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Development and Application of Otolith Microstructure Analysis of glass-phase American eels (*Anguilla rostrata*) in the Saw Kill

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**Development and Application of Otolith Microstructure Analysis of glass-
phase American eels (*Anguilla rostrata*) in the Saw Kill**

Senior Project Submitted to
The Division of Science, Mathematics, and Computing
of Bard College

by
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Annandale-on-Hudson, New York

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Long live *Anguilla rostrata*.



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Abstract

The American eel, *Anguilla rostrata*, is a catadromous teleost fish that reaches adulthood in the freshwater streams and rivers of North America and then migrates to the Sargasso Sea to spawn en masse. After hatching, the larval eels migrate along the Gulf Stream as they mature into their secondary juvenile stage, the glass eel. It is at this stage that they enter the Hudson River Estuary. While the American eel has been the subject of numerous scientific studies dating back multiple decades, relatively little is known about their mass-spawning event in the Sargasso or their subsequent hatching and larval migration. These aspects of their life history could be investigated through analysis of their otolith microstructure.

Otoliths are the three pairs of small structures formed within the inner ear of finfish, created by regular variations in the depositions of calcium carbonate and organic matrix fibers (Morales-Nin, 2000). These variations create nested translucent and opaque rings, or increments, which are endogenously regulated and deposited daily (Wang, 2000). Significant life events, such as hatching and the metamorphosis from the larval stage to the glass eel stage, create visual disruptions, or checks, in the microstructure, which can be used to investigate their life history.

The available manuals on otolith processing for light or scanning electron microscopy require the use of equipment that may be cost-prohibitive to some interested in conducting an otolith study. Over the course of this project I successfully established a protocol for the removal and processing of the sagittal pair of otoliths from glass-phase eel specimens using low-budget and low-tech tools and techniques, which can be replicated by any party with access to compound and

dissecting microscopes. Use of these methods created a database of images that can be used to examine and interpret the life histories of the American eels migrating up the Hudson and into the Saw Kill through analysis of the otolith microstructure to determine their age and growth rates.

Introduction

Life History of *Anguilla Rostrata*

Anguilla rostrata is one of 19 species of anguillid eels, and the only species to inhabit North American estuaries, streams, rivers and lakes, ranging geographically from Venezuela to Greenland (Jessop, 2002). They're a commercially and scientifically important species, historically supporting commercial fisheries with landings in the millions of pounds and acting as both predator and prey species within a large range of ecosystems (ASMFC). While a 2011 review from the US Fish and Wildlife Service resulted in a 2015 announcement that *A. rostrata* populations are stable, the limited information about them, particularly about the oceanic phases of their life cycle, combined with the increasing rates of environmental and anthropogenic threats make ongoing studies, monitoring and protective efforts necessary to ensure their long-term stability.

American eels share their spawning site with one other Atlantic eel, *Anguilla anguilla*, or the European eel, and their respective spawning seasons overlap in March and April (Bonhommeau, 2010). While the two species differ very slightly physically, with the number of vertebrae being the only morphological distinction (Albert, 2006), they are separated by mitochondrial lineages (Wielgoss, 2014). Evidence of hybridization has been found in eels inhabiting Icelandic freshwater habitats (Pujolar et al, 2014), with an overall hybrid proportion of 15.5% in 2006. 30% of those hybrids were found to be second generation or later, indicating that the American-European hybrids are fertile and capable of participating in migration and spawning activities (Albert, 2006).

The larval American Eels, or leptocephali, are carried at depth of 50 to 300 meters (Bonhommeau, 2010) from the Sargasso Sea to the continental Americas along the Gulf Stream, maturing into the second juvenile stage, or glass eels, once they have reached a minimal threshold size (Albert, 2006). The exact duration of this larval migration remains contested, with estimates varying between seven months and over two years, depending on the methods used. Estimations made using floating ocean circulation models predict a longer migration, but may be insufficient to model this migration due to the depth of the larvae and observation of both vertical and horizontal swimming by leptocephali (Bonhommeau, 2010).

Glass eels are unpigmented and sexually undifferentiated with limited swimming efficiency compared to adult eels, and tend to migrate into freshwater streams and estuaries by riding flood tides and anchoring themselves during ebb tides (ASFMC, 2009). Glass eels are primarily nocturnal, and are presumed to detect freshwater olfactorily by following the scent of decaying freshwater detritus (ASFMC, 2009). While at this life stage the eels are targeted as prey by numerous fish species, including the more mature elver and yellow eels, as well as by humans, who require wild-caught juvenile eels to replenish aquaculture farm stocks.

The third juvenile stage, or elver stage, is characterized by increased size and green and brown pigmentation, and are found to progressively increase in size with increasing latitude (Powles, 2002). At this stage the eels become more diurnally active, and begin migrating up freshwater streams (ASFMC, 2009), though an unknown proportion of elvers may inhabit coastal and estuarine waters until sexual maturation (Jessop, 2002). Elvers mature into yellow eels, or sexually immature

adults, which are characterized by yellow-green pigmentation and subject to environmental sex determination (Oliveira, 2002). It is at this stage the eels spend the majority of their lives, maturing for up to 9 years for males and up to 30 years for females before beginning the downstream silvering process and migration back to the Sargasso Sea. Juvenile eels are known to migrate seasonally between estuarine and freshwater environments before their spawning migration, and elvers that had a delayed entrance to freshwater via extended marine or estuarine residency were found to produce silver eels around 380 times higher than those that directly entered freshwater rivers and streams (Jessop, 2002), indicating an increased freshwater mortality rate.

In contrast with most fish, which continuously grow throughout their lives, American eels grow to sex-specific threshold sizes. Their rate of growth is habitat-dependent, with eels residing in salt water growing up to 2.2 times faster than freshwater anguillid eels. Faster growing eels in Canada were found to achieve the migrating size threshold and depart in under half the time taken by slow-growing freshwater residents, which implies that studies done on silver eels migrating downstream may over-represent higher age categories (Lamson, 2009). There is also a sexual disparity in growth rate, with female eels growing significantly faster than males after four years and showing a decreased reduction in growth rate over time (Oliveira, 2002). This is due to the species having sexually dimorphic life history strategies, with females benefitting from a growth rate that permits maximum size for fecundity and males benefitting from one that permits the shortest maturation time.

The final life stage, or silver-phase, is morphologically triggered by the downstream migration, when the migratory swimming allows the lipid mobilization necessary for sexual maturation (Palstra, 2010). The spawning migration of American eels requires a journey of up to 4,000 kilometers, which necessitates an extremely energy efficient swimming method. Migratory swimming is the trigger for silvering in eels, specifically through the lipid mobilization necessary for sexual maturation (Palstra, 2010). The necessity of this migratory swimming means that eels that cannot migrate are unable to reproduce, and subsequently prevents captive eel breeding as either an economic or preservative measure. Vitellogenesis, or yolk formation, occurs in the final stages of female sexual maturation, which requires the reabsorption of vertebrae to create phospho-calcium reserves. Vitellogenesis is inhibited by swimming, and can only occur once the females have arrived at the breeding grounds in the Sargasso. Subsequently, females require roughly 2 months of post-migration maturation to create viable oocytes.

Current Threats to *Anguilla rostrata*

With lifespans of up to 30 years in females, the longevity and complex life cycle of *A. rostrata* exposes them to a wide range of habitats and threats, making them difficult to monitor and protect. Overfishing, invasive species, pollution, habitat loss and river damming all contribute to the decline in American eel populations (Fig. 1), resulting in a 2012 Benchmark assessment that found eel stocks to be depleted and recommended a reduction in mortality at all life stages (American Eel Technical Committee, 2014). Glass eels in particular are at risk, since a decrease in availability of Japanese and European eels on the market led to a ten-

fold increase in price since 2010, which in turn increased the rate of harvest in the United States. Spawning is impossible to reproduce in captivity due to the complex migrational component, so eel aquaculture stocks are dependent on yearly inputs of wild-caught glass-phase eels. The majority of anguillid aquaculture takes place in freshwater, which reduces the growth rate, extends the time needed for the farmed eels to reach market size, and potentially raises the environmental impact per kilogram of eel produced (Lamson, 2009).

In addition to its economic value as a food and bait source, *A. rostrata* is a species that is highly sensitive to North Atlantic climatic changes, particularly in regards to the strength and position of the Gulf Stream, making it an “ideal candidate for tracking ongoing and past effects of climatic change” (Wirth, 2002). The potential for American eels to be used as an ecological barometer for the health of the North Atlantic further solidifies the importance of maintaining and monitoring eel populations.

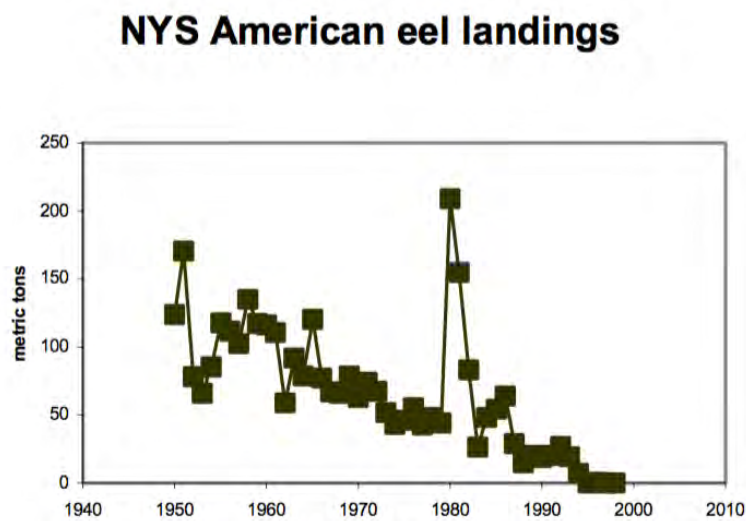


Fig. 1. The decline of American eel landings in New York State since 1950 (Limburg, 2002)

One of the contributing factors to the American eel population decline was the introduction of the parasitic nematode *Anguillicoloides crassus* into the immunologically naive *A. rostrata* populations from previously infected *A. anguilla* stocks. The native host, *Anguilla japonica*, has an effective defense mechanism against the nematodes, as shown in a 2009 study which found that Japanese eels were able to effectively encapsulate nematodes in granuloma-like cysts in both intestinal and swimbladder walls by initiating antibody production and elevating macrophage activity (Heitlinger, 2009). This acquired immunity isn't seen in European or American eels (Knopf, 2006), possibly due to the genetic divergence found between the *A. crassus* that parasitize Asian eels and those that parasitize European eels (Weclawski, 2013).

A. crassus impairs the function of eel swimbladders and therefore impacts the success of spawning migration. The swimbladder is a crucial organ for this migration as it allows the eels to go from a nocturnal depth of 100-300 meters to a diurnal depth of 600-1000 meters by maintaining neutral buoyancy (Pelster, 2015). As a result of infection by *A. crassus*, the swimbladder epithelium thickens and gas deposition is reduced, inhibiting the eel's ability to maintain buoyancy and move between depths. This impairs the eel's migration and could potentially make spawning impossible (Pelster, 2015).

The diminished swimming efficiency caused by an *A. crassus* infection can lead to a late arrival in the Sargasso Sea and incomplete vitellogenesis (Palstra, 2010). This can have a significant negative effect on eel populations, as female eels take up to 30 years to reach migration maturity where male eels only require

between 4 to 9 years due to different patterns of gonadal development.

Unfortunately, *A. crassus* disproportionately targets female eels, as they are up to ten times larger than males due to their maximum-growth life strategy, and are therefore more likely to ingest the infected copepods and fish acting as intermediate hosts for the infective stage of the nematode since their increased size allows for an increased niche breadth, which is exemplified by size-dependent piscivory at lengths around 400mm (Oliviera, 2002).

