

Abstract

Analyzing stable isotopes in archival tissues, such as fish eye lenses, is a technique used to document shifts in feeding ecology, diet, habitat use, and to reconstruct life history. Fish eye lenses have a structure similar to onions, consisting of multiple layers, or laminae. These laminae represent the chronology of the fish's life, much like tree rings. Lenses are protein-rich, which makes them an ideal structure for analyzing light isotopes such as $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and $\delta^{34}\text{S}$. These light isotopes are primarily integrated into the lens tissue through the fish's diet, similar to muscle tissue. As more research emerges using eye lenses to reconstruct the life histories of fishes, the need for a reproducible method of delamination grows. For this study, two different individuals peeled lenses from each eye of the same adult Chinook Salmon (*Oncorhynchus tshawytscha*), for a total of 10 fish. Lens lamina number, diameter (mm), and mass (mg) of individual laminae were recorded. Laminae were then submitted for isotopic analysis of both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$. Isotope values were used as a validation reference to compare delamination patterns between both peelers. They were then plotted using both the lamina number (assigned by peeler) and lens diameter to compare the difference between the two. Throughout the experiment, analyzing the laminae based on lamina number resulted in significant variability among peelers. However, when lens diameter was used instead of lamina number, the isotope values throughout the lenses of the same fish were nearly identical. Using lens diameter removes subjectivity between peelers, thereby increasing the reproducibility of the technique and providing a more robust interpretation of the lens.

Introduction

Monitoring animal movements across continents or hemispheres is widely employed in the field of conservation biology due to its significance in determining which geographic regions and habitats warrant conservation efforts. This method is particularly powerful for animals with large home ranges or complex life cycles that depend on different habitats throughout ontogeny.

Studies that track migration with traditional extrinsic markers are often complicated, costly, and invasive to the animals. Stable isotope analysis (SIA) offers an alternative way of studying animal movement patterns, diet histories, and their natal origins without requiring the subsequent recapture of the same individual [1,2]. SIA has successfully been used to create isoscapes, or geographic maps that depict where unique isotopic signatures or unique food web patterns occur within the landscape [3,4]. This method is particularly useful for organisms that may be too small for tracking devices but can also complement studies that use traditional extrinsic markers [5]. Lenses have emerged as intrinsic markers to reconstruct the life history of fishes by analyzing the $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and $\delta^{34}\text{S}$ found in each sequential lamina. This has been informative in determining detailed dietary isotope trajectories over time [4,6–12].

In previous studies, lenses have been represented using both laminae number as well as diameter [13,14]. When studying eye lenses, lamina number is a more accessible metric to use compared to lens diameter. Measuring the lens necessitates specialized and often costly equipment, such as microscope cameras. Lamina Number is assigned by the peeler and is heavily influenced by each peeler's different tendencies. Thus, interpreting the lens using lamina number can lead to errors, especially if there are multiple lens peelers. The application of the delamination of eye lenses is a growing research tool. Yet the peeler to peeler variation has not yet been effectively quantified. For this reason, there is a need to expand upon the methodology of this field via assessing peeler to peeler inconsistencies when it comes to lamina number. This study strives to offer a more refined and reproducible method for lens delamination by reducing the intrinsic human effects of the delamination process.

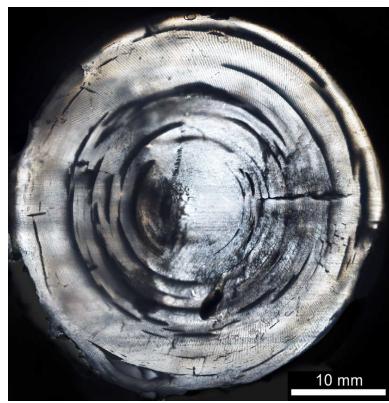
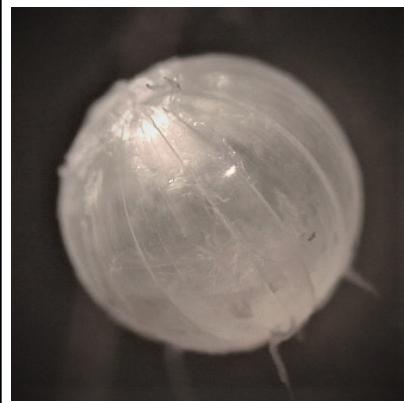
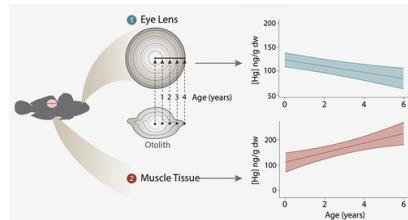


Photo 1: A picture of a Chinook Salmon eye lens. Taken on a Leica S9i with the accompanying software, LAS X.

