

Evaluation of Pacific Lamprey *Entosphenus tridentatus* statoliths for age determination across their life history

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Short Title: Pacific Lamprey statolith size and age

Abstract

There is a lack of fundamental biological and ecological information, such as age and migration patterns, for anadromous Pacific Lamprey (*Entosphenus tridentatus*). This ecologically and culturally important fish is native to the North Pacific Ocean and its adjacent freshwater tributaries and has experienced declines in abundance and distribution throughout large portions of its range. We used samples of known-age hatchery and wild individuals to evaluate the potential of statoliths (apatite concretions found within the auditory capsules of lampreys) to provide estimates of individual size and age throughout ontogeny. Statolith size was assessed with comparisons of morphometrics (area, perimeter, length, and width) in relation to fish length. We observed a strong, positive relationship between statolith size and fish length for larvae, which were hatchery reared. However, no relationship existed between statolith size and fish length for any of the post-metamorphic life stages, which indicates that the structure slows dramatically or ceases growth after metamorphosis. Statoliths were also assessed as age recording structures. Traditional band-reading methods applied to statoliths of known-age lamprey (1–9-year-olds) resulted in accurate age estimates for 28.3% ($\pm 45.4\%$ SD) of the fish, and 57% of mean age estimates fell within one year of true age. Within-reader average percent error (APE) was $16.8 \pm 4.4\%$, lower than among-reader APE, which averaged $28.4 \pm 1.0\%$. While we found that the statolith was not useful for traditional ageing techniques, its inclusion in morphometric-based age classifications was promising. Using a Random Forest (RF) analysis to age fish based on body length, freshwater origin, and statolith area, we accurately assigned 76% of the larval and freshwater juveniles to their known age. However, a RF model using only nonlethal predictor variables correctly classified 70% of the known-age samples, providing a potential nonlethal approach to ageing larval and early juvenile Pacific Lamprey.

Age data are often required to derive population demographic rates such as growth, recruitment, and mortality, which are needed for conservation risk assessments and the development of effective management plans (Ricker 1975, IUCN 2022). Anatomical hard structures, such as otoliths, scales, bones, and fin rays, are commonly used to elucidate age in fish. Otoliths are used often because they provide the most accurate age estimations across the widest age ranges of any structure (Secor et al. 1995). The accuracy of otolith-based age determination relies on well-supported assumptions that otoliths: (1) grow continuously even through periods of reduced somatic growth (Maillet and Checkley 1990; Campana and Thorrold 2001); (2) do not undergo resorption or remodeling (Campana and Neilson 1985); and (3) consistently accrete growth rings (Panella 1971; Campana 1999). Unfortunately, lampreys are a cartilaginous group of fish that lack otoliths and the other common hard structures used for age determination in teleost fishes. Here, we evaluate statoliths, which are an analogous structure to the otolith found within lampreys, for their potential to provide information on individual age.

Statoliths are apatite concretions within the auditory capsules of lampreys. While most fish have three pairs of otoliths, lampreys possess one pair of statoliths originally referred to as “microstatoliths” and numerous smaller structures called “statocysts” (Carlström 1963). Examinations of microstatoliths (hereafter “statoliths”) viewed from a lateral aspect reveal internal patterns of alternating translucent and opaque bands, which are similar to the annual growth rings found in otoliths (Jearld 1983; Jebbink and Beamish 1995). Medland and Beamish (1991) examined Sea Lamprey (*Petromyzon marinus*) and Southern Brook Lamprey (*Ichthyomyzon gagei*) statolith growth in larvae and found that their statoliths developed annual growth rings. They also found that the Sea Lamprey statoliths did not deposit growth rings

during or after metamorphosis until active feeding began, wherein statolith growth resumed. Furthermore, Sea Lamprey statoliths did not grow at or below 8 °C, nor did visible bands form when both lamprey species were held at constant temperatures.

Statoliths have been studied in different lamprey species as a potential age recording structure with mixed results. Volk (1986) enumerated the alternating bands on the statoliths of larval Sea Lamprey from the Laurentian Great Lakes (North America) and found that they correlated well with the approximate annual age of the samples up to four years, whereafter the bands became too difficult to interpret. Studies on reader-based age determination demonstrated that some species are easier to age than others: the average percent error was 16.7% for larval Pacific Lamprey (*Entosphenus tridentatus*), 33.0% for larval Western Brook Lamprey (*Lampetra richardsoni*) (Meeuwig and Bayer 2011), and 24.3 to 36.2% for larval Sea Lamprey, although reader precision declined in older larvae (Dawson et al. 2009). Later studies focused on the morphology of the structure, including length, width, and height, as a potential age determination tool. However, the inclusion of statolith morphometrics did not improve estimates of proportions at age compared to estimates that only used length-frequency data (Potts et al. 2015).

However, none of these statolith age determination studies used known age samples, instead relying on other means of establishing “known-age” populations through lampricide treatments, length-frequency modes, and DNA-based parentage assignments, all of which have inherent issues that can influence age estimates. Lampricide treatments are not 100% effective (e.g., McDonald and Kolar 2007) so one cannot assume that all larvae caught in a system after treatment are the result of new colonization. Lampreys also display little to no modal separation in size among age classes, making age estimations based on length-frequency distributions inaccurate (Dawson et al. 2009). DNA-based parentage assignment is a relatively new technique

that, while promising, has limitations. The accuracy and precision of the age estimate for a candidate offspring is dependent on the level of certainty regarding when its parents were spawned. Although a high level of certainty for the spawn date of fish is possible when adults overwinter in tanks prior to translocation (Hess et al. 2022), the potential for fish to overwinter for a second year in the wild could decrease the accuracy offspring age estimates (Hess et al. 2015). Furthermore, age validation through mark-recapture studies or the identification of true known-age fish is required to verify the accuracy of age techniques (Beamish and McFarlane 1983). Currently, there is no established method for determining the age of lampreys, but management and conservation of native lampreys would benefit from accurate age estimates throughout their life history.