The expansion of the eel trade for restocking and human consumption was responsible for much of the distribution of *A. crassus* throughout Japanese and European eel populations, with the path of infection overlapping heavily with the trade routes taken by lorries transporting eels within continental Europe. The ability of *A. crassus* to osmoconform, or match their body fluid solute concentration with the blood plasma of the organisms they infect, allowed nematodes infecting any species of host to cross salinity boundaries and survive in both marine and brackish waters in addition to fresh water. Regurgitation of fish by fish-eating birds may also have contributed to the spread of *A. crassus*, and a lack of intermediate hosts or temperatures below 4°C are the only known barriers to its dissemination (Kirk, 2003). The presence of both first generation hybrids and backcrosses found in Icelandic eel populations indicates that interbreeding occurs between European and American eels, and possibly indicates an intermediate migratory behavior (Pujolar et al, 2014), which could have provided an additional pathway for *A. crassus* to enter American eel populations after infecting the European eels.

A study of European eels found that 89% of them were infected with one of 13 species of parasite, and *A. crassus* was the most common, making up 56% of infections (Gerard, 2013). A study of the Hudson River estuary tributaries found an *A. crassus* infection prevalence of 39%, over half the prevalence found in European populations despite their much more recent introduction (Machut, 2008). Infected eels have been found along the East Coast as far north as Cape Breton, Nova Scotia, with a parasite prevalence range of 3-30% in 2009 (Aieta, 2009) and up to 67% in 2012 (Denny et al. 2013). These findings indicate that despite the previous assumption that lower water temperatures may reduce the impact of *A. crassus* (Machut, 2008), American eels located in northeastern tributaries are still vulnerable to infection by *A. crassus*.

Eels in the Hudson

Despite the large number of studies performed involving American eels, much of the oceanic stage of their life history remains unknown – for example, while their spawning event is known to occur in the Sargasso Sea, it has never been witnessed or documented, and the exact timeline of the leptocephali hatching, metamorphosis, and migration back to continental waters is still relatively unknown for both American and European eels. Given the multitude of threats to these eels and the severity of the anthropogenic influence on American rivers like the Hudson, consistent ongoing studies are needed in order to best support their populations across the wide range of environments they encounter and inhabit. For example, anguillid eels are highly susceptible to the bioaccumulation of lipophilic contaminants, with an increasing contaminant burden extending the duration of the

continental phase of their lives (Lamson, 2009). This susceptibility means that eels inhabiting the Hudson may show reduced growth and extended maturation time when compared to eels inhabiting less urbanized streams due to their exposure to the polychlorinated biphenyl (PCBs) polluting the Hudson's water, which extends the duration of their exposure to both natural and anthropogenically driven threats.

The Saw Kill is an excellent example of an anthropogenically affected rural stream, given its upstream dams, the presence of water treatment plant runoff, and its mouth's location (Fig.2) within Tivoli South Bay (Findlay, 2010). The Tivoli Bays contain a number of habitats, including freshwater tidal marsh, tidal swamp, and mudflats (NYSDEC), which have been cut off from the Hudson by a man-made embankment built in 1850 to support a railroad line (HRVNHA, 2008). Migrating glass eels entering the bays from late March to early June must do so through small drainage openings in the embankment, contrasting with both the streams and other wetlands that directly contact the Hudson. This distinctive location is an important sampling site to use in order to increase the breadth of studied eel-inhabited environments and better understand how they arrive to and function across a range of habitats.



Fig. 2. Map of the Saw Kill entering Tivoli South Bay (NYSDEC). The railway embankment is indicated by the green stripe to the left, separating South Bay from the Hudson, and the eel-sampling site is indicated by the symbol of the house, representing the Bard Field Station.

Otolith Overview

Interpretation of *A. rostrata* early life history could be done with the use of otolith analysis. Otoliths are small, bipartite acellular calcium carbonate structures located in the vestibular labyrinth of the inner ear of all vertebrates, and have been the focus of numerous studies for over 135 species of fish due to the large amount of information they can provide (Secor, 1992). Finfish have three pairs of otoliths, the largest of which are the sagittae, and are the most frequently used in otolith studies.

The two smaller pairs, the lapilli and asterisci, are largely overlooked due to the additional inconvenience of their size and the reduced clarity of the micro and macrostructures. Otoliths prove to be useful in determining the age and examining the development of finfish due to the way the calcium carbonate deposition is regulated by circadian rhythm, which produces distinct daily increments (Miller, 1994). The increments are accompanied by checks, or stress marks, which form within the microstructure during times of stress or significant life events, such as hatching and metamorphosis, and provide valuable information about the life history of individual specimens.

Otolith formation within the inner ear is caused by variations in the deposition of keratin-like organic matrix fibers and calcium carbonate crystals, which create alternating opaque and translucent increments (Morales-Nin, 2000), referred to as D-zones and L-zones for dark and light zones, respectively (Kalish, 1987). The increments are endogenously regulated by daily calcium metabolic and neuropeptide secretion rhythms. When cut by a transverse plane, the otolith resembles a tree stump, with a dark, rounded primordial granule composed of vaterite at the core surrounded by expanding ring-like increments (Miller, 1994). Otoliths have both macro and microstructure, with macrostructure being comprised of yearly concentric annuli in adult fishes, and microstructure being comprised of daily and sub-daily increments (Kalish, 1987). The sulcus acusticus is a groove on the medial surface of saggital otoliths where the otolith was in contact with a sensory epithelium (Morales-Nin, 2000), and is typically the area of the otolith with

greatest incremental clarity, and therefore the ideal place to make the transverse plane for image analysis.

Growth rings have been historically used to age fish in vertebrae and scales from as far back as the 1700s. Otoliths were slightly more recently used, with the annual ring formation first being observed in 1899 (Jones, 1992). However, the presence of regular daily growth increments in the otoliths of marine fish wasn't demonstrated until as recently as 1971 (Bonhommeau, 2010), after which its use increased rapidly throughout the 1980's, finally resulting in the validation of daily increment deposition glass-phase American eels in 2001 (Cieri, 2001). Otoliths can be used in a variety of studies, including visual analysis for age studies, and microchemistry analysis, which can allow researchers to determine the type of habitat the fish inhabited at the time of deposition for each increment by examining patterns in their strontium to calcium ratios (Jessop, 2002). Given the historically recent validation of daily increments in American eels, the large gaps of knowledge about their early life history, and the lack of studies performed on glass-phase eels migrating up the Hudson, analysis of the otolith microstructure of glass eels in the Saw Kill has the potential to provide useful insight into the life history of these organisms.

Methods/Results

Collection and Preservation

I collected 20 glass eels from a fyke net set across the mouth of the Saw Kill as it enters Tivoli Bays on Bard College Campus on May 11th, 2015 (Table 1). This sampling date occurred three days after the largest peak of glass eels caught in the Saw Kill, which was caused by a large new moon tide (Fig. 3). This high flood tide could have had an effect on the glass eel cohort that I collected by “bunching” eels that may have otherwise had more stratified arrival times, allowing them to enter the fyke net all at once rather than over the course of several days. The strength of the tide could also have prevented some glass eel specimens from entering streams downriver from the Saw Kill site, carrying them further up the Hudson than they would have otherwise traveled. Initial examination of the Hudson River Eel Project fyke net data from 2015 seemed to indicate that sampling sites downriver from the Saw Kill had lower than average glass eel capture during the days following the new moon tide (G. Ballou, pers. comm. April 25, 2016).

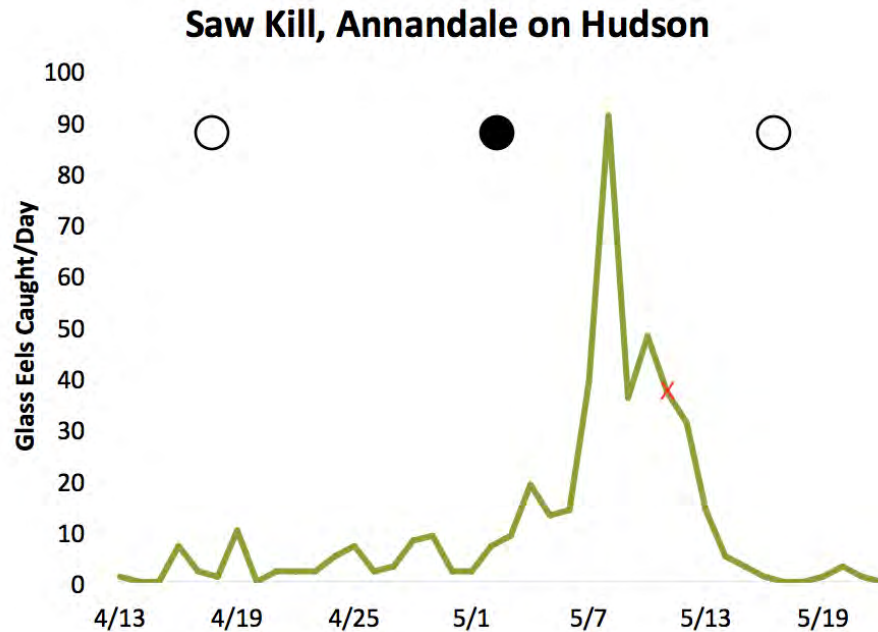


Fig. 3. Glass eel capture data at the Saw Kill site for the migrational season of 2015. The red X indicates the sampling date for this project, and the circles represent new and full moons (Bowser, 2015).

The fyke net was installed at the deepest point of the Saw Kill mouth to maximize the number of eels caught, and to ensure that the net would remain partially submerged during low tides (Fig. 4). The net's design features 13.5-foot wings that guide glass and elver eels through a screen of $\frac{1}{4}$ inch mesh and into a funnel trap. The funnel trap's small opening is suspended in the center of a cylindrical netting bag, which can be easily accessed for collection. The small size of the conical opening excludes any larger organisms that may prey on the eels from entering the net, and the instinct for glass eels to swim to the bottom of their environment combined with their migrational drive to swim against the current prevents them from exiting back through the suspended funnel. The collection was

performed under the supervision of Erik Kiviat, who holds the necessary endangered/threatened species license from the New York DEC.



Fig. 4. The fyke net installed at the mouth of the Saw Kill as it enters Tivoli Bays at low tide

The eels were humanely euthanized with clove oil at the recommendation of Robert Schmidt (R. Schmidt, pers. comm., April 27, 2015). Clove oil, or eugenol, acts as an anesthetic, and was found to induce anesthesia more rapidly and at lower concentrations than the commercial powdered anesthetic Tricaine mesylate, or MS-222 (Grush, 2004). Exposure to high concentrations of clove oil caused a fatal overdose in the eels via hypoxia. The eel specimens were then placed on a sylguard plate and measured to the nearest half of a millimeter, fully submerged in individual vials of DI water, and frozen at -81 °C until the time of dissection.

Site	Saw Kill
GPS coordinates	N 42.01817 W 073.91756
Date	5/11/15
Time of Sampling	2:00 PM
Glass eels in fyke	37
Elvers in fyke	7
Number eels weighed	20
Total weight (g)	3
Air Temp F°	86
Water Temp F°	70
Time of Low Tide	3:12 PM
Tide Stage	Low
Cloud Cover	Clear
Precipitation	None

Table 1. Environmental and cohort data from day of capture (G. Ballou, pers. comm., April 25, 2016)

Dissection

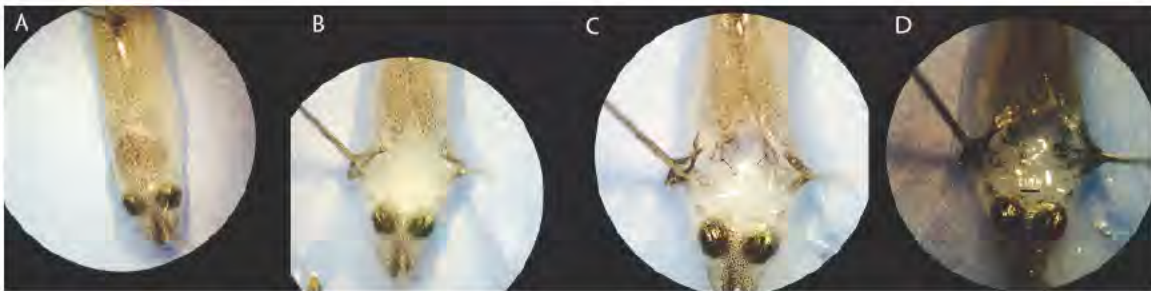


Fig. 5. Dissection process for otolith recovery. The black arrows in 2C indicate the locations of the sagittal otoliths within the cranial wall.