Photo 2: Cross section of a Chinook Salmon lens.



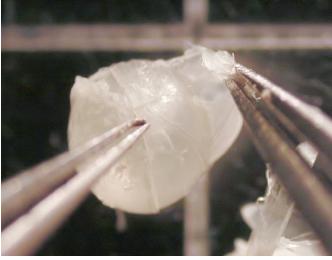
Methods

Lens Technique

For this study, lenses were extracted from both the left and right eyes of 10 adult fall-run Chinook Salmon (*Oncorhynchus tshawytscha*). Lenses were extracted by creating an incision in the eyes and then removed with a pair of forceps. Each lens was then stored in a 1.5mL eppendorf tube and frozen until delamination could take place.

Both peelers delaminated one of the lenses from the same fish, for all 10 fish used in this study, using methods developed by [7] and [13]. Each individual lamina was peeled all the way to the core and measured to the nearest thousandth of a millimeter using a Leica S9i imaging microscope with LAS X imaging software. Laminae were then stored in pre-weighed 8x5mm tin capsules (Elemental Microanalysis pressed tin capsules) and, once dried, were weighed to the nearest hundredth of a milligram on a Mettler Toledo Semi-Micro Balance (MS105DU). Individual lamina that exceeded the weight limit (>2.0mg) for isotope analysis were homogenized and subsampled to meet the 0.6mg-6 mg dried weight criteria for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analysis. All samples were then submitted to the CAMAS Stable Isotope Laboratory at Idaho State University for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analysis. Individuals worked independently and without communication with one another.

Lens Delamination Process:

			
1) Lens delamination or peeling begins by locating the seam in the middle the lens.	2) Work all the way around the lens, peeling each section of the lamina off the lens. When lenses became too dry and brittle for delamination, a small amount of DI water was added to the sample to aid in peeling of the lens.	3) The lens should have four main quadrants, as seen in the photo (apart from one that began to split into two).	4) Once the lamina is removed, it can be moved to a tin capsule to finish drying. Then repeat steps 1-4 for the next lamina.

Statistical Analysis:

In order to spot any initial differences between the two peelers, boxplots were used to visually compare the average total number of laminae per eye peeled by the two peelers. A t-test was subsequently conducted on the data from the boxplot to determine if the difference was statistically significant. Following this comprehensive overview, the details of the aspects of analysis were investigated. Specifically, the average width of each lamina between the two peelers was examined, with the aim of identifying potential patterns or trends. To quantify the observations, a linear regression model was constructed using peelers and lamina width as factors, followed by an anova test on this model, primarily intended to ascertain the potential impacts of peelers on the width of individual lamina. After the validity of using lamina number as a potential sample tracking metric was concluded/proven, the introduction of lamina diameter as an alternative tracking method was decided upon. Subsequently, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ isotope values of each sample were plotted against these two parameters, and a visual investigation was conducted to determine which method better facilitated the alignment of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ isotope values from the same pair of eyes processed by the two different peelers.

Analysis:

Patterns in laminae and diameter isotopic variation were compared visually between peelers using plots created in R. All analyses were performed using R version 3.4.4 (R Core Team, 2018). The four fish with the largest variation in lamina number between individuals were used to display the variation in data.

Results:

Differences between peelers:

Peeler 1 and 2 displayed distinct variations in their peeling methods and outcomes. Notably, Peeler 1 exhibited a tendency to peel thicker laminae compared to Peeler 2, resulting in a mean total lamina count of 24.1, while Peeler 2, who tended to peel thinner laminae, achieved a higher mean total lamina count of 27.7 (see Figs. 1 and 2). Peeler 1's total lamina count did not exceed 26, whereas Peeler 2 acquired a maximum of 33 laminae. A t-test found there to be a

statistically significant difference in the average lamina number produced per eye between the two peelers ($t = 3.33$, $P < 0.05$).

