjusting these settings may allow you to see bands better without having to polish excessively. If the sample is too dark, try a high exposure and if it is almost transparent, try a low exposure.
5. Remember to place the label back on the slide when complete.

VII. Known Age Lobster Care: 1-month old benthic juveniles and a 2-year old sub-adult were transported from the Scripps experimental aquarium to NOAA SWFSC experimental aquarium and acclimated then placed in a recirculating system tank. Starting measurements consisted of carapace length, width, total length (CL, CW, TL: measured to nearest mm with caliper) and weight (W: measured to nearest g with scale). Measurements were re-taken 7-days post-molt along with information on the molting history. The lobsters were also tagged. The benthic juveniles were marked on their carapace with distinct colored glue adhesive and the sub-adult was tagged with white abalone tag #391. The 2 benthic juveniles and 1 sub-adult were moved from a recirculating system and were acclimated to the ambient flow-through tank. Lobster separation at these stages is important. The juveniles are kept in a tackle box set up with a tubes for flow through water in the individual compartments. PVC tubes were set in the tank for the lobsters to hide in and metal wire mesh was glued to the bottom of the tackle box container to allow for a substrate to hold on to when the lobsters molted. To achieve acclimation, the tackle box container floated in the new tank before adding new water and the sub-adult was separately acclimated. The tanks were

cleaned and the lobsters were fed an assortment of squid or anchovy on Mondays, Wednesdays, and Fridays. Observations of molting persist daily.

VIII. Validation: Future validation relies on the rearing of known-age lobsters and an estimated age by length-frequency analysis.

IX. Reader Precision: The zygocardiac ossicle samples were the best for band analysis. For the first round of band count analysis, 10 samples were used. To prevent bias, a code was created to rename all of the samples and they were randomized in Excel. Three independent readers who had no knowledge of the lobster's size or other reader estimates followed a protocol created for assessing band counts. Further analysis will be necessary to determine reader precision.

Discussion:

Preparation for Sectioning: During the first round of samples, we only mounted the ossicles and eyestalks with a thin layer of Devcon 2-ton epoxy. For the zygocardiac ossicles that method worked well, but for the pterocardiac ossicles, mesocardiac ossicles, and the eyestalks a thicker application of resin was needed due to the obscure shape and therefore, no sufficient sections were available for analysis. To combat this, the eyestalks were filled with resin which helped during sectioning but in a future study, encasing the ossicles and eyestalks in a cube of resin is recommended. Additionally, the first round of zygocardiac sections and eyestalk sections were a 360 micrometer thickness which we found to be too thick and required extensive polishing/sanding. Only one eyestalk showed bands but without proper documentation it was lost. All zygocardiac ossicle sections showed bands but the readability varied. For the thicker samples, high exposure was necessary. 180 micrometer sections for eyestalks is recommended or future studies. For this study we mounted the ventral side down, allowing for the dorsal side to be analyzed. Analyzing the ventral side by mounting the dorsal side down should be considered.
Sectioning: The most important part of using the Isomet slow-speed saw is watching the water level. When more attention was paid to keeping the water level right, the better the samples came out.
Mounting with cytoseal: For the first round, cytoseal was used for mounting. We found that the sections were lost easily during polishing and it led us to want to try a different adhesive. Because cytoseal is highly toxic and had to be applied under a fume hood or snorkel, the chance of losing the sections was higher because we had to wait for many samples to be sectioned before mounting. If one were to use this method in the future, thinner sections (180 micrometer thickness) is recommended to prevent over-sanding/ the loss of the section.
Mounting with crystalbond: For the second round of samples, we focused our time on sectioning the zygocardiac ossicles. They were all mounted on the paper successfully with the Devcon 2-ton epoxy, but this time we used crystalbond to mount the sections onto the slide. When using the crystalbond it is important to not burn the section as it will prevent readability. Additionally, make sure the section is parallel to the slide. In some cases, when the section was thicker than 180 micrometers, bubbling would occur when the section came in contact with the crystalbond. This was mitigated by moving the section around in the crystalbond. Crystalbond has not been attempted with eyestalks or pterocardiac/mesocardiac ossicles. Expect bubbling. The crystalbond prevented the sections from falling off the slides during polishing but if the section was not