Each individual specimen was removed from frozen storage, thawed, mounted on a sylguard plate with .2mm minutien pins, and examined under a dissecting microscope (Fig. 5A). A dorsoventral incision through the top of the cranium was made with micro scissors to expose the brain (Fig. 5B), which was

removed using a .25mm Tungsten dissecting needle and watchmaker's forceps to allow visualization of the semi-circular canals. The translucency of the specimens allowed direct visualization of the sagittal otoliths (Fig. 5C), which were carefully teased out of the cranium walls and maneuvered into the empty cavity (Fig. 5D). The otoliths were then placed directly on the sylguard and excess tissue was removed with the dissecting needle to reduce inhibitions during analysis and interpretation.

While under the dissecting microscope, the otoliths were examined to determine whether or not they were vateritic. Vateritic otoliths grow chaotically and can produce incorrect data if analyzed, and must be excluded from analysis (ICES, 2011). Vateritic otoliths are generally identifiable by their distinctive a-symmetrical shape with multiple growth points, in contrast with the uniform shape and smoothness of a normal glass eel otolith, and polishing a vateritic otolith will reveal structural inconsistencies that inhibit image analysis (Supplemental Fig. 4). The removed otoliths were then rinsed in DI water, air dried, and placed in labeled 2mL microcentrifuge tubes for dry storage. 13 of the specimens also had their lapillus otoliths removed and stored for potential future examination. All eel specimens were preserved in 70% ethanol after dissection.

Specimen #	Sample location	Collection Date	Length before freezing (mm)	Otoliths recovered (all)	Sagittae stored
1	Saw Kill	5/11/15	65	1	1
2	Saw Kill	5/11/15	60	2	2
3	Saw Kill	5/11/15	56	2	2
4	Saw Kill	5/11/15	54	3	2
5	Saw Kill	5/11/15	62	2	1
6	Saw Kill	5/11/15	55	4	1
7	Saw Kill	5/11/15	54	4	2
8	Saw Kill	5/11/15	53	4	2
9	Saw Kill	5/11/15	53	3	2
10	Saw Kill	5/11/15	55	4	2
11	Saw Kill	5/11/15	57	2	2
12	Saw Kill	5/11/15	55	3	2
13	Saw Kill	5/11/15	59	2	2
14	Saw Kill	5/11/15	60	3	2
15	Saw Kill	5/11/15	60	4	2
16	Saw Kill	5/11/15	54	4	2
17	Saw Kill	5/11/15	57	2	2
18	Saw Kill	5/11/15	55	2	2
19	Saw Kill	5/11/15	58	3	2
20	Saw Kill	5/11/15	53	4	2

Table 2. Sample collection and eel morphological data. Samples with more than 2 recovered otoliths also had at least one of their lapilli otoliths removed, cleaned, and stored.

Processing Overview

Otolith processing typically requires at least three steps after the initial dissection in order to retrieve data from the macro or microstructure: mounting, polishing, and imaging (Fig. 6). Mounting the otoliths is the process of affixing them to a glass microscope slide with adhesive, either directly or after embedding the otolith in epoxy. The adhesive used must be strong enough to keep the otolith secure during the polishing process, and cannot be opaque, as this would inhibit future analysis using light microscopy. The mounted otoliths are then polished with

fine grit lapping film to create a transverse plane parallel to the slide that exposes the primordium and growth increments for analysis – it is important to note that the inadequacies of certain embedding or mounting methods may not become apparent until the polishing phase. After mounting and polishing, the otoliths can be etched and stained if needed to increase the contrast between increments and improve visualization during the image analysis stage.

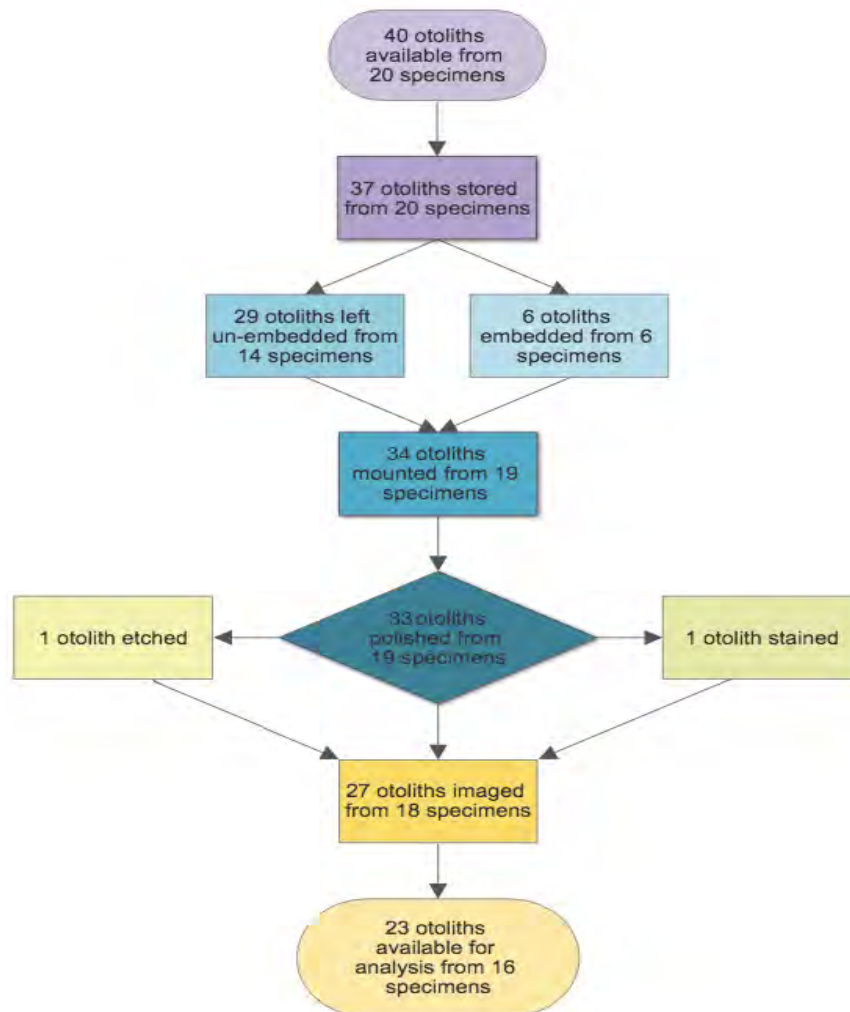


Fig. 6. Overview of otolith processing steps

Embedding

Bob Schmidt provided a manual for otolith removal and preparation by Secor et al. for this project (R. Schmidt, pers. comm., June 12, 2015). The manual recommends embedding the otolith in epoxy to increase the ease of handling during the sectioning and polishing stages of processing. Sectioning required the use of an Isomet saw which, increased the risk of shattering the otoliths, and was only recommended for otoliths longer than 3mm, and was therefore not incorporated into any of the tested methods in this study. To determine if embedding the otoliths would assist the polishing process without sectioning, I selected 1 sagittal otolith from 5 specimens with complete recovered pairs and embedded them in ACE brand epoxy before mounting them on glass slides (Secor et al., Brothers 1975, Ralston 1988, Miller 1996). Due to the lack of adequate molds for otoliths of such a small size, I allowed the epoxy to set on one of three surfaces: the palm of a nitrile glove, the dissecting field of a sylguard plate, and a glass slide.

Method 1: I placed a droplet of liquid epoxy on the palm of a nitrile glove laid flat on the lab bench, placed one otolith in it with the sulcus side down, and gently tapped the otolith with a dissecting needle until it was completely submerged within the epoxy to prevent air bubbles from being trapped beneath it (Secor et al.). After being allowed to set, the epoxy was to be flipped and mounted on the slide with the sulcus acusticus side facing up for polishing, as the increments are clearest near the sulcus (Kalish, 1987). I chose the glove for its flexible material, which would hypothetically peel away from the epoxy once set, reducing the risk of chipping or cracking the epoxy and otolith. However, the act of manually securing

the embedded otolith and peeling the glove away put excess pressure on the otolith, destroying it and causing it to be lost from the dataset.

Method 2: I allowed an epoxy droplet containing the second selected otolith to set on the sylguard dissecting plate in lieu of the nitrile glove. The plane of the plate was more consistent and level than that of the nitrile glove, and the flexible material and density of the sylguard allowed me to remove the embedded otolith by using the dissecting needle to gently push the sylguard material down and away from the epoxy instead of trying to “peel” the otolith off. I found this to be the simplest and most effective embedding method barring method 4.

Method 3: I allowed an epoxy droplet containing the third embedded otolith to set on a clean glass slide in lieu of either the nitrile glove or sylguard plate. While this method provided a consistent plane for the otoliths to sit on and allowed the clearest visualization of the process, the epoxy was more difficult to remove from the glass, due to its inflexibility and the fragility of the otolith. This increased both the methodological difficulty and the potential risk of damaging the otoliths. When comparison between the first three embedded otoliths found method 2 to be the most effective and easiest method to employ, I subsequently embedded the remaining previously selected otoliths following method 2.

Method 4: I selected an additional otolith, left it un-embedded, mounted it directly onto a glass slide, polished, and examined under a compound microscope to allow comparison between embedded and un-embedded otoliths (Cieri, 2001). Secor et al. described the embedding stage as increasing the ease of handling the otoliths when flipping them over after polishing one side of the transverse plane in

order to polish the other side, creating a thinner section for improved visualization of the microstructure. However, it was unclear if it would be necessary to polish these otoliths on both sides of the transverse plane given their small size¹, and the embedding process appeared to exacerbate their fragility, increasing the time spent on each individual specimen, the risk of damage to the otoliths, and the severity of the damage (Fig. 7).

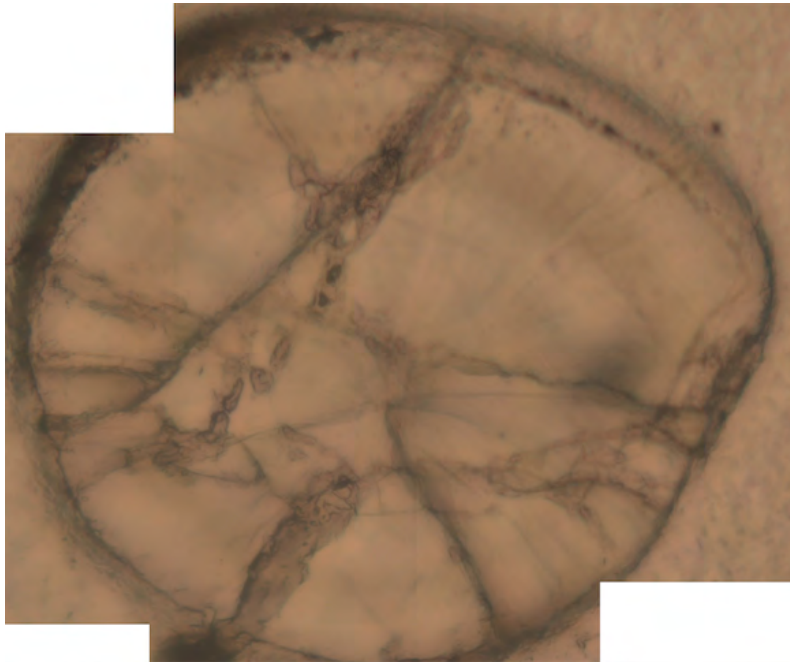


Fig. 7. Example of a shattered otolith embedded in epoxy using method 3 and mounted using method 1. While the cracking did not occur until the polishing phase, it was caused by the epoxy “flexing” under polishing pressure, creating fractures in the otolith and eliminating it from the image dataset.