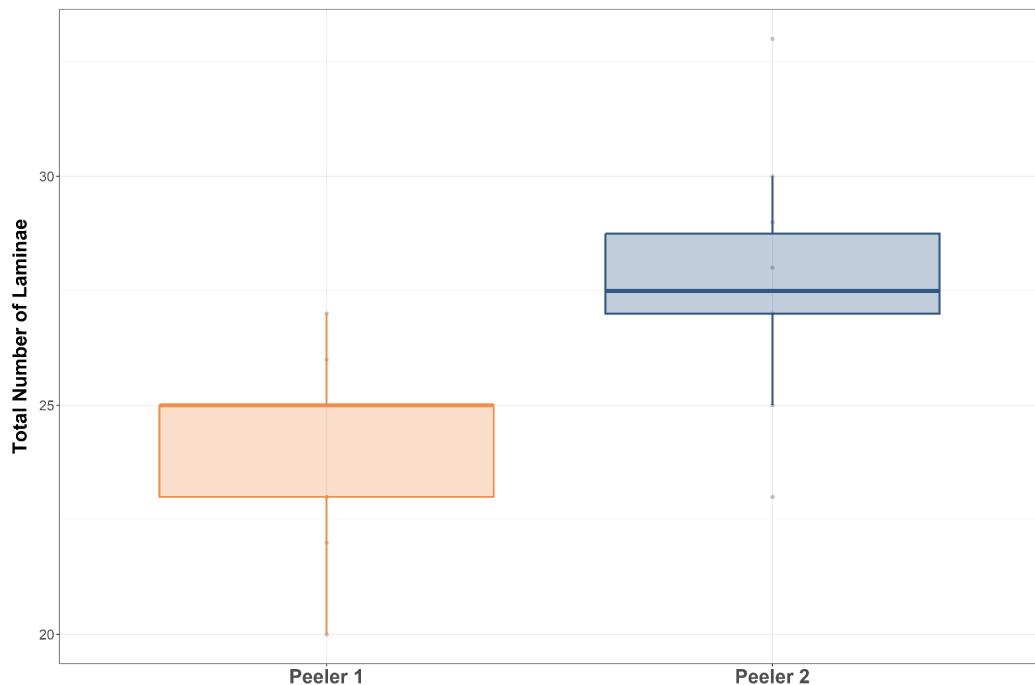


Figure 1: A boxplot displays the difference in total laminae between peelers. Peeler 1 has an average total laminae of 24.1, and Peeler 2 has an average total laminae of 27.7. The box denotes the median and interquartile range, while the whiskers extend to 1.5 times the interquartile range.