perfectly parallel, portions of the section could be sanded off which interrupted readability as well. Under the microscope these samples looked very different than samples in previous studies (Figure 3 and 4). Previous studies showed samples as very dark, much like many of the samples in round 1 (Figure 5,6,7,8). For round 2 we cut thinner sections of the zygocardiac ossicle (180 micrometer thickness). Using the crystalbond bands were not shown unless the samples were almost transparent with a very low exposure on the microscope. These samples also featured many scratches evident on the cytoseal. It didn't appear to interrupt band counts but if using this methodology, emphasize polishing. When the samples weren't sanded away distinct bands were shown, therefore, there is promise in this methodology in the future. It would be helpful to compare with the cytoseal samples.

Figure 3.

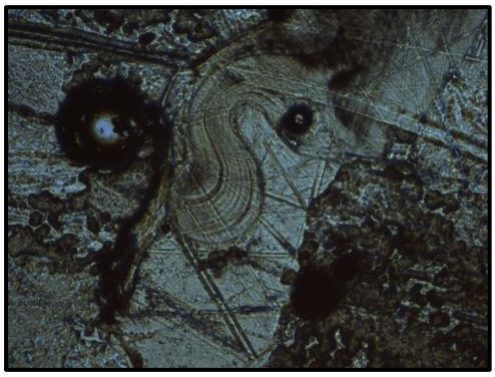

Figure 3. 180 micrometers thick transverse section of zygocardiac ossicle, mounted with crystalbond

Figure 4.

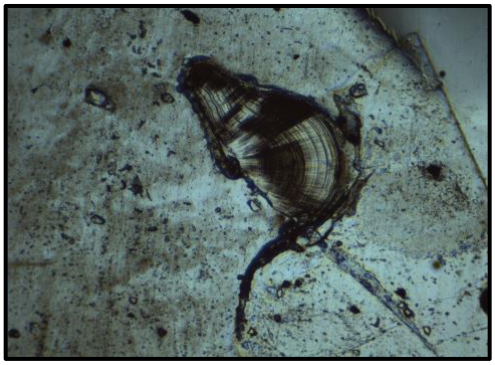

Figure 4. 180 micrometers thick transverse section of zygocardiac ossicle, mounted with crystalbond

Polishing/Analyzation: Remember to be patient with polishing. This part of the methodology is very tedious and checking the section under the microscope often is very necessary. When in doubt, stick to the finer grit lapping film. If the crystalbond samples appear to not be parallel to the slide, place the slide on the hot plate as stated in the methodology, let the crystalbond heat up, and re-orient the section and let dry. During the first round of samples, mineral oil was used to smooth and brighten the image, but in actuality the mineral oil blended bands together or resulted in the sections becoming completely transparent. Therefore, the use of mineral oil is not advised in the future. If it is experimented with, remember to document the sample before the application.

Figure 5.

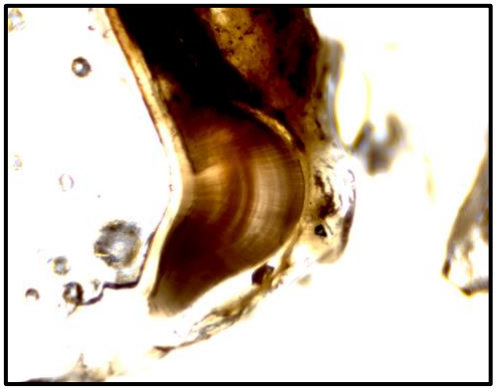

Figure 5. LJK 2 not treated with mineral oil, 360 micrometer transverse section of zygocardiac ossicle

Figure 6.