The abundance and distribution of Pacific Lamprey have declined throughout their native range (Murauskas et al. 2016; Clemens et al. 2017; 2021). An anadromous fish native to the North Pacific Ocean and its adjacent freshwater tributaries, Pacific Lamprey is distributed along the west coast of North America from northern Alaska to southern California, reaching inland through ocean-draining rivers and streams (Clemens et al. 2017). They spend their larval stage burrowed in freshwater fine sediment, filter-feeding on detritus, diatoms, and algae (Hammond 1979; Potter 1980). After a protracted freshwater residence of approximately 3-10 years, larvae metamorphose into juveniles that migrate to the ocean (McGree et al. 2008; Hess et al. 2022), where they begin parasitic feeding. There is minimal information on marine residency of Pacific Lamprey, but recent estimates based on full-sibling genetic analysis range from 4 to 7 years (Hess et al. 2022).

Pacific Lamprey then migrate back into freshwater to spawn, typically over-wintering once or twice before spawning, although some adults may spawn immediately after returning

from the ocean (Clemens et al. 2013; Porter et al. 2017; Parker et al. 2019; Hess et al. 2022). Pacific Lamprey play ecologically important roles in freshwater systems by facilitating the conversion of particle-borne nutrients to biomass as larvae (Kan 1975; Evans and Lampman 2019), providing a seasonally available food source during migration (Merrell 1959; Poe et al. 1991), bringing marine-derived nutrients to freshwater food webs (Weaver et al. 2015; Dunkle et al. 2020), and acting as a potential predation buffer for migrating salmonids (Close et al. 2002; Arakawa et al. 2020).

We describe the morphology of Pacific Lamprey statoliths throughout their ontogeny, which we define here in four stages: larvae, freshwater juvenile, marine juvenile, and adult. We use the terms “larvae” (the eyeless, filter-feeding, freshwater life stage) and “juvenile” (the eyed, parasitic, post-metamorphic life stage) as in Clemens (2019). However, we differentiate “freshwater juveniles,” which are migrating downstream prior to ocean entry, and “marine juveniles”, which occupy marine waters. Specifically, we compared several statolith morphometrics across life stages to evaluate the structure’s size and shape over time. Additionally, we evaluated the efficacy of the structure for age determination, using known-age artificially propagated and parentage-based tagged wild samples, through band interpretation and morphometrics.

METHODS

Samples. —Known-age larvae were provided by two hatcheries: Prosser Hatchery and the Walla Walla Community College Water and Environmental Center (WEC). Prosser Hatchery, a Yakama Nation Fisheries (YNF) facility studying artificial propagation techniques of Pacific Lamprey, provided two years of samples from three brood years which resulted in six age classes

(1-, 2-, 4-, 5-, 6-, and 7-year-olds). The WEC facility supplied additional hatchery-reared larvae, which included age-0 and -1 fish (Moser et al. 2019). Wild freshwater juveniles of known age (7-, 8-, and 9-year-olds) were provided by the Columbia River Inter-Tribal Fish Commission (CRITFC). Ages were known based on DNA parentage-based tagging study conducted in the Snake River basin, Idaho (Hess et al. 2015; 2022).

For morphometric evaluation of the statolith across life stages, samples of unknown age were acquired from several sources. Freshwater juveniles, casualties from a screw trap on a tributary (Ahtanum Creek) of the Yakama River, were also provided by the YNF. Marine juveniles collected as by-catch from the National Oceanic and Atmospheric Administration's (NOAA) At-Sea Hake Observer Program (ASHOP) in the summers of 2017 and 2018 were provided by the Northwest Fisheries Science Center (Weitkamp et al., this volume). Adult samples, the majority of which were collected during the 2016 tribal harvest at Willamette Falls, Oregon, were provided by CRITFC. Additional adult samples collected in the Klamath River Estuary were also provided by CRITFC with assistance from the Yurok Tribe. All samples were stored at -20 °C until dissection. Measurements of total length (0.1 mm for larvae and juveniles and 0.1 cm for adults) were collected prior to dissection.

Sample preparation, imaging, and measurement. — Statoliths were extracted using a stereoscope and fine-tipped forceps. Incisions were made into the partially thawed lamprey heads to isolate the otic capsule, wherein the viscous gel encompassing the statolith could be removed. The statolith was then separated from the gel using forceps and nanopure water. When possible, both left and right (paired) statoliths were extracted. The average success rate of extracting at least one statolith from each lamprey age-1 or older was >90%. Statoliths could not be extracted from the sub-yearling samples (<1 cm total length) provided by WEC as the otic capsule was not

yet apparent. Similarly, statoliths extracted from samples that were frozen for a long period of time (>4 years) and subjected to thawing and refreezing were too fragile to image after extraction. After extraction, statoliths were placed base-down onto double-sided tape attached to paper and stored in tissue culture trays.

Dorsal and lateral images were acquired of one statolith from each individual on a Zeiss compound light microscope (Primostar) with a mounted Zeiss digital camera (Primostar HD full-Köhl HD IP Camera 5MP) using ZEN Blue software (ZEN 3.1 blue edition). All images were taken at 100x magnification with a combination of reflected and refracted light. Statolith orientation terminology (lateral and dorsal) follows Meeuwig and Bayer (2011) and does not describe the orientation of the structure with respect to the lamprey body, as the actual anatomical orientation is uncertain (Figure 1). Dorsal images were acquired with the statolith resting on its base. For lateral images, statoliths were balanced on edge using a cover slip and viscous immersion oil as described in Volk (1986). Statolith condition was then ranked between one and three, with one being “excellent” and three being “damaged”.