I examined the un-embedded otolith under 40x magnification and verified that the individual increments of the microstructure were visible and determined that polishing one side of the otolith was sufficient for clear visualization of the core

¹ The otoliths were on average roughly 1mm in diameter along the transverse plane (S. Fig. 4)

² Though the cracking didn't occur until the polishing phase of processing, there was

and microstructure for analysis. When the previous embedding methods proved to be either of no benefit or actively detrimental during the polishing phase of processing, I left all the remaining otoliths un-embedded and mounted them directly onto the slides (Table 3).

Method Used	Otoliths Used	% of Otoliths Cracked ²	% of Otoliths Lost to Dataset	Otoliths Available for Analysis
Embedding 1	1	100%	100%	0
Embedding 2	4	100%	75%	1
Embedding 3	1	100%	100%	0
Embedding 4	29	89%	24%	22

Table 3. The total number of otoliths used for each embedding method, the percentages of otoliths that cracked and were lost to the dataset over the course of processing, and the number of otoliths available for analysis after processing.

Mounting

Method 1: Following methods outlined by Secor et al., I affixed the four remaining sagittal otoliths embedded in epoxy to glass slides using Crystalbond 509 thermoplastic resin. I heated a ceramic hotplate to the resin's flow point of 121 °C and pressed the resin stick against it until it liquefied. I applied the liquid resin to a clean, heated glass slide using the edge of another glass slide to create a thin and even layer of media, then placed the embedded otolith in the Crystalbond with the sulcus acusticus side up and set the slide aside to allow the resin to cool and re-harden. While this method was not prohibitively complicated during the mounting phase, it proved to be inadequate during the polishing phase of processing. Despite

² Though the cracking didn't occur until the polishing phase of processing, there was a clear causative relationship between embedding the otoliths in epoxy and severe damage that caused them to be eliminated from the final dataset

having a flow point of 121 °C, the Crystalbond began to soften and shift during the polishing process. This softening both reduced its adhesion to the embedded otoliths, allowing the epoxy to move and risk becoming dislodged, and caused the resin to start to “smear” during polishing instead of being polished away. The smearing resin reduced the effectiveness of the lapping film by coating its abrasive face and began to obscure the transverse plane of the otolith, which required additional polishing with new lapping film to remove. Subsequently, 3 of the 4 otoliths mounted using this method were lost to the dataset from damage sustained during polishing, and I found this method to be inadequate for this study.

Method 2: To continue to test the effectiveness of embedding the otoliths, I concurrently placed one un-embedded otolith directly onto the liquid Crystalbond with its sulcus acusticus side up (Cieri, 2001). However, I found this method to be inadequate, as the Crystalbond’s adhesion to the otolith’s smooth surface was insufficient, resulting in the otolith becoming non-destructively dislodged during the polishing phase and replaced in storage. The otolith was later re-mounted using method 4.

Method 3: Given the issues of working with Crystalbond, Dr. Aude Lochet recommended the use of Loctite super glue to affix the otoliths directly to the slides (A. Lochet, pers. comm., November 19, 2015). I mounted one otolith on a slide using Loctite liquid superglue, but it was immediately non-destructively dislodged during polishing and was replaced in dry storage. The otolith was later re-mounted using method 4.

Method 4: An additional otolith was affixed directly to the slide using Loctite gel super glue. The gel super glue maintained excellent adhesion to the otoliths during polishing, and was substantially faster and simpler to use than the Crystalbond. The increased adhesion may have been due to the gel super glue's ability to hold a drop-like shape, which increased the surface area of contact with the otolith, as opposed to the liquid form, which spread out along the surface of the slide, though this remains conjecture. I subsequently mounted the otoliths of all remaining specimens directly to the slides with the sulcus side up using Loctite gel superglue (Table 4).

Method Used	Otoliths Used	% of Otoliths Cracked ³	% of Otoliths Lost to Dataset	Otoliths Available for Analysis
Mounting 1	4	100%	75%	1
Mounting 2	1	100%	100%	0
Mounting 3	1	100%	0%	0
Mounting 4	30	90%	26%	22

Table 4. The number of otoliths used, the percentage cracked and lost to the dataset, and the number available for analysis for each method attempted during the mounting phase of processing. The 30 otoliths mounted using method 4 include the two otoliths that were initially mounted using methods 2 and 3 but were non-destructively dislodged during polishing and subsequently re-mounted.

Polishing

Method 1: I placed a glass slide with a mounted embedded otolith on the sylguard plate under a dissecting microscope to allow clearer visualization of progress during polishing. The otolith was polished by placing lapping film over the slide and moving it in a circular pattern while applying light pressure with an index

³ Cracking occurred during the polishing phase of processing

finger. However, this method completely obscured the otolith from sight during polishing, which increased the risk of accidentally dislodging it from its mounting media without noticing or over-polishing it and destroying the microstructure. Additionally, the large size of a fingertip in comparison with the otolith reduced the polishing precision that was necessary to ensure that the best plane is created for analysis. This method was inefficient and enhanced the risk of over-polishing the outer edge of the otolith, and was rapidly abandoned in favor of method 2.

Method 2: I then folded the lapping film to create an edge that would allow direct visualization of the otolith during polishing, which was obscured by the lapping film in method 1. While the edge allowed improved visual access, it also increased the difficulty of handling the film and reduced the amount of pressure I was able to place on it during polishing, which significantly lengthened the polishing process and was entirely insufficient for polishing through the epoxy of embedded otoliths. This method was abandoned in favor of method 3.

Method 3: I wrapped the lapping film around the edge of an unused glass slide (Supplemental Fig. 2), which enabled more effective and precise polishing while still allowing constant visualization of the otolith under the dissecting microscope. The edge of the slide provided a flat polishing plane in contrast with the use of a fingertip, and this reduced the risk of creating an uneven transverse plane through the otolith. A crooked transverse plane risks exposing some increments while eliminating others, which would impede image analysis and cause the sample to be lost to the dataset. An additional benefit was that the increased precision

allowed me to adjust the angle of polishing accordingly if the otolith had accidentally been embedded or mounted at an angle.

I found this method to be effective and practical for the first otolith, and it was subsequently used for all other otoliths regardless of embedding or mounting methods (Table 5). The glass slides were placed on a sylguard plate under a dissecting microscope (Supplemental Fig. 3) and the otoliths were polished incrementally with 5, 1 and 0.3 micron lapping film, with frequent examinations under a compound microscope at 40x magnification until the core and daily increments were clearly visible. Regular checking with a compound microscope is necessary to prevent over-polishing, which would eliminate the core and increments and render the otolith unusable for image analysis (Fig. 8)

Method Used	Otoliths Used	% of Otoliths Cracked	% of Otoliths Lost to Dataset	Otoliths Available for Analysis
Polishing 1	1	0%	0%	0
Polishing 2	1	0%	0%	0
Polishing 3	33	90%	30%	23

Table 5. The number of otoliths used, the percentage cracked and lost to the dataset, and the number available for analysis for each method attempted during the polishing phase of processing. Note: Some of the otolith loss and cracking at this stage was due to insufficient embedding or mounting methods, which only became apparent during the polishing phase.

Though the majority of otoliths cracked during the polishing phase, most of these were not severe enough to eliminate the otoliths from the dataset. Of the 10 otoliths lost during polishing, 4 were lost as a result of being previously embedded in epoxy (Table 3) and 3 were lost due to over-polishing (Supplemental Fig. 1), meaning only 3 otoliths were cracked severely enough to be eliminated from the

dataset. The otoliths that were lost due to over-polishing were not severely cracked enough to be eliminated from the dataset (Fig. 8), and could have been analyzed if the microstructure was still present.

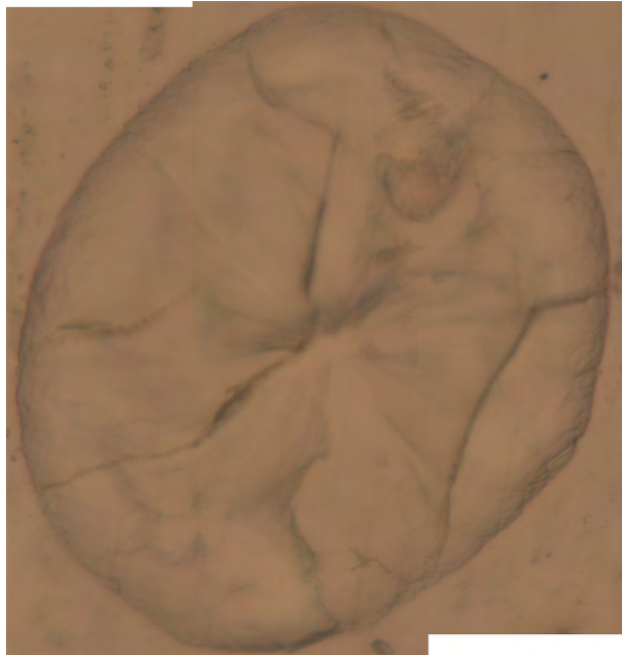


Fig. 8. Example of an otolith that was over-polished. No microstructure is visible for image analysis, eliminating it from the image dataset.

Etching

Method 1: Etching the otolith involves dissolving either the organic matrix or the calcium carbonate with an acid to improve visualization of the other component of the increments. I etched one otolith for 3 minutes with 5% EDTA and then rinsed it with DI water (Ralston 1988, Cieri 2001, Jessop 2008). However, this method produced a film over the otolith's surface, which required the otolith to be re-polished with lapping film. Given that re-polishing the otolith after every etching attempt puts the otolith at increased risk of becoming over-polished (Fig. 8) or

cracked and being eliminated from the image dataset, no further otoliths were etched using this method.

Method 2: I examined an un-etched, polished otolith under a compound microscope, and the primordium and daily increments were clearly visible across multiple magnifications. Given the clear visualization of microstructure and the lack of clear benefit from etching with EDTA, I left the remaining otoliths un-etched after polishing (Table 6).

Method Used	Otoliths Used	% of Otoliths Cracked ⁴	% of Otoliths Lost to Dataset	Otoliths Available for Analysis
Etching 1	1	100%	0%	0 ⁵
Etching 2	27	88%	14%	23

Table 6. The number of otoliths used, the percentage of otoliths cracked and lost from the dataset, and the number of otoliths available for analysis for each method attempted during the etching phase of processing. The 27 otoliths left un-etched include the otolith that was initially etched and subsequently re-polished.

Staining

Method 1: I stained a polished otolith for 5 minutes with 5% Toluidine blue and then rinsed it with DI water (Secor et al, Stirrat 2001). Visual comparison between the stained otolith and an unstained otolith showed no difference in the distinction between increments, indicating that the stain had not been given adequate time to set.

⁴ The otoliths had previously cracked during the polishing phase, and there was no causative relationship between etching and otolith damage.

⁵ This otolith was re-polished and left un-etched, and therefore couldn't be analyzed after etching despite not being eliminated from the dataset

Method 2: I re-stained the otolith for 30 minutes with 5% Toluidine blue and rinsed it with DI water (Stirrat, 2001). Visual comparison between the stained otolith and an un-stained otolith under a compound microscope found that while the increased staining time had allowed some pigmentation to take hold, staining did not improve the distinction between increments, even obscuring them in some areas, and offered no substantial benefit over leaving the otoliths unstained (Fig. 9).