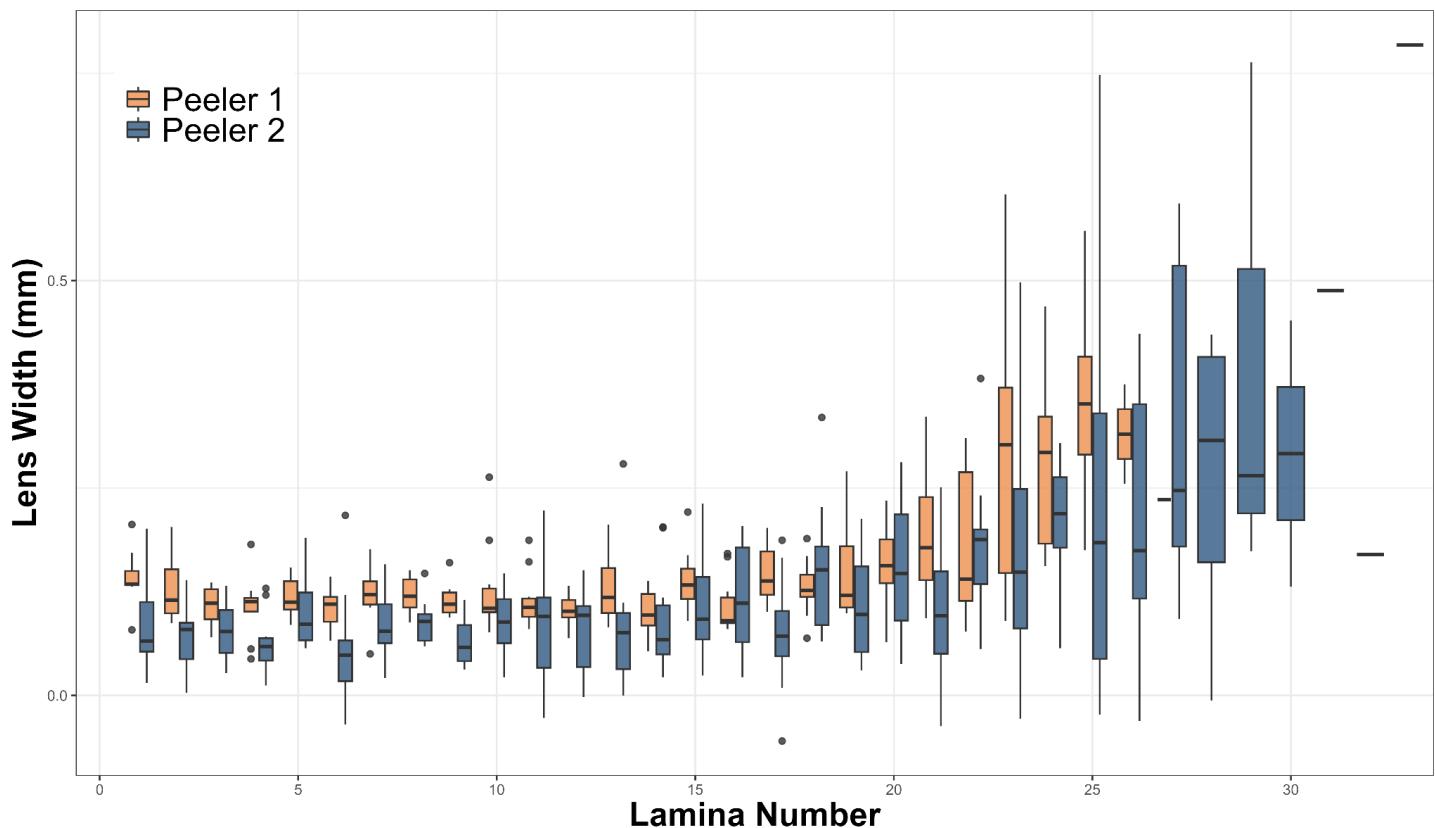


Figure 2: A boxplot showing average lens widths for each lamina between peelers. The box denotes the median and interquartile range, and whiskers denote 1.5 times the interquartile range.

Analytical Impacts:

The differences between peelers had implications for subsequent analyses. Figure 3 illustrates that at identical lamina numbers, Peeler 1 consistently recorded larger lens diameters than Peeler 2. This trend is further supported by analyzing the average lamina mass of each lamina by peeler, where Peeler 1 removed laminae with greater mass than Peeler 2 for the same lamina number on average (see Fig. S1, supplemental). It is evident that both peelers presented distinct trends in their peeling methods that significantly influenced the assigned lamina number for the same lens diameter ($F(1, 500) = 134.71, p < 2.2e-16$). Due to the human influence linked with lamina number assignment, the same lamina number might correspond to different lens diameters for fish delaminated by different peelers, thus causing a mismatch with isotope values.