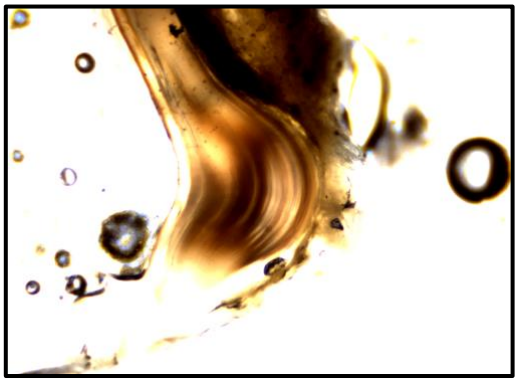

Figure 6. LJK 2 treated with mineral oil, 360 micrometer transverse section of zygocardiac ossicle

Validation/Reader Precision: As of now, there is not a known age lobster in the sample. 3 lobsters are being reared for age-determination in the future but we need to account for sufficient growth and survival. Before they are sacrificed, there needs to be less variability in methodology which can be accomplished by extending the length of the project. We do have an age estimate for specimen LJK 2 as it was found tagged, but further band analysis and validation is needed. In terms of reader precision, there is high variability in the band counts. A deeper analysis of distinguishing the differences between thick and thinner bands is needed as well as an understanding of how far the bands persist on the zygocardiac ossicle. This may be achieved with further research, outreach to previous studies, and ultimately, analysis of known-age *P. interruptus.*

Figure 7. Variability in band count

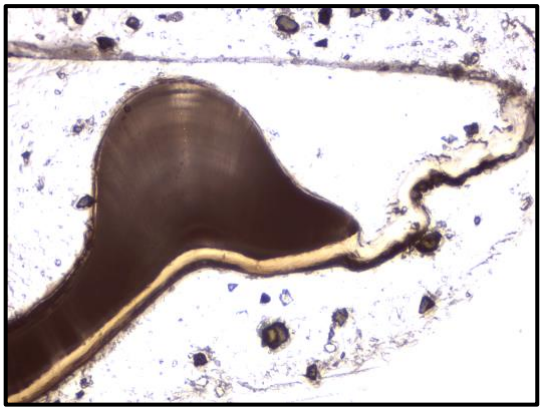

Figure 7. Sample L07181T2, 360 micrometer zygocardiac section, 6 band counts (white dots)

Figure 8. Variability in band count

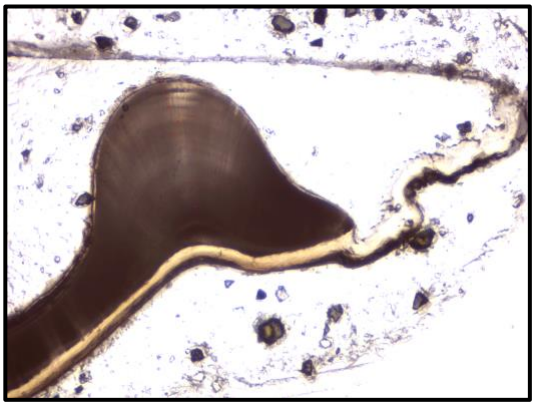

Figure 8. Sample L07181T2, 360 micrometer zygocardiac section, 12 band counts (red dots)

Conclusion:

Accurate age-determination of *Panulirus interruptus* would be groundbreaking for the management of the southern California fishery that has existed for over a century. Though the spiny lobster fishery has been relatively stable the last few decades, commercial landings dropped in the 1970s due to the landing of sub-legal lobsters. Management practices were applied by the CDFW to limit the landing of sub-legal *P. interruptus* (CDFW, 2011).The CDFW's role in management heavily relies on accurate data sources and relevant research. As of now, there is promise in future *P. interruptus* research as bands have been observed on the zygocardiac ossicles of the gastric mill. Age-determination would provide the groundwork for thorough models that will support a sustainable fishery, guide management decisions, and provide comprehensive knowledge of the life history of *P. interruptus*.

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