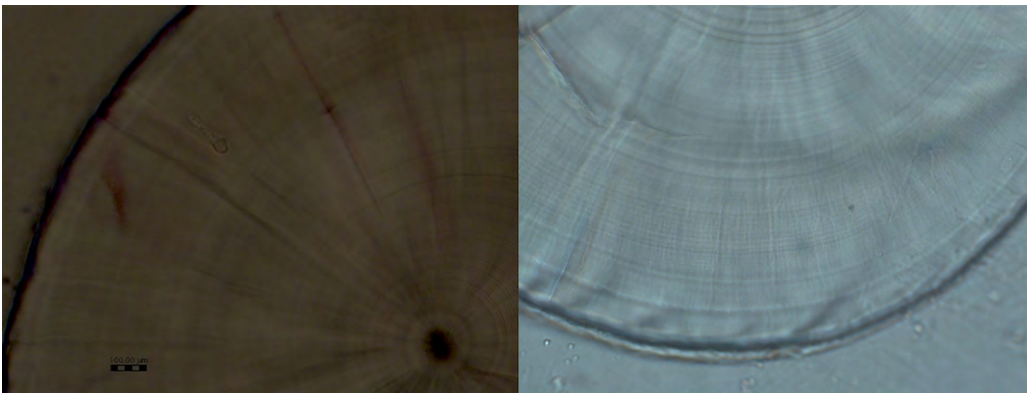


Fig. 9. Visual comparison between an otolith stained using method 2 and an unstained otolith. Staining with 5% Toluidine blue provided no visible benefit to the visualization and interpretation of the daily increments.

Method 3: Given the clear visualization of the microstructure of unstained otoliths and the lack of substantial benefit from staining the otoliths, I left all the remaining otoliths unstained after polishing (Table 7).

Method Used	Otoliths Used	% of Otoliths Cracked ⁶	% of Otoliths Lost to Dataset	Otoliths Available for Analysis
Staining 1	1	100%	0%	0
Staining 2	1	100%	0%	1
Staining 3	26	88%	15%	22

Table 7. The number of otoliths used, the percentage cracked and lost to the dataset, and the number available for analysis for each method attempted during the staining phase of processing. Note: The otolith initially stained to test method 1 was re-stained to test method 2.

Imaging

After the final phase of polishing was complete, I examined and photographed the otoliths with the ProgRes C5 camera mount from Jenoptik. I determined the best transect or axis available for increment analysis under 40x magnification when the entirety of the otolith was visible (Fig. 10), and images were taken at 63x and 100x magnification under oil immersion (Campana, 1992).

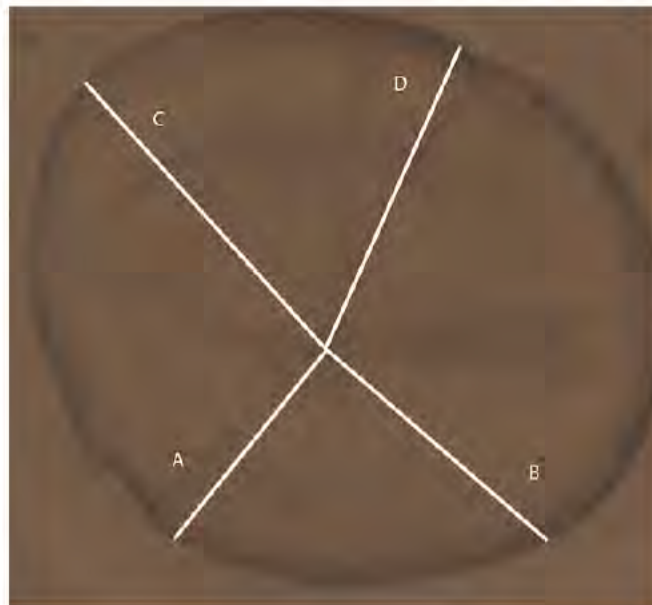


Fig. 10. Possible transects of analysis from the otolith core to the edge.

⁶ The otoliths had previously cracked during the polishing phase, and there was no causative relationship between staining and otolith damage.

Transect A (Fig. 10) creates the shortest radius, which is the least likely to exhibit a complete increment sequence, and will be the most difficult to analyze due to the “crowding” of the increments (Campana, 1992). Transect C creates the longest radius, but is the most likely to contain growth anomalies. Transects B and D are of similar length, but transect B avoids the dark bands radiating from the core, making it the best choice for accurate age and growth analysis. I took the images along as many viable transects as possible as well as at various foci to ensure the best visualization of all increments (Fig. 11). Focal variations are essential for achieving the best visualization to differentiate between the daily and subdaily increments, and for compensating for the focal plane variation of the increments themselves (Campana, 1992).

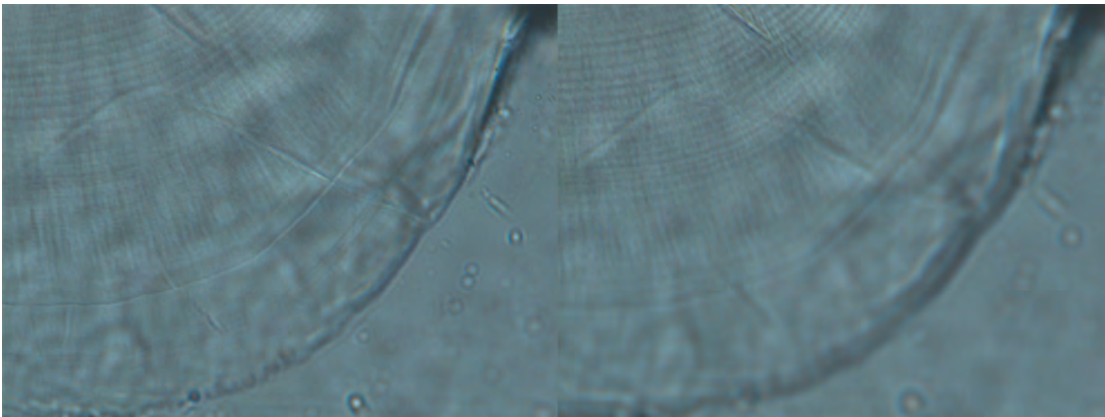


Fig. 11. Examples of the variation in increment visibility at different foci under 100x magnification.

Evaluation

After completing the imaging phase, I examined the pictures taken at both 63x magnification to determine if the otolith was suitable for analysis, or if it had

sustained too much damage and needed to be withdrawn from the final dataset. Cracked otoliths were evaluated by determining if the crack would interfere with visual analysis of the transects from the core to the outer edge (Fig. 12). If the crack was restricted to one side of the otolith, particularly with either the largest or shortest diameter, as those transects are the least suitable for analysis, then the otolith was still viable and could be kept in the dataset (Fig. 12A).

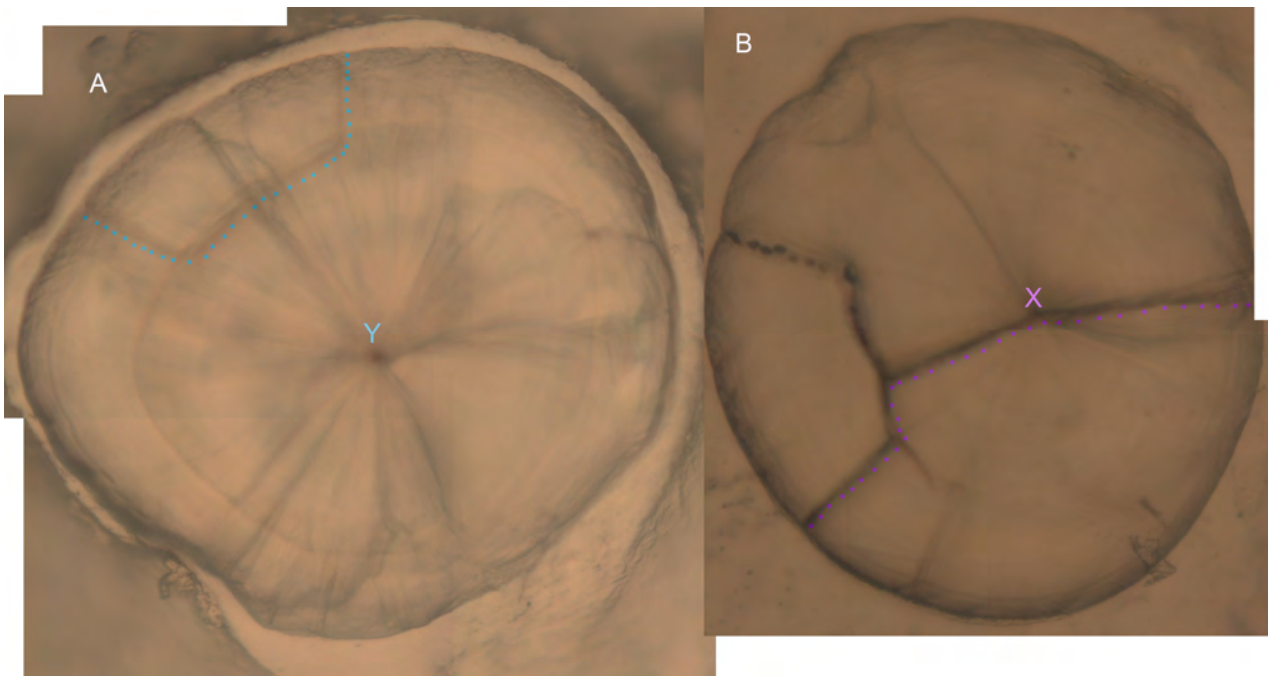


Fig. 12. Examples of cracked otoliths from two different specimens at 63x magnification. The crack in A is restricted to the outside edge of the otolith and doesn't cross the primordium (Y), so a complete transect can still be made from the core to an un-damaged part of the outer edge. The crack in B crosses the primordium (X), potentially obscuring the core and preventing analysis.

If the crack obscured or cut through the core of the otolith, I examined the images taken at 100x magnification to confirm that analysis was inhibited. In some cases the crack was either clean and far enough to the side of the core to still permit analysis, or a change in focus allowed the visualization of the core underneath the

crack if it was shallow. However, in three cases it created fracturing effect that obscured the core and the increments immediately surrounding it regardless of the focus, eliminating them from the dataset (Fig. 13).

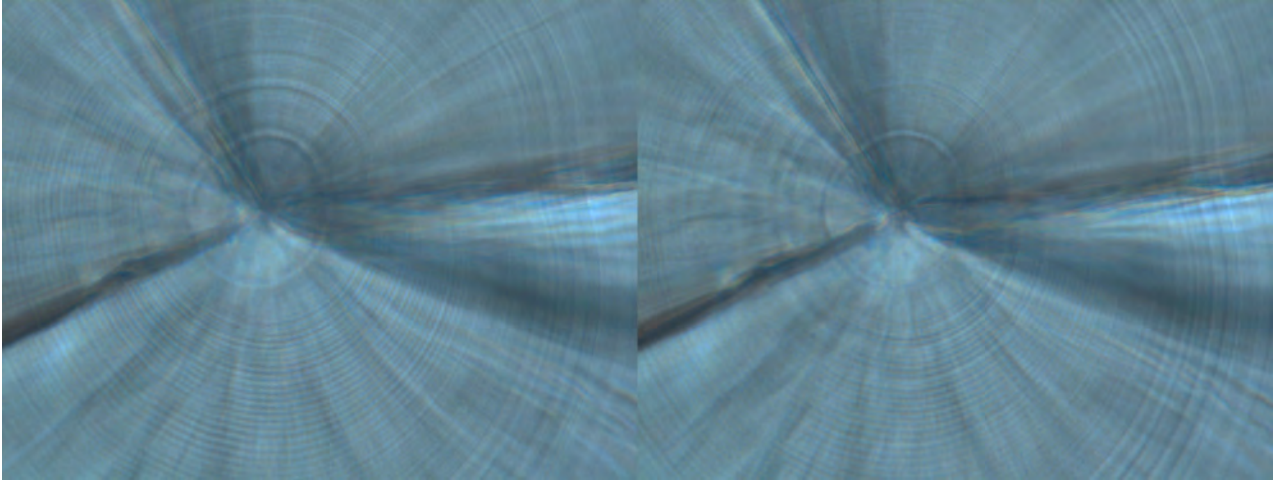


Fig. 13. The primordium of the otolith from Fig. 12B at two different foci under 100x magnification.