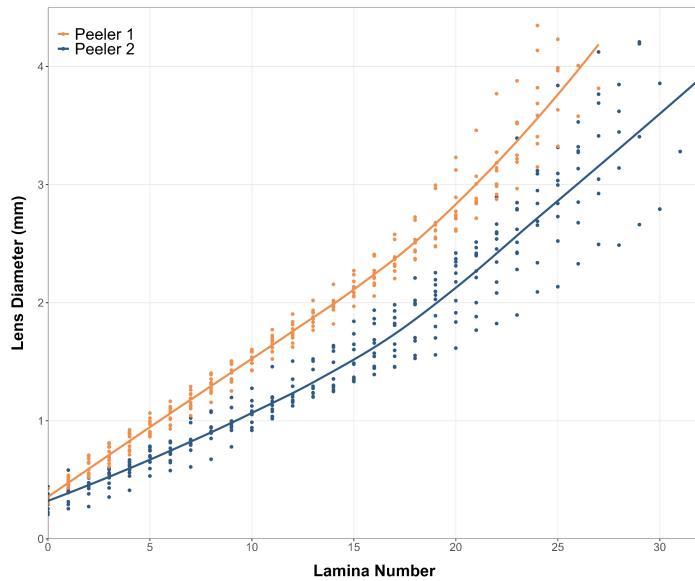


Figure 3: A scatter plot of lamina number plotted against the lens diameter (mm) of each peeler.

Resolutions:

A comparative analysis was then performed using $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values from two lenses of the same fish. This analysis evaluated the utilization of assigned lamina numbers and measured lens diameters as metrics for comparison, as shown in Figures 4.1, 4.2, 4.3, and 4.4. The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values served as grounding references for assessing the relative accuracy between lamina number and lens diameter. When plotting the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ trajectories against lens diameter, a much more consistent alignment between the two peelers was evident (Figs. 4.3 and 4.4). This suggests that the discrepancy between the isotope trajectories is due to the use of lamina number as a metric.