Dorsal images of the statoliths were measured to generate morphometrics (Figure 1), which were then compared across life stages. Dorsal images were analyzed using the ShapeR package (Libungan and Pálsson 2015) in R (R Development Core Team, R version 3.6.2). The “detect.outline” function was used to capture statolith outlines, which we then used to extract four morphological measurements — area, length, width, and perimeter. All images were oriented in the same direction to ensure that the Feret diameters of each statolith would be comparable.

Statolith morphology across ontogeny. —To determine how statolith morphology related to fish size, we compared statolith morphometrics (area, perimeter, length, and width) to fish

total length for each life stage (larvae, freshwater juvenile, marine juvenile, and adult). First, we performed non-parametric Kendall rank correlation tests between each statolith metric and lamprey length for each life stage as data did not meet assumptions for parametric analyses. All analyses were performed using base functions in R software (R Core Team 2019).

Reader-based age determination. —A subset of ~40% ($n = 60$) of the statoliths from our known-age samples was selected to evaluate reader-based age interpretation and included hatchery larvae ($n = 50$) and freshwater juveniles ($n = 10$) from age-1 to age-9. Statolith banding patterns were examined by three readers using lateral images, which were coded to ensure that the sample's age remained unknown during interpretation. Hatchery and wild samples were read in separate groupings, as the hatchery statoliths were substantially larger than the wild samples. A set of alternating light and dark bands was assumed to represent one year of growth, as per previous statolith ageing studies (Volk 1886; Dawson et al. 2009; Meeuwig and Bayer 2011). All statoliths were evaluated twice by each reader to assess within-reader precision through comparisons of average percent error (APE) (Beamish & Medland 1988). Age bias plots were constructed between readers and true ages to evaluate interpretation bias. The mean age estimated of all three readers was then compared to the true sample ages to evaluate accuracy.

Morphometric-based age determination. —We used Random Forest (RF) analysis to evaluate the utility of statolith morphometrics for age discrimination. RF is a nonlinear machine learning technique that fits classification trees to many (e.g., 5000) bootstrapped subsamples from a data set, then combines predictions from all trees to determine the class of each observation. We employed subsampling with replacement. Each subsample used approximately two-thirds of the dataset for the creation of each tree, leaving out one-third of the observations (i.e., out-of-bag, OOB). These OOB observations were used for internal cross-validation of each

tree, where they were used to determine misclassification rates. The predicted misclassifications for these OOB observations are then averaged over all cases to obtain the model's OOB estimate of error (Cutler et al. 2007, Strobl et al. 2008). The final predicted class of each observation is determined by majority vote of the OOB predictions, with ties settled randomly (Cutler et al. 2007).

The RF technique is useful for data sets with multicollinearity, such as our statolith morphometric measurements. In our application it is preferable to other approaches, such as linear models, which exclude potentially valuable data due to collinearity between variables (Breiman 2001). The RF approach has utility for classification because it is nonlinear, simultaneously evaluates collinear variables, and has built-in cross-validation (Breiman 2001; Cutler et al. 2007; Strobl et al. 2008).

To evaluate an alternate ageing, RF analysis was used to predict the age of our known-age larval ($n = 95$) and freshwater juvenile ($n = 45$) samples. Fish age was treated as a discrete variable for classification. We evaluated the potential to objectively age the known-age larvae and freshwater juveniles using nine different variables: fish total length; life stage; hatchery or wild origin; origin river/stream; origin river basin; statolith area; statolith perimeter; statolith length; and statolith width. The RF analysis was performed using the “randomForest” package (Liaw and Wiener 2002, package 4.6-14) in R (R Core Team 2019). In all RF models described here, 5000 classification trees were generated from the data, which were used to calculate the prediction error of each class (age group) and OOB error (average misclassification rate) for each model. Several different random seeds were tested, which impacts model subsampling, to ensure our results were stable. Model selection was conducted through identification of important predictor variables and comparisons of model OOB error rates.

Predictor variable importance was assessed using the mean decrease in accuracy and mean decrease in Gini (also known as “Gini Impurity”), which measures a predictor variable’s importance for estimating the value of a given observation across all trees in the forest.

RESULTS

Statoliths were successfully extracted from 338 individual Pacific Lamprey: 113 larvae, 47 freshwater juveniles, 37 marine juveniles, and 141 adults (Table 1). Of those, there were 158 known-age samples: 113 age-1 to age-7 larvae and 45 age-4 to age-9 freshwater juveniles. The average success rate of extracting at least one statolith from each lamprey age-1 or older was > 90%. Statoliths ($n = 14$) ranked with a condition of three (damaged) were removed before further analysis.

The relationship between statolith morphometrics and lamprey total length varied with life stage. The larval life stage displayed a linear relationship between statolith size and fish length, and all statolith metrics for larvae increased with fish length (Table 2). However, there was no apparent relationship between statolith metrics and body length for the freshwater juvenile, marine juvenile, or adult samples. The statolith size plateaued after metamorphosis, whereafter there was no relationship between statolith metrics and fish length (Figure 2). The Klamath Estuary adult samples ($n = 7$) also had notably smaller statoliths compared to the Willamette River adult lamprey of the same size (Figure 2).