While at this stage I also took stock of the structural anomalies that were present in some of the otoliths. One such anomaly was the presence of an accessory growth center, or a growth center that's formed outside of the otolith's core and typically produces a new plane of growth, at the outer edge of an otolith (Fig. 14). Accessory growth centers are generally associated with some form of life-history transition, and can inhibit the interpretation of the daily increments if they interfere with the axis of analysis (Kalish, 1987). However, in this instance the location of the accessory growth center does not interfere with the integrity of the rest of the otolith, and does not inhibit analysis.

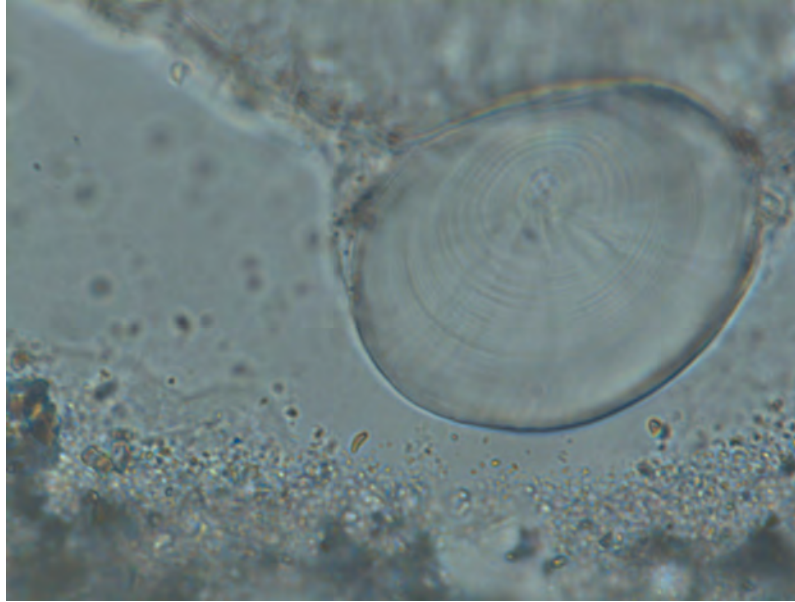


Fig. 14. An accessory growth center on the outer edge of an otolith. Increments within the accessory growth center are visible, and can be analyzed to determine how recently the center was created, indicating the time of a significant life history event.

An additional structural anomaly I found was an otolith with multiple primordial granules (Kalish, 1987). While it's not abnormal for otoliths to have multiple primordial granules, the granules generally fuse together during the early stages of otolith growth to form the core, so it's less likely to see them remaining distinct from each other. This structural formation does not restrict analysis and interpretation of the daily increments, and the crack in this specimen provides a good example of a crack through the core that does not inhibit analysis, as the primordia and surrounding increments are still clearly visible.

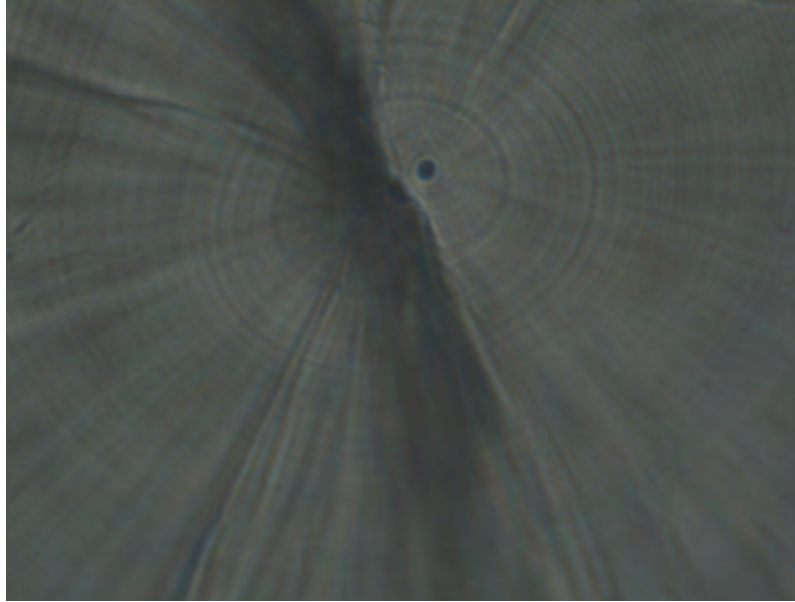


Figure 15. An otolith with multiple granules in the primordium.

Interpretation and Analysis

The image database I created over the course of this project contains images of 27 otoliths from 16 specimens, which are available for analysis. Further analysis would consist of using editing software like Photoshop to stitch together the images with the most distinct visible increments to create a complete transect from the core to the edge of the otolith, then using image-processing software to count the individual daily increments and measure the distances between them. The recommended software for this kind of analysis is Image Pro Plus, though this software is highly expensive and may be cost prohibitive (A. Lochet, pers. comm., November 19, 2015). A suitable alternative is the ObjectJ plugin for ImageJ, a free image analysis software plugin that was designed for analyzing tree rings and otoliths. This software allows the user to use a point and click technique to mark the outer edge of each increment, and non-destructively numbers the increments,

allowing the user to track their progress without compromising the image (Fig. 16). ObjectJ also produces a text output with the number of increments measured and the distance between each increment, allowing the user to estimate the rate of growth at different stages of life.

The most pressing issue for image analysis is determining which increments are daily and which are subdaily, as confusion between the two would produce erroneous results that could jeopardize the integrity of the study. While this is primarily an issue of familiarity with otolith imagery, differentiation between the two can be improved by taking the regularity of the true daily increments into account. Daily increments form consistently, and should produce a regular sequence with smooth transitions and visual prominence over subdaily increments. By locating a section of clear daily increments and using their form and regularity to inform their interpretation of the rest of the otolith, the analyst can improve their chances of successfully interpreting the otolith microstructure (Campana, 1992).

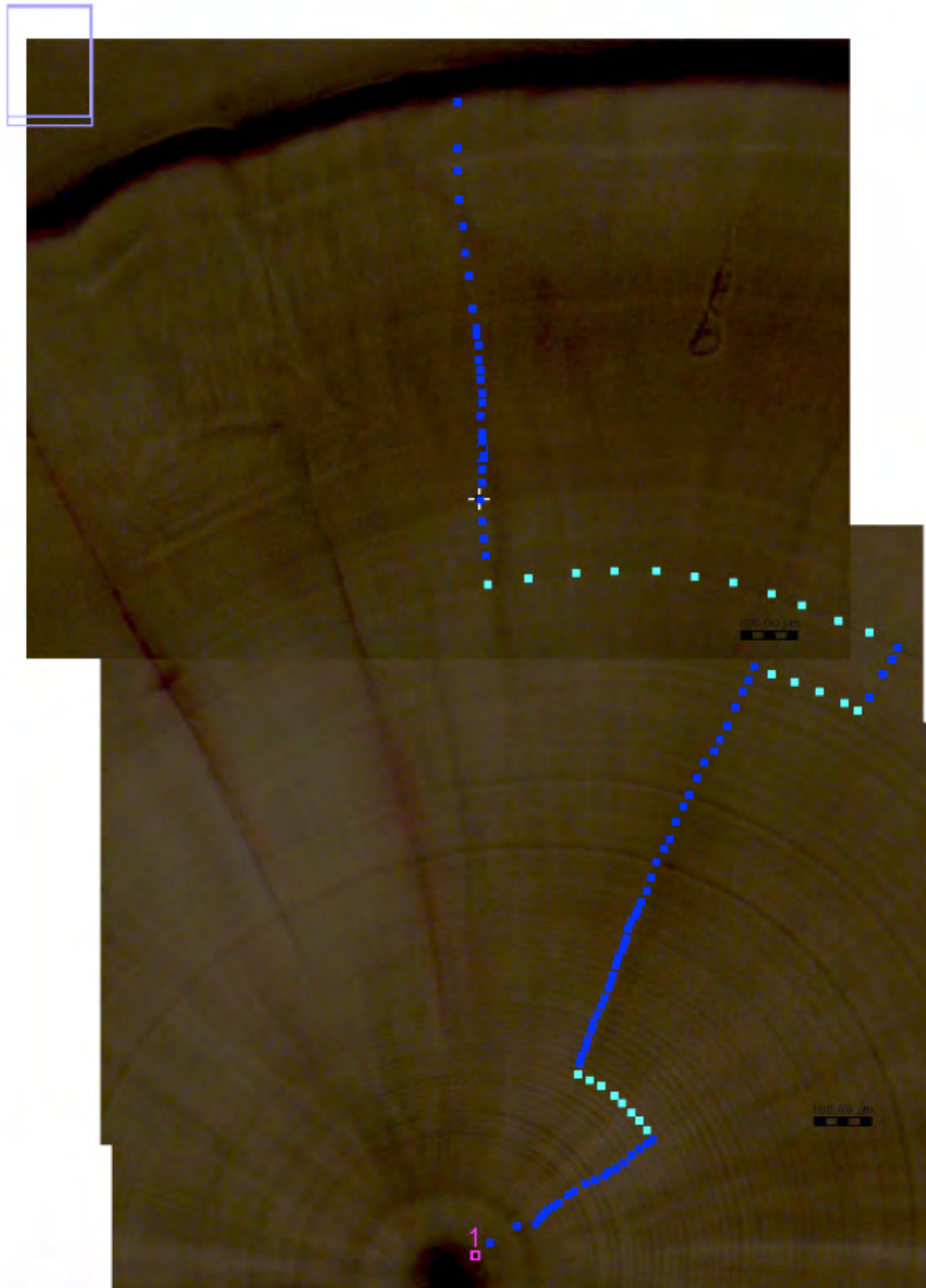


Fig. 16. Example of an otolith with daily increments marked and counted using ImageJ's ObjectJ plugin. The dark blue dots indicate distinct daily increments, and the light blue dots indicate moving along a single increment to an area with clearer visualization. This example of analysis followed the path of clearest visualization to count the increments, but would not be suitable for determining the rate of growth as the path deviated from a single straight transect.

Discussion/Conclusion

Evaluation of Methods

While this project was successful in creating a viable database of images for future study and analysis, the process of refining the protocol and even the finalized version of the protocol produced a large number of cracked and lost otoliths, leaving only 23 of the initial 40 otoliths available for analysis. Five of the otoliths were lost due to mishandling (Supplemental Fig. 5), or my initial inability to manipulate objects of such a small size despite the use of a dissecting microscope. In several cases the otoliths were lost when I put too much pressure on the watchmaker's forceps while trying to pick them up to remove them from the specimen's cranium or transfer them to dry storage, effectively catapulting them off of the sylguard plate and onto the lab bench where they were unable to be recovered due to their minute size. This was also an issue when I was manipulating them on the sylguard plate to clean off the excess tissue. While some collateral damage is to be expected when attempting to use fine motor skills in a new way for the first time, it can be mediated by using extreme restraint in the amount of pressure used to manipulate these structures, and by moving and transferring the otoliths by allowing them to "stick" to one of the forcep tips instead of trying to grasp them between both tips.

Though the cracks do not inhibit analysis in many cases, it's important to minimize the amount of damage done to all samples in order to improve both the ease of analysis and the reliability and reproducibility of results. The majority of the damage was caused during the polishing phase of processing, when applying too much or uneven pressure caused the otoliths to crack. While this issue was largely

due to my inexperience with otolith handling and processing, it's possible that an alternative polishing method may provide better results in future studies.