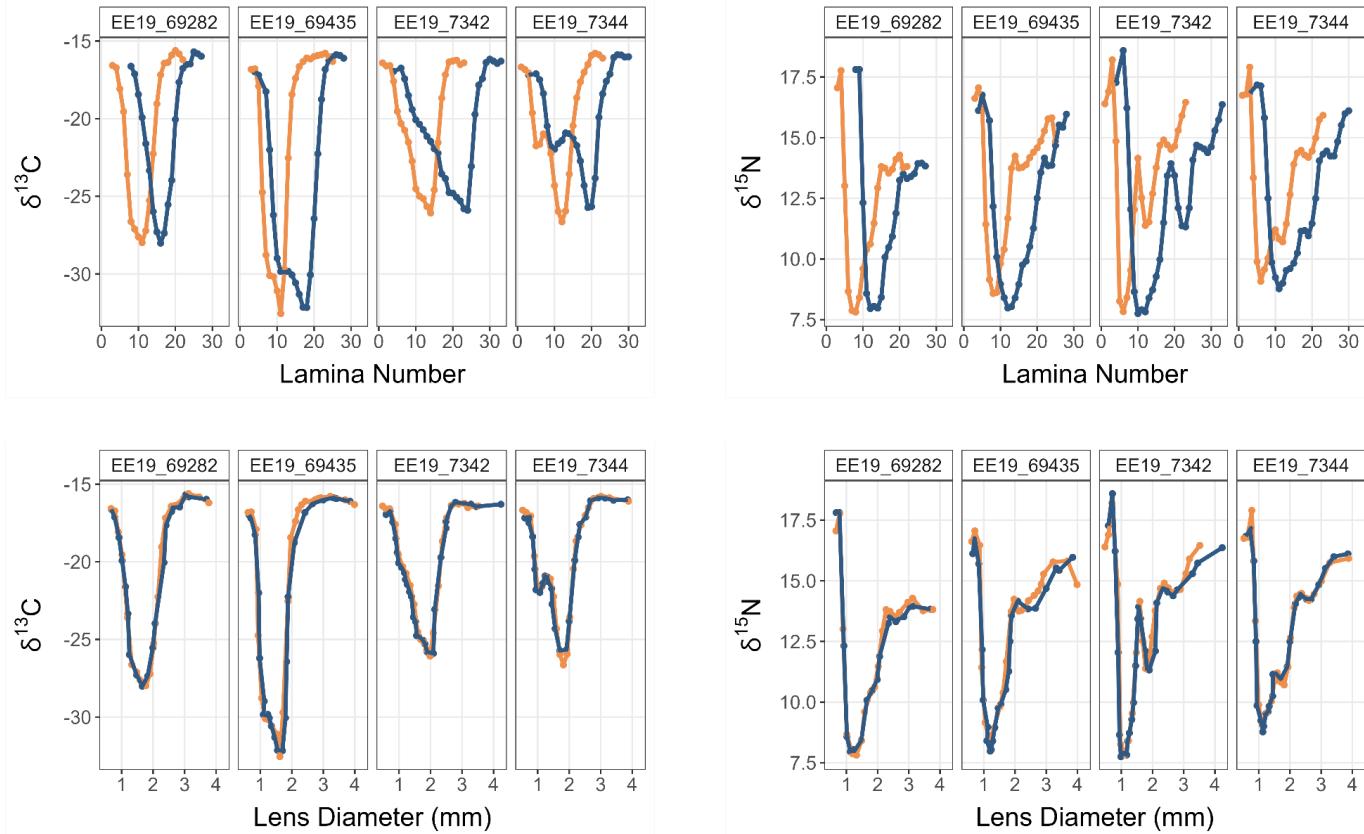


Figure 4.1 (top left), Figure 4.2 (top right): Scatter plots of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values from consecutive laminae plotted against lamina number for 4 of the most distinctive fish from the total sample size of 10.

Figure 4.3 (bottom left), Figure 4.4 (bottom right): Scatter plots of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values from consecutive laminae plotted against lens diameter (mm) for the same fish from Figures 4.1 and 4.2.

Discussion:

Stable isotope analysis using fish eye lenses is becoming increasingly popular as a way of understanding and interpreting a fish's life history. The data from lens isotope studies is valuable in elucidating diet history as well as habitat use. This tool, in combination with other endogenous records, such as otoliths, has the ability to provide a more holistic understanding of a fish's life history to better understand population level needs. As the amount of research increases, the need to standardize for reproducibility grows. This study demonstrated how to delaminate lenses in a way that is reproducible.

When plotting lamina number against lens diameter, it became evident that the innate human differences between peelers influenced the assigned lamina number for the same lens diameter, resulting in confounding interpretations of the lens (see Fig. 3). Due to the minimal variation of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in the respective laminae of the left and right eyes, the isotope trajectory for both lenses is expected to match (Wallace). With lamina number on the x-axis, there was a noticeable lag in isotopic trends between the peelers, despite the similarities in shape between them (Figs. 4.1 and 4.2). This implies that the isotope values for the laminae received would correspond to different time periods in the fish's life, making it challenging to extract valuable conclusions when focusing solely on analyzing a specific life stage (see Figs. 4.1 and 4.2). Given the complexities associated with lamina numbers, lens diameter was introduced as an alternative metric for processing and analyzing isotope data. When the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values were plotted with lens diameters instead of lamina number, the trends between peelers were aligned, demonstrating that lens diameter is crucial when comparing the isotope values of lens laminae when there is more than one peeler. The results underscore

the significant impact that different peelers have on the eye lens peeling process, thereby emphasizing the need for an alternative approach to ensure quality control. Lens diameter offers a more dependable marker for growth categorization, overcoming the limitations associated with the use of lamina number. The adoption of lens diameter corrects for biases introduced by human effects that are evident in assigned lamina numbers. Using lens diameter instead of lamina number as a comparison metric enhances the reliability and reproducibility of lens delamination.

However, despite the differences between peelers, the use of lamina number is still a valid method available when measuring tools are unavailable or inaccessible due to high cost. Imaging microscopes can be costly, while other measuring tools exist, they might not provide as accurate or high of resolution of measurements in the lens as an imaging scope. Consistency was observed within individuals, suggesting that lamina number can be used if there is one person consistently delaminating lenses. Then isotopic variation and trends can still be interpreted for that specific study. Additionally, categorizing by lamina number is a useful naming system for keeping individual lamina organized for sample submission, storage, and in a database. Delaminating lenses generates many samples per individual fish; having a consistent naming system, such as lamina number is key for organization.

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Supplemental Information:

Eye Lens Peeling Notes:

1. In some adult fish, there is sometimes a lamina that isn't fully formed, the PFL or partially formed laminae, that contains living cellular organelles [15].

2. In our study, we found that the lens had four main quadrants that can be peeled off given enough moisture. Too much moisture caused too much of the lamina to be peeled off at once, while too little moisture made the lens too difficult for peeling and for the four main quadrants to split into multiple pieces. With the right balance of moisture, multiple, discrete laminae can be removed from the lens. This allows for higher resolution isotope data that can be interpreted from each individual fish.