Our mean ageing accuracy, based on enumeration of visible bands within statoliths, was of 28.3% ($\pm 45.4\%$ SD). Our age estimates for hatchery larvae ($30\% \pm 46.3\%$ SD; $n = 50$) were more accurate than for wild freshwater juveniles ($20\% \pm 42.2\%$ SD; $n = 10$). Overall, 57% of the mean age estimations fell within one year of true ages. Mean among-reader average percent error (APE) ($28.4\% \pm 1.0\%$ SD) was greater than within-reader APE ($16.8\% \pm 4.4\%$ SD) % SD).

Some bias was observed at all ages (Figure 3). All three readers overestimated age-1 and age-2 lamprey whereas older ages (age-7, -8, and -9) were underestimated. The least bias was for intermediate ages (age-4, -5, and -6). There was overlap in lamprey length across all ages examined for the hatchery larvae and wild freshwater juveniles (Figure 4) and no clear modal separation across age classes.

We used RF to determine the usefulness of statolith morphometrics for age discrimination. Four predictor variables, among the nine tested, were important for age classification: fish total length, origin river/stream, statolith perimeter, and statolith area (Table 4). In all RF models, fish total length was the most important predictor variable based on the mean decrease in accuracy and mean decrease in Gini (Figure 5). Statolith perimeter and area had similar variable importance indices, but statolith area performed better in model classifications. An RF with total length alone (RF5) yielded a relatively low OOB accuracy (54.29%) whereas the model that included total length and origin (RF4) increased the accuracy to 70.00%. The RF model (RF1) that included the top four predictor variables achieved an OOB estimate of accuracy of 75.00%. However, the model (RF3) that included three predictor variables (total length, fish origin river/stream, and statolith area) performed similarly with an OOB accuracy estimate of 76.43%.

The age and origins of lamprey also influenced our ability to age them correctly. The predicted age classes with the highest classification accuracies in RF1 were at age-1 ($n = 29$; 100% accuracy) and age-4 ($n = 24$; 86% accuracy) (Table 5). RF1 performed the worst on the age-7 ($n = 10$) samples with an accuracy of ~30%. The remaining age classes (2-, 5-, 6-, 8-, and 9-year-olds) had classification accuracies that ranged from ~37–79% and sample sizes of 7–20 fish each.

DISCUSSION

Previous assessments of lamprey statolith morphology to determine individual age primarily focused on the larval life stage, precluding the post-metamorphic and parasitic phases. The present research represents, to the best of our knowledge, the first study to evaluate Pacific Lamprey statolith morphology throughout ontogeny. Our results indicate that statolith growth ceases, or dramatically slows, after metamorphosis. Even though Pacific Lamprey experience rapid somatic growth during this period, especially during their parasitic marine residency (Weitkamp et al. 2015, this volume; Clemens et al. 2019), we observed no relationships between statolith size and fish length for any of the post-metamorphic life stages. Generally, the relationship between somatic and hard-part growth is tightly coupled (Francis 1990; Morita and Matsuishi 2001). Some structures, such as teleost otoliths, continue to grow even through slow or absent somatic growth (Mosegaard et al. 1988; Fey 2006). For unknown reasons, the statoliths in our dataset do not appear to be influenced by somatic growth in the post-metamorphic life stages.

Volk (1986) hypothesized that statolith growth slowed with increasing age in larval Sea Lamprey, which resulted in successively smaller statolith bands. Meeuwig and Bayer (2011) also suggested that statolith growth slowed over time based on curvilinear trends found between statolith metrics and fish length for larval Pacific Lamprey and larval Western Brook Lamprey. However, neither study examined older, post-metamorphic lamprey to determine trends between statolith growth and somatic growth. Hess et al. (2022) showed that growth in body size was relatively linear for ages 1 – 4 in larvae and then plateaued for older ages 4 – 12. Our results corroborate these growth patterns for larval Pacific lamprey and indicate that statolith growth may in fact cease during or shortly after metamorphosis.

Why statoliths would cease to grow after metamorphosis is uncertain. Medland and Beamish (1991) found that temperature affected growth of larval Sea Lamprey statoliths, which

stopped growing at or below 8°C. Pacific Lamprey are known to parasitize Pacific Hake (*Merluccius productus*) and other species with relatively deep depth distributions (Weitkamp et al., *in review*), which could keep them in relatively cool waters although some variation in thermal exposure would be expected. The sole use of hatchery larvae in this study could contribute to the strong positive relationship between statolith and body size as studies of hatchery salmon otoliths indicate that artificial rearing results in wider and more uniform growth rings (Zhang et al. 1995; Barnett-Johnson et al. 2007; Claiborne et al. 2014). Meeuwig and Bayer (2011), however, also found a strong positive relationship between statolith size and lamprey length for wild, larval Pacific Lamprey and larval Western Brook Lamprey.

Lampreys are a basal group of fishes that possess rudimentary skeletal and nervous systems, precursors to the systems found in modern vertebrates. The simplicity of their vestibular system, consisting of two otic capsules, may limit statolith size. Pacific Lamprey also become parasitic after metamorphosis, so perhaps a reliance on the balance and orientation of their host species supplants the necessity for statolith growth, although they need these abilities to return to freshwater to reproduce. Additionally, there may be size or shape constraints on their head and oral disc due to their parasitic lifestyle (Pawluk et al. 2015) that could affect statolith growth.