An alternative method of polishing that wasn't used in this study requires adhering the lapping film to a flat surface, placing a slide with a mounted otolith face down on the lapping film, placing the pointer and middle finger on either side of the otolith, and polishing by moving the whole slide in gentle circular motions (Campana, 1992). This may provide a more even polishing plane and reduce the risk of cracking as well as avoid the issue of over-polishing the edges of the otoliths that arose in this study – however, the mounting media must have extremely secure adhesion to avoid dislodging the otolith, and the otolith cannot be mounted at an angle, as this method doesn't allow the precision needed to compensate.

The issue of cracking during polishing was exacerbated if the otolith had been embedded in epoxy resin, as polishing through the resin required more pressure than the otoliths could stand. Being embedded in resin may have also changed the angle at which the pressure was directed at the otolith, causing weaker areas (e.g. the thinner outer edges) to crack. Given these results, the embedding of glass eel otoliths in epoxy resin is not recommended unless the researcher has access to the precision tools necessary for sectioning after embedding.

Future Studies

Future analysis could be performed on this image dataset to address a number of unknown facets of the life history of American eels inhabiting the Hudson watershed. These images could be used in a stand-alone study to determine the age of the eels at the day of capture, providing an estimate of how long it took for them

to complete their larval migration. This potential study could also include an intra-cohort comparison of the rate of growth these eels had along their migration, which would be determined by measuring the length of the individual increments while counting them. The researcher could also look at stress checks in the microstructure and create a timeline of stressful or significant life events in the eels' histories – for example, stress checks can be caused by temperature fluctuations (Miller, 1994), like encountering the thermal pollution caused by nuclear power plants like Indian Point. If consistent stress checks were recently formed, it could be an additional indication of Indian Point's impact on the Hudson.

In addition to the saggitae, 12 specimens had at least one lapillus otolith removed during dissection and placed in dry storage. While the processing of otoliths for light microscopy renders them unusable for microchemistry studies (Jones, 1992), a microchemistry analysis could be performed on the stored lapilli to provide important environmental context about the eels' migration. These kinds of intra-cohort studies have never been performed for the Hudson River's American eel population, and the data they would provide could be useful in maintaining and monitoring their population. Since the larval life stage is one of the most significant sources of eel mortality (Bonhommeau, 2010), understanding the duration and conditions of this migration is pivotal to understanding *A. rostrata* population dynamics. Establishing early life growth rate is especially important for understanding the life history of long-lived species like *A. rostrata*, as they may undergo growth rate changes of the course of their lives due to internal and external changes (Oliveira, 2002).

An additional study could be performed by using the preserved bodies of the eels to look for evidence of *A. crassus*. While preserving the eels in ethanol caused them to become opaque, reducing the effectiveness of visual examination during dissection of the swim bladder and intestines to confirm the presence of nematodes, a DNA extraction could be performed to establish the presence or absence of an infection. If an infection with *A. crassus* is confirmed, the researcher should examine the results of the otolith image analysis to try to determine the age of infection. If infected eels consistently show an increased age at time of capture and/or a decreased growth rate after a stress check, this would imply that *A. crassus* is parasitizing these eels before they reach freshwater. Additional DNA extractions could also be performed on the gut contents of the preserved glass eels, which could indicate the vector host being used by *A. crassus* to enter migrating eel populations. The data could also be used in conjunction with the analysis of otoliths from glass eels collected during previous and/or future migrations to determine if the age of arrival and rate of growth remains consistent across migration cohorts. If significant differences are found in either the age or rate of growth of glass eels arriving in freshwater streams off of the Hudson, it could indicate significant changes in the health and function of the Hudson or the Gulf Stream.

It has also been found that the size of the immigrating glass eels decreases over the course of the spring migration in the Saw Kill and other streams feeding into the Hudson, with glass eels entering the Saw Kill at the end of the run having significantly shorter total lengths on average than the eels that arrived early in the season (R. Schmidt, pers. comm., April 10, 2015). An otolith study could be

performed to investigate this pattern in order to determine if this disparity in size is simply because it takes longer for the smaller eels to complete migration, or if there is a correlation between hatching later in the season and having a shorter total body length, and to see if this size disparity is consistent across migrations. A similar study of elver stage eels in North Carolina found that the shorter eels were in fact older than the longer eels, indicating that the former hypothesis is highly likely (Powles, 2002). The New York State Museum has glass eel specimens from the 2004, 2005, 2006 and 2007 spring migrations preserved in ethanol by Robert Schmidt, which would be suitable for these kinds of studies – specimens preserved earlier are kept in formalin, which degrades otolith structure and renders them unusable for otolith analysis. Each of these studies, either performed individually or in conjunction with each other, would provide valuable data about the life history and health of American eel populations in small Hudson tributaries.

Despite the large number of studies conducted on *A. rostrata* in a range of habitats, there is still much to be determined about its oceanic, migrational, and early life history, and otolith analysis is an extremely useful tool in the pursuit of that information. This project was successful in refining a replicable protocol for processing otoliths of juvenile eels for image analysis using materials that are easy to access and manipulate and are not cost-prohibitive. It was also successful in creating a database of images that can be used for either an independent study of intra-cohort growth rates of glass eels in the Saw Kill, or as part of a larger study of the life history of eels all along the Hudson. The preserved eel specimens will be stored in the Bard Field Station and all mounted and stored otoliths and images will

be kept in the RKC for later use and reference if future Bard students or members of the Hudson River Eel Project decide to pursue one of the experiments outlined above.

Bibliography

- Aieta, A. E., & Oliveira, K. (2009). Distribution, prevalence, and intensity of the swim bladder parasite *Anguillicola crassus* in New England and eastern Canada. *Diseases of Aquatic Organisms*, *84*, 229–235. <http://doi.org/10.3354/dao02049>
- Albert, V., Jonnson, B., & Bernatchez, L. (2006). Natural hybrids in Atlantic eels (*Anguilla anguilla*, *A. rostrata*): evidence for successful reproduction and fluctuating abundance in space and time. *Molecular Ecology*, *15*, 1903–1916. <http://doi.org/10.1111/j.1365-294X.2006.02917.x>
- American Eel Technical Committee. (2014). Atlantic States Marine Fisheries Commission 1050. *Atlantic States Marine Fisheries Commission*.
- ASMFC. (2009). Atlantic Coast Diadromous Fish Habitat: A Review of Utilization, Threats, Recommendations for Conservation, and Research Needs. *Habitat Management Series*, *9*.
- Bonhommeau, S., Castonguay, M., Rivot, E., Sabati, R., Pape, O. Le, Ouest, A., ... Halieutique, L. E. (2010). The duration of migration of Atlantic *Anguilla* larvae. *Fish and Fisheries*, *11*(3), 289–306. Retrieved from <http://archimer.ifremer.fr/doc/00011/12208/9033.pdf>
- Bowser, C. (2015). Hudson River Eel Project 2015.
- Brothers, E. B., Mathews, C. P., & Lasker, R. (1976). Daily growth increments in otoliths from larval and adult fishes. *Fishery Bulletin*, *74*(1), 1–8. <http://doi.org/10.1006/jfbi.1993.1006>
- Campana, S. (1992). Measurement and Interpretation of the Microstructure of Fish Otoliths. *Aquatic Science*, *117*, 59–71.
- Cieri, M. D., & McCleave, J. D. (2001). Validation of daily otolith increments in glass-phase American eels *Anguilla rostrata* (Lesueur) during estuarine residency. *Journal of Experimental Marine Biology and Ecology*, *257*(2), 219–227. [http://doi.org/10.1016/S0022-0981\(00\)00333-6](http://doi.org/10.1016/S0022-0981(00)00333-6)
- Denny, S. K., Denny, A., & Paul, T. (2013). Distribution, prevalence and intensity of *Anguillicoloides crassus* in the American eel, *Anguilla rostrata*, in the Bras d'Or Lakes, Nova Scotia. *BioInvasions Records*, *2*(1), 19–26.
- Findlay, S., Burns, D., Urban-mead, R., & Lynch, T. (2010). Water Resources of Dutchess County, NY. *Natural Resource Inventory of Dutchess County*.

- Gerard, C., Trancart, T., Amilhat, E., Faliex, E., Virag, L., Feunteun, E., & Acou, A. (2013). Influence of introduced vs. native parasites on the body condition of migrant silver eels. *Parasite*, *20*(38). <http://doi.org/10.1051/parasite/2013040>
- Grush, J. (2004). The Efficacy of Clove Oil As An Anesthetic for the Zebrafish, *Danio rerio*. *Zebrafish*, *1*(1): 46-53
- Heitlinger, E. G., Laetsch, D. R., Weclawski, U., Han, S., & Taraschewski, H. (2009). Massive encapsulation of larval *Anguillicoloides crassus* in the intestinal wall of Japanese eels. *Parasites & Vectors*, *2*(48), 1–11. <http://doi.org/10.1186/1756-3305-2-48>
- Hudson River Valley National Heritage Area. (2008). *Windows on History*.
- ICES. (2011). Report of the Workshop on Age Reading of European and American Eel (WKAREA2)
- Jessop, B., Shiao, J., Iizuka, Y., & Tzeng, W. (2002). Migratory behaviour and habitat use by American eels *Anguilla rostrata* as revealed by otolith microchemistry. *Marine Ecology Progress Series*, *233*(October 2015), 217–229. <http://doi.org/10.3354/meps233217>
- Jones, C. M. (1993). Development and application of the otolith increment technique. *Canadian Special Publication of Fisheries and Aquatic Sciences*. Retrieved from http://www.marinebiodiversity.ca/otolith/english/Abstracts/S_C-Chapter1.pdf
- Kirk, R. S. (2003). The impact of *Anguillicola crassus* on European eels. *Fisheries Management and Ecology*, *10*: 385–394
- Knopf, K., & Mahnke, M. (2004). Differences in susceptibility of the European eel (*Anguilla anguilla*) and the Japanese eel (*Anguilla japonica*) to the swim-bladder nematode *Anguillicola crassus*. *Parasitology*, *129*(04), 491-496
- Knopf, K. (2006). The swimbladder nematode *Anguillicola crassus* in the European eel *Anguilla anguilla* and the Japanese eel *Anguilla japonica*: differences in susceptibility and immunity between a recently colonized host and the original host. *Journal of helminthology*, *80*(02), 129-136
- Lamson, H., Cairns, D. K., & Shiao, J. (2009). American eel, *Anguilla rostrata*, growth in fresh and salt water: Implications for conservation and aquaculture. *Fisheries Management and Ecology*, *16*, 306–314. <http://doi.org/10.1111/j.1365-2400.2009.00677.x>
- Limburg, K. E., & Schmidt, R. E. (2002). Importance of Tributaries to the Hudson River Eel Population : Implications for Restoration and Management, 1–26.