Lamina Number VS mass:

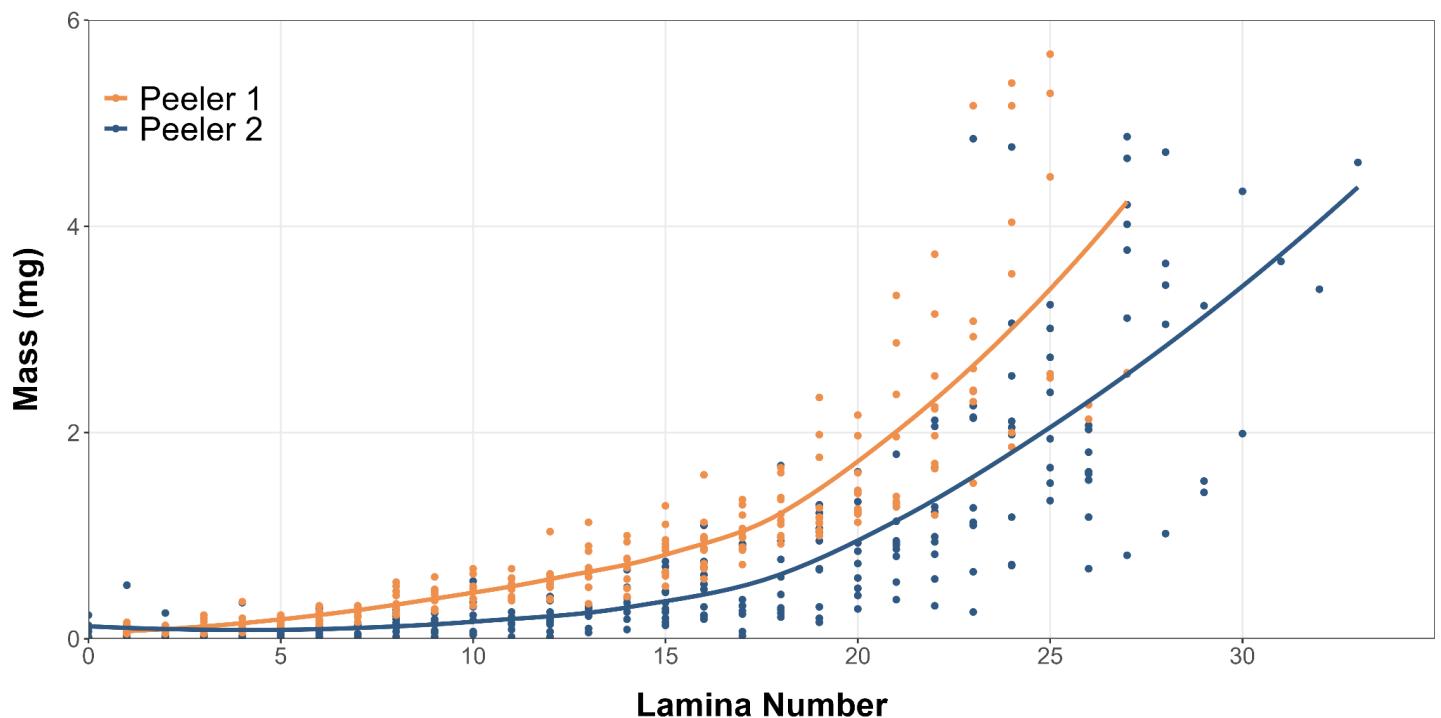


Figure S1: Mass (mg) of each lamina plotted against lamina number. Laminae were weighed using a microbalance. Peeler 1 (orange) peeled fewer and heavier lamina on average. Peeler 2, however, had a tendency to peel lighter lamina, resulting in more lamina.

Anova result based off of model lm(lens_dm, layer_no*initial, lowest_max_layer)

Analysis of Variance Table						
Response: lens_dm	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
layer_no	1	365.89	365.89	8560.38	< 2.2e-16	***
initial	1	29.71	29.71	695.13	< 2.2e-16	***
layer_no:initial	1	5.76	5.76	134.71	< 2.2e-16	***
Residuals	500	21.37	0.04			

Signif. codes: 0 '****' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1						

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Reviewers:

Matt Young