One unexpected observation was the extreme difference in statolith sizes between adult fish of the same length from the Klamath River Estuary and Willamette River. We offer the following two potential explanations for this difference in statolith size: 1) the duration of the larval freshwater stage is shorter on average for the Klamath River versus Willamette River Pacific Lamprey; and 2) there were differences in collection conditions that decreased the quality of statoliths in the Klamath River relative to the Willamette River collections. The first

explanation would require some degree of population substructure such that adults collected in the Klamath River were associated with a difference in larval rearing compared to those collected in the Willamette River. Our current understanding of Pacific Lamprey migration is that the adults do not precisely natal home when returning to freshwater to spawn, as supported by a lack of genetic stock structure (Hess et al. 2013), although, Snake River origin Pacific Lamprey do exhibit strong preference to follow mainstem passage routes toward their interior Columbia River natal origins (Hess et al. 2022). However, despite some degree of preference for navigating themselves toward their natal origins, it has been shown that relatively few migrants per generation are needed to generate genetically homogeneous populations (Mills & Allendorf 1996, Palumbi 2003). The consistent difference in statolith sizes observed between these two adult groups raises questions on whether there could be some level of spatial structure, either between river systems or inland versus coastal regions, which could explain the pattern seen in our returning adult statoliths. Alternatively, our second hypothesis would imply that treatment of these adult samples during harvest and followed by cycles of freezing and thawing could impact the quality of the statoliths. Obviously, a more comprehensive evaluation of statoliths from returning adults across many river systems and some controls on storage and handling of statoliths would be needed to parse-out potential spatial patterns in statolith metrics and clarify which alternative hypothesis is most likely.

At this time, the only potential methods of assessing Pacific Lamprey age are length-frequency analysis, parentage-based tagging, statolith band interpretation, and prediction or likelihood-based statistical models. Length-frequency analysis has been proven to be an inaccurate form of age estimation in lampreys due to subjectivity and overlapping in lengths between age classes, especially for older individuals (Dawson et al. 2009; Meeuwig and Bayer

2011). Visual inspection of the length-frequency plots of our known-age samples clearly displays a lack of modal separation in length between the different age groups (Figure 3). Parentage-based tagging is an exciting new technology but requires a relatively long span of years to develop a baseline of parent genetic data to to assign a broad range of ages to offspring and requires controlled conditions for ascertaining a precise spawn timing of the candidate parents to estimate precise ages (Hess et al. 2022).

Our attempt to validate statolith band interpretation and morphometric-based prediction techniques using true known-age samples highlights several considerations. The rough surface of the statolith coupled with its small size (<370 μm wide for the largest statolith in our collection) and fragility makes it a less-than-ideal structure for reader-based age interpretations. Unlike otoliths, preparing cross-sections or polishing the statoliths to improve the visibility of growth rings was not successful due to their small size and propensity to break, crack, or crumble. Instead, they were imaged whole at a lateral angle, making their utility as age-determining structures difficult.

Our within-reader APE ($16.8 \pm 4.4\%$) falls within the APE ranges reported in previous statolith studies. Studies on reader-based age determination reported APEs of 2.4% for Mountain Brook Lamprey (Medland and Beamish 1987), 2.8-5.2% for Sea Lamprey, 1.4-2.7% for American Brook Lamprey (Beamish and Medland 1988), 16.7% for Pacific Lamprey, 33.0% for Western Brook Lamprey (Meeuwig and Bayer 2011), and 24.3-36.2% for larval Sea Lamprey (Dawson et al. 2009). Unfortunately, the mean accuracy of the age estimations we generated was only 28%, which is far from ideal and too low to be effective for management purposes.

The most effective RF model correctly classified 75% of our known-age lamprey samples based on fish length, origin stream or river, and statolith area. The identification of 'origin

system' as important in the RF models indicates that geographic variability is influential for age classification in our samples, highlighting a need for system-specific validated age techniques. Although statolith mass was not measured in this study, otolith mass has been shown to correlate positively with fish age (Boehlert 1985). Thus, it stands to reason that mass as a metric could also be useful in statolith-based age determination, although whether it would perform better than the metrics tested here is uncertain. Unfortunately, due to the apparent cessation in statolith growth discussed previously, statoliths are likely not a viable age recording structure for later life stages. Nonetheless, our findings suggest that statolith metrics are useful for age classification of earlier life stages, which encompass a considerable portion of the Pacific Lamprey's considerable lifespan.

We also observed high classification accuracies in RF models that exclusively used nonlethal predictor variables (i.e., length, origin stream or river), which opens a potential avenue for a nonlethal approach to Pacific Lamprey age determination. While statolith morphometrics have proven to be useful for age discrimination, their extraction requires euthanizing the lamprey. The abundance and distribution of Pacific Lamprey are in decline, so lethal sampling should be a last resort. The RF model that only used fish length and origin system correctly classified ~66% of our known-age samples, which warrants further exploration. Specifically, the collection of additional nonlethal data such as fish mass and collection season could increase the RF's accuracy. Our RF models also indicated that origin was an important predictor, highlighting a need for system-specific validated age techniques. Water temperature and larval density, for example, are known to strongly impact growth rates of larval lamprey (Rodríguez-Muñoz et al. 2003; Lampman et al. 2016; Dawson et al. 2021) and these types of attributes may help explain the influence stemming from the origin. RF analysis, paired with parentage-based tagging of

freshwater juveniles from different systems, could contribute to a larger-scale validation of age at metamorphosis and migration and lay the groundwork for a nonlethal method of estimating larval Pacific Lamprey age.

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TABLE 1. Summary of Pacific Lamprey samples wherein at least one statolith was extracted from an individual. The sources, origins (H = hatchery, W = wild), and relevant data on life stage (L = larval, FJ = freshwater juvenile, MJ = marine juvenile, and A = adult), collection location, total length (TL, cm), age range (years), and sample size (*n*) are presented.