- Machut, L. S., & Limburg, K. E. (2008). *Anguillicola crassus* infection in *Anguilla rostrata* from small tributaries of the Hudson River watershed, New York, USA. *Diseases of Aquatic Organisms*, 79, 37–45. <http://doi.org/10.3354/dao01901>
- Miller, J. a, & Simenstad, C. a. (1994). Otolith microstructure preparation, analysis, and interpretation: procedures for a potential habitat assessment methodology, (August), 1–32.
- Morales-Nin, B. (2000). Review of growth regulation processes of otolith daily increment formation. *Fisheries Research*, 46(MAY 2000), 53–67. [http://doi.org/10.1016/S0165-7836\(00\)00133-8](http://doi.org/10.1016/S0165-7836(00)00133-8)
- NYSDEC. (n.d.). Tivoli Bays. Retrieved May 13, 2016, from <http://www.dec.ny.gov/lands/92370.html>
- Oliveira, K. (2002). Sexually Different Growth Histories of the American Eel in Four Rivers in Maine. *Marine Sciences Faculty Scholarship*, (119), 203–211.
- Palstra, A. P., & Thillart, G. E. E. J. M. Van Den. (2010). Swimming physiology of European silver eels (*Anguilla anguilla* L.): energetic costs and effects on sexual maturation and reproduction. *Fish Physiol Biochem*, 36, 297–322. <http://doi.org/10.1007/s10695-010-9397-4>
- Pelster, B. (2015). Swimbladder function and the spawning migration of the European eel *Anguilla anguilla*. *Frontiers in Physiology*, 5(January), 1–10. <http://doi.org/10.3389/fphys.2014.00486>
- Powles, P. M., & Warlen, S. M. (2002). Recruitment season, size, and age of young American eels (*Anguilla rostrata*) entering an estuary near Beaufort, North Carolina. *Fish Bulletin*, 100, 299–306.
- Secor, D. H., Dean, J. M., & Laban, E. H. (1992). Manual for Otolith Removal and Preparation for Microstructural Examination. *Copeia*, 2.
- Stirratt, H. (Atlantic S. M. F. C. (2001). Proceedings of the Workshop on Aging and Sexing American Eel. *Atlantic States Marine Fisheries Commission*, (72), 63.
- Wang, C. H., & Tzeng, W. N. (2000). The timing of metamorphosis and growth rates of American and European eel leptocephali: A mechanism of larval segregative migration. *Fisheries Research*, 46(1-3), 191–205. [http://doi.org/10.1016/S0165-7836\(00\)00146-6](http://doi.org/10.1016/S0165-7836(00)00146-6)
- Weclawski, U., Heitlinger, E. G., Baust, T., Klar, B., Petney, T., Han, Y. S., & Taraschewski, H. (2013). Evolutionary divergence of the swim bladder nematode *Anguillicola crassus* after colonization of a novel host, *Anguilla*

anguilla. *BMC Evolutionary Biology*, 13(78), 1–11. <http://doi.org/10.1186/1471-2148-13-78>

Wielgoss, S., Gilabert, A., Meyer, A., & Wirth, T. (2014). Introgressive hybridization and latitudinal admixture clines in North Atlantic eels. *BMC Evolutionary Biology*, 14(61), 1–18.

Wirth, T., & Bernatchez, L. (2003). Decline of North Atlantic eels: a fatal synergy? *The Royal Society*, (September 2002), 681–688. <http://doi.org/10.1098/rspb.2002.2301>

Supplemental Figures

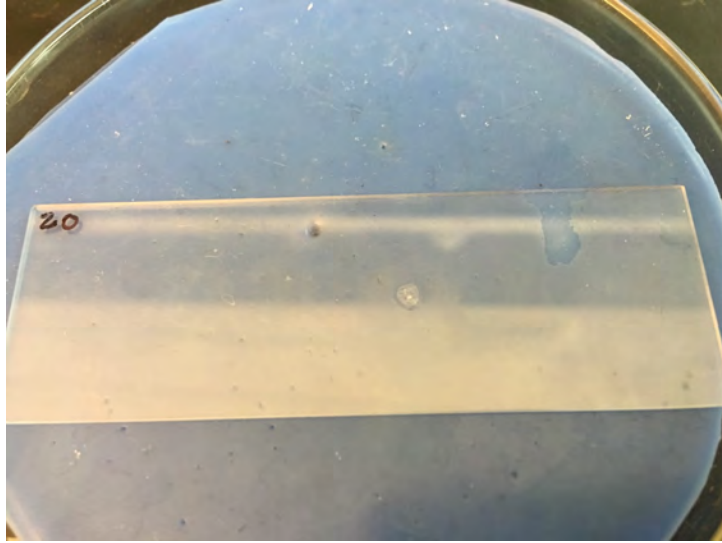
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2	1	NA	NA	NA	NA	NA	NA	no	yes	embedding	mishandling
3	NA	NA	NA	NA	NA	NA	NA	no	yes	transfer	mishandling
4	2	2	3	2	3	yes	yes	no	yes	polishing	over polishing
5	4	4	3	2	3	no	no	yes	no	NA	NA
6	4	4	3	2	3	yes	no	yes	no	NA	NA
7	3	1	3	NA	NA	yes	yes	no	yes	polishing	cracking
8	2	1	3	2	3	yes	no	yes	no	NA	NA
9	2	1	3	2	3	yes	yes	yes	yes	polishing	cracking
10	2	1	3	2	3	yes	yes	no	yes	polishing	over polishing
11	4	3	3	2	3	yes	no	yes	no	NA	NA
12	4	4	3	2	1 and 2	yes	no	yes	no	NA	NA
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17	4	4	3	2	3	yes	no	yes	no	NA	NA
18	4	4	3	NA	NA	yes	no	no	yes	polishing	over polishing
19	4	4	3	2	3	yes	no	yes	no	NA	NA
20	4	4	3	2	3	yes	yes	yes	yes	polishing	cracking
1B	NA	NA	NA	NA	NA	NA	NA	no	yes	dissection	mishandling
2B	4	4	3	2	3	yes	no	yes	no	NA	NA
3B	NA	NA	NA	NA	NA	NA	NA	no	yes	transfer	mishandling
4B	4	4	3	2	3	yes	no	yes	no	NA	NA
5B	NA	NA	NA	NA	NA	NA	NA	no	yes	dissection	mishandling
6B	NA	NA	NA	NA	NA	NA	NA	no	yes	dissection	mishandling
7B	4	4	NA	NA	NA	NA	NA	no	yes	mounting	mishandling
8B	4	4	3	2	3	yes	no	yes	no	NA	NA
9B	4	4	3	2	3	yes	yes	no	yes	polishing	cracking
10B	4	4	3	2	3	yes	no	yes	no	NA	NA
11B	4	4	3	2	3	yes	no	yes	no	NA	NA
12B	4	4	3	2	3	yes	no	yes	no	NA	NA
13B	4	4	3	2	3	yes	no	yes	no	NA	NA
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16B	4	4	3	2	3	yes	no	yes	no	NA	NA
17B	4	4	3	2	3	yes	no	yes	no	NA	NA
18B	4	4	3	2	3	yes	no	yes	no	NA	NA
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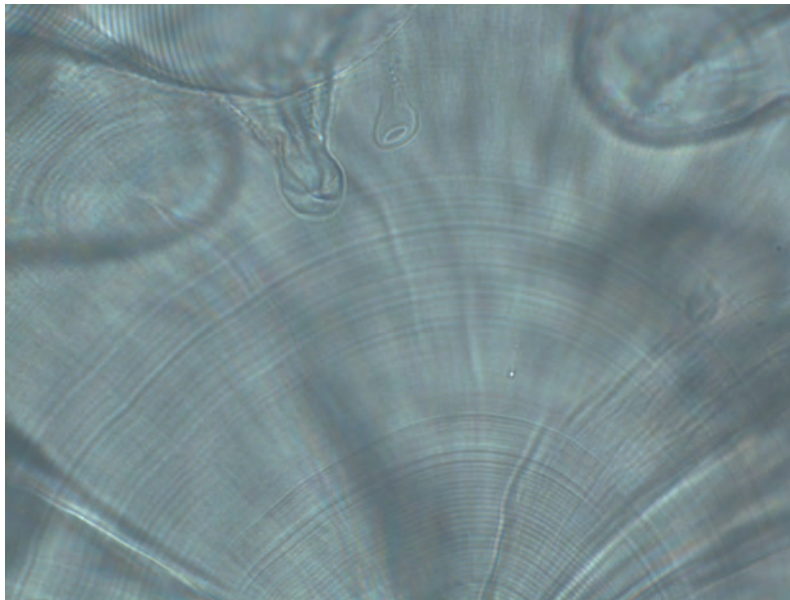
Supplemental Figure 1. Complete methods table for all otoliths in all specimens



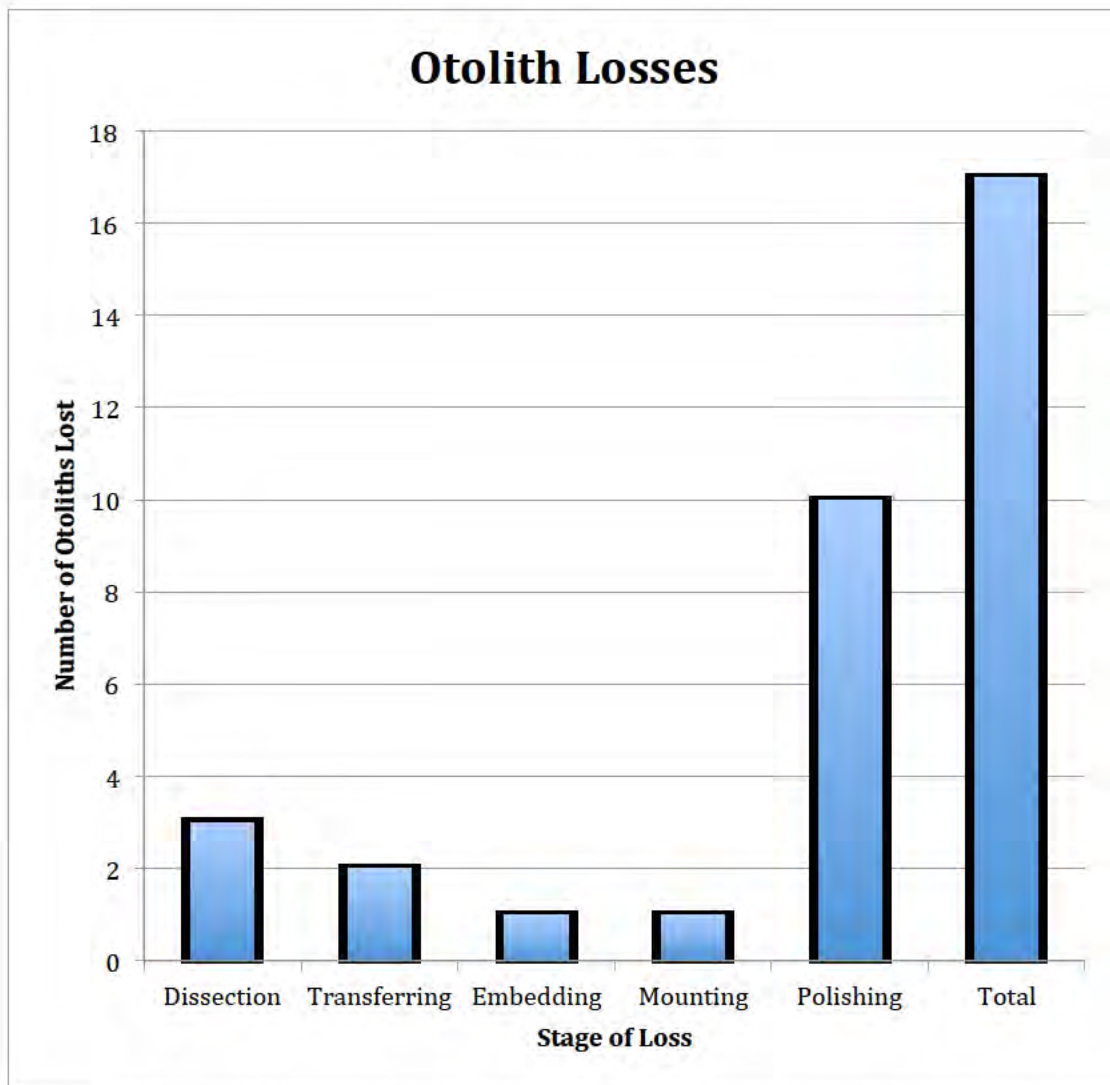
Supplemental Figure 2. The lapping film setup used for polishing method 3.



Supplemental Figure 3. An un-embedded otolith mounted with Loctite gel super glue on a glass slide held on a sylguard plate for polishing under a dissection microscope



Supplemental Figure 4. An example of a polished vateritic otolith with structural anomalies that inhibit analysis



Supplemental Figure 5. Number of sagittae lost at each phase of processing.