Source	Origin	Stage	Location (state)	TL range (cm)	Age range	<i>n</i>
YNF ^a	H	L	Prosser Hatchery (WA)	(3.2 – 12.7)	(1–7)	95
WEC ^b	H	L	Walla Walla Comm. College (WA)	NA	(< 1–1)	18
CRITFC ^c	W	FJ	Snake River Basin (ID)	(11.0 – 15.7)	(7–9)	41
YNF ^a	H	FJ	Ahtanum Creek (WA)	(13.3 – 16.4)	NA	6
ASHOP ^d	W	MJ	Pacific Ocean	(11.9 – 64.4)	NA	37
CRITFC ^c	W	A	Klamath Estuary (OR)	(65.0 – 69.5)	NA	7
CRITFC ^c	W	A	Willamette Falls (OR)	(44.8 – 69.5)	NA	134
Total						338

^aYakama Nation Fisheries.

^bWater and Environmental Center, Walla Walla Community College (Moser et al. 2019).

^cColumbia River Inter-Tribal Fish Commission (Hess et al. 2015).

^dNational Oceanic and Atmospheric Administration Northwest Fisheries Science Center At-Sea Hake Observer Program (Weitkamp et al., this volume).

TABLE 2. Correlation coefficients and *p*-value (in parentheses) between metrics (area, perimeter, length, and width) and lamprey total length (TL), separated by life stage.

	Larvae	Freshwater Juvenile	Marine Juvenile	Adult
Statolith Area: TL	0.614 (<0.001)	-0.026 (0.797)	-0.168 (0.147)	-0.097 (0.098)
Statolith Perimeter: TL	0.627 (<0.001)	-0.017 (0.869)	-0.153 (0.188)	-0.071 (0.227)
Statolith Length: TL	0.588 (<0.001)	-0.025 (0.811)	-0.192 (0.097)	0.010 (0.872)
Statolith Width: TL	0.554 (<0.001)	-0.019 (0.854)	-0.093 (0.428)	-0.111 (0.059)

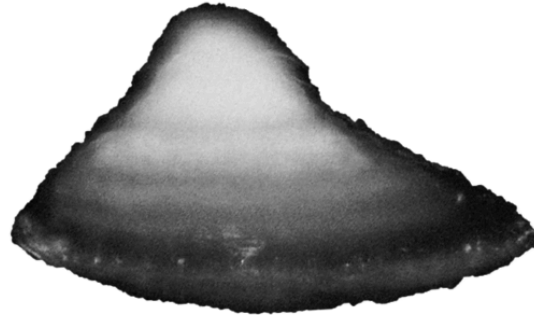
TABLE 4. Random Forest (RF) models generated to predict the age of known-age larval and freshwater juvenile Pacific Lamprey. Predictor variables included total length (TL), statolith metrics (perimeter and area), and the river/stream system where the sample was collected (origin). The first three models (RF1, RF2, and RF3) had the highest out-of-bag (OOB) accuracies.

Model	Predictor Variables	OOB Accuracy
RF1	TL + S Perimeter + S Area + Origin System	75.00%
RF2	TL + S Perimeter + Origin System	74.29%
RF3	TL + S Area + Origin System	76.43%
RF4	TL + Origin System	70.00%
RF5	TL	54.29%

TABLE 5. Pacific Lamprey known versus the age predicted for hatchery larvae and freshwater juveniles using Random Forest analysis (Model RF3), which included total length, origin system, and statolith area as predictors. The proportion of fish misclassified for each age class is also shown. Correct classifications are in bold along the diagonal.

Actual Age	Predicted Age								Proportion Misclassified
	Age-1	Age-2	Age-4	Age-5	Age-6	Age-7	Age-8	Age-9	
Age-1	29	0	0	0	0	0	0	0	0.00
Age-2	0	5	2	0	0	0	0	0	0.29
Age-4	2	0	24	0	2	0	0	0	0.14
Age-5	2	1	2	3	0	0	0	0	0.63
Age-6	0	0	2	0	15	2	0	0	0.21
Age-7	0	1	1	0	5	3	0	0	0.70
Age-8	0	0	0	0	0	0	14	6	0.30
Age-9	0	0	0	0	0	0	5	14	0.26

Lateral view



Dorsal view

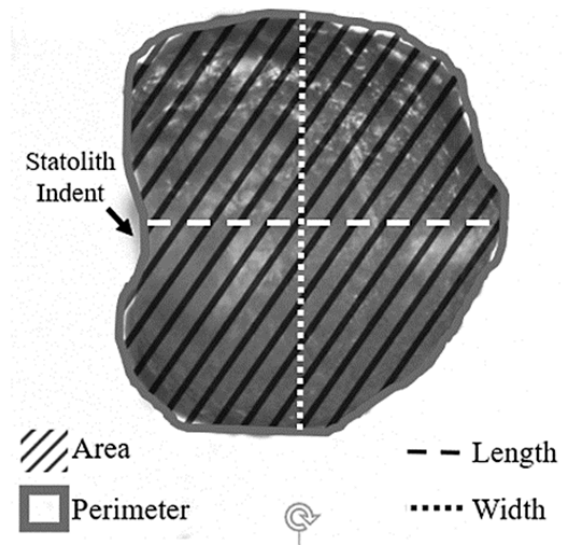


FIGURE 1. Images of the lateral (top) and dorsal (bottom) aspects of a statolith from a 9-year-old freshwater juvenile Pacific Lamprey, collected in the Snake River Basin, Idaho. The dorsal image also includes the statolith morphometrics used in this study: area, perimeter, length, and width.

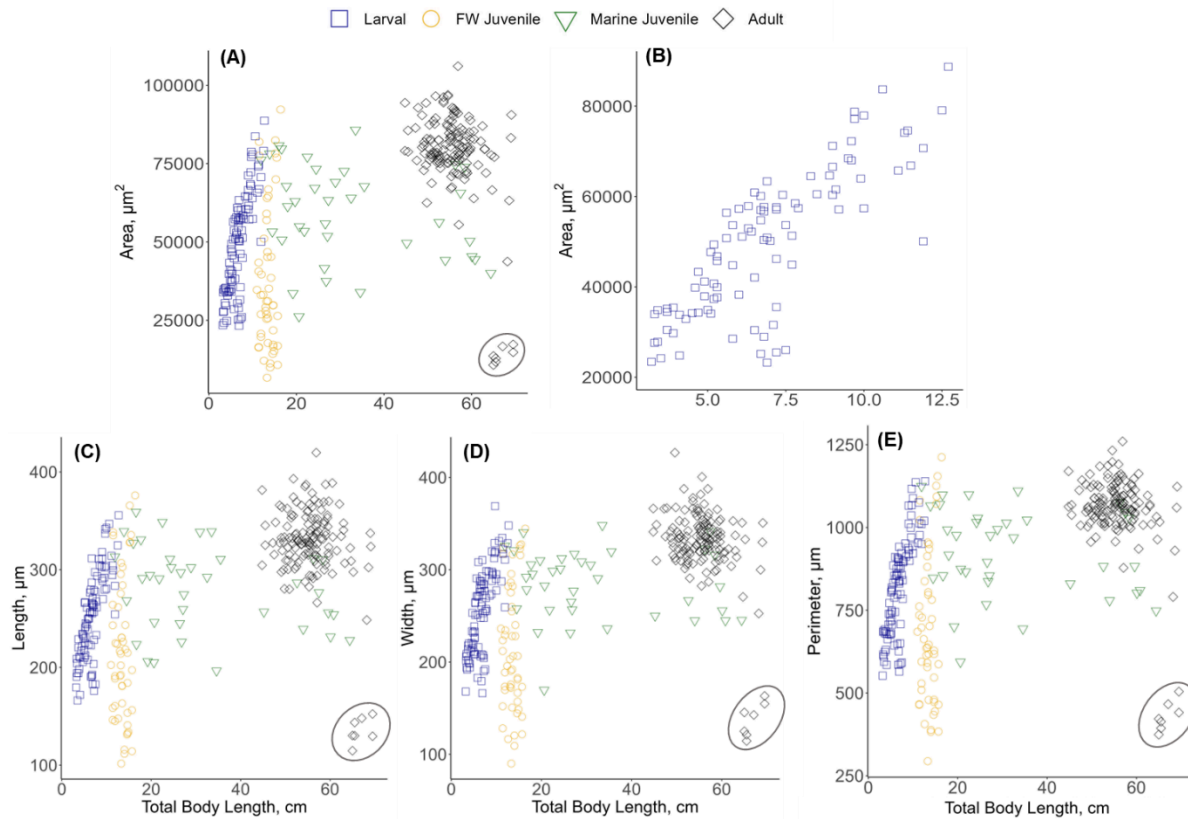


FIGURE 2. Relationships between Pacific Lamprey statolith morphometrics and fish total length (cm). (A) Statolith area for all life stages (larval, freshwater juvenile, marine juvenile, and adult); (B) Statolith area only for larvae; and (C) Statolith perimeter; (D) Statolith length; and (E) Statolith width for all life stages. The adults collected from the Klamath River estuary are circled in (A), (C), (D), and (E).

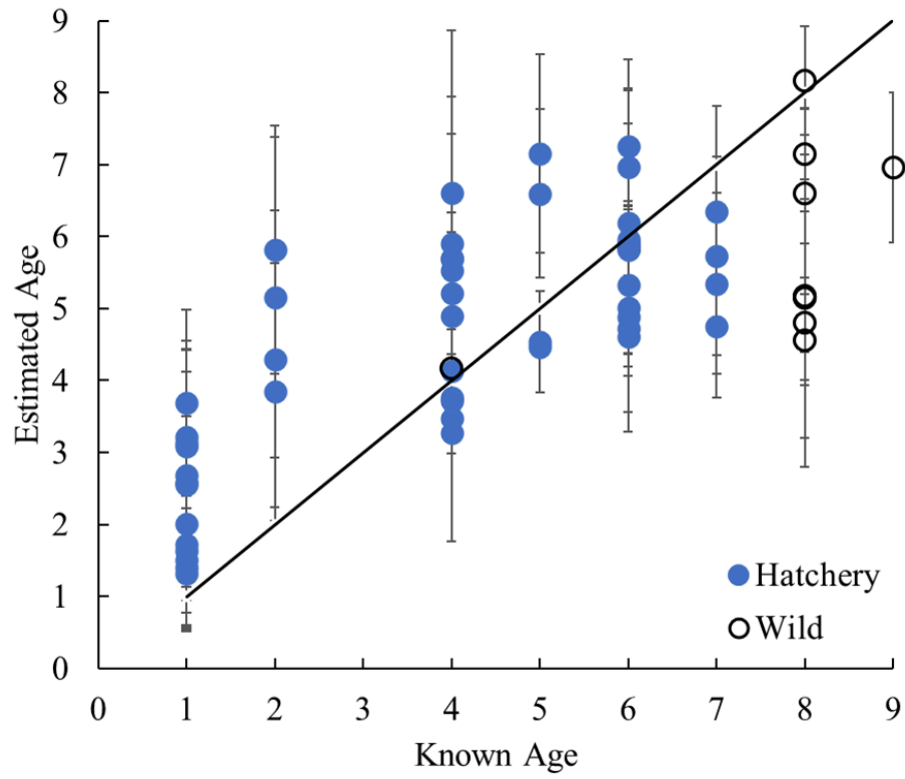


FIGURE 3. Age bias plots for hatchery larvae and wild, freshwater juvenile Pacific Lamprey.

Mean age (\pm SD) are included with a 1:1 line. Data points were jittered for visibility.

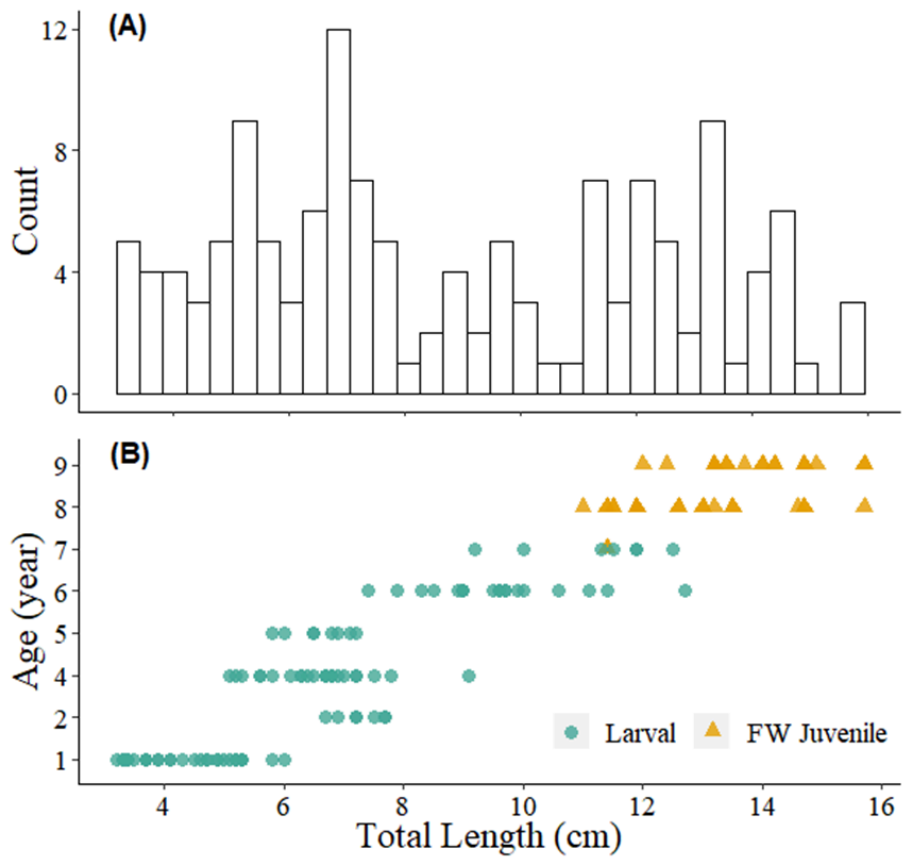


FIGURE 4. (A) Overall length-frequency distribution and (B) length-age distribution with life stage distinguished by symbol color and shape for known-age Pacific Lamprey hatchery larvae and wild freshwater juveniles.

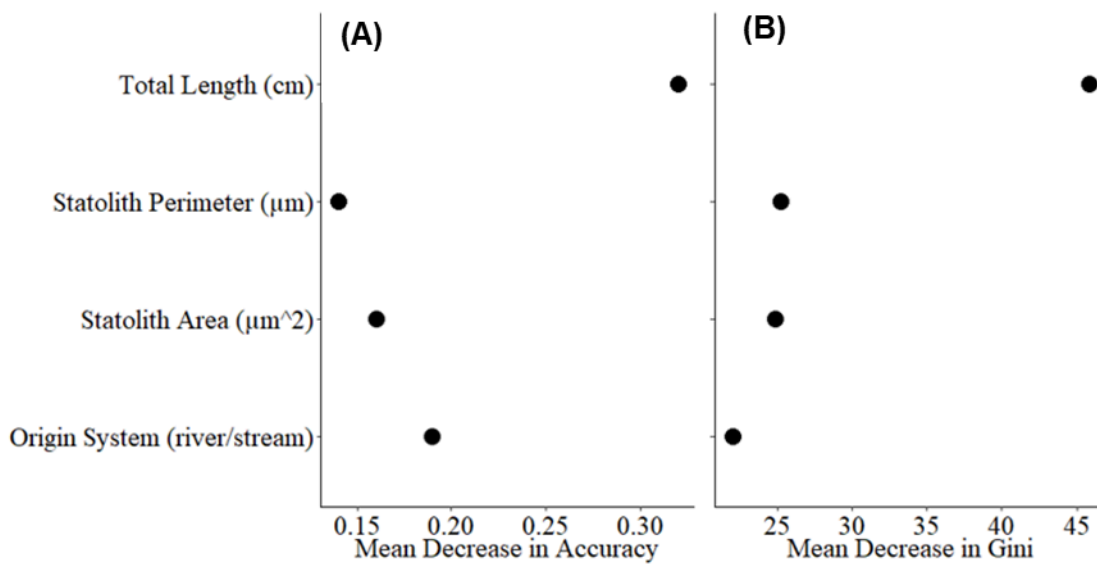


FIGURE 5. Conditional variable importance indices for predicting Pacific Lamprey ages using the Random Forest approach and four predictor variables (Model RF1). Panel (A) represents the mean decrease in accuracy and panel (B) represents the mean decrease in the Gini index.