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BRIEF COMMUNICATION

Staining protocols affect use of otolith to estimate the demography of the damselfish sergeant major (*Abudefduf vaigiensis*)

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Abstract

This study assessed the otolith (sagittae, lapilli, and asterisci) increment deposition rate in the range-shifting damselfish, *A. vaigiensis*, using different concentrations of Alizain Red S and evaluated the impact of staining on increment width. Daily increment deposition was verified in all otolith types and presented clearer fluorescent markings in the lapilli and sagittae than the asterisci, with high stain concentration showing the best clarity. Higher stain concentrations were found to decrease increment width, suggesting care is needed when using stained otoliths as a proxy for growth for this species.

KEYWORDS
Abudefduf vaigiensis, Alizarin Red S, climate change, growth, otolith validation, vagrant fish

The ongoing warming of marine environments facilitates the poleward expansion of species (Poloczanska et al. 2013). Throughout the austral summer and autumn, the East Australian Current delivers a suite of larval tropical marine fishes (vagrants) to temperate areas on the east coast of Australia (Booth et al., 2007). This annual recruitment event currently ends in near-complete mortality, with winter temperatures acting as a bottleneck to survivorship (Figueira & Booth, 2010). Given winter water temperatures are expected to increase due to climate change, the annual recruitment phase is likely to result in the establishment of viable tropical vagrant populations on temperate reefs and increased winter survivorship, potentially affecting long-term competitive processes with temperate species and food chains (Coni et al., 2022). Despite the expatriation of tropical reef fish to temperate communities, little is known about their in situ performance, such as growth patterns and early life histories. Performance is mediated by temperature and potentially interacts with predation pressure to dictate survival above critical thermal minima. Given that age, growth, and mortality are key to understanding the population dynamics of fish, ecologists need accurate methods to estimate these metrics.

Otoliths are calcium carbonate structures found within the inner ear of fish. Otolith growth is a continuous precipitous process that results in the deposition of concentric light and dark bands that can be observed microscopically. These increments are typically deposited both daily and annually, enabling accurate estimates of age to the resolution of a day (Panella, 1971). Increment widths can be coupled to somatic growth and thus daily increment width can be used as a proxy for growth (Molony & Choat, 1990). Microstructural analysis of otolith increments can reveal insights into ecological dynamics such as pelagic larval duration, larval dispersal, and early growth and survivorship. Otolith microstructural analysis in range-extension ecology can provide metrics that allow researchers to examine the drivers of the success (or permanent residency) of vagrant populations under climate change (Booth et al., 2011).

Most fish species have three pairs of otoliths: the sagittae, lapilli, and asterisci. Properties, such as deposition rate and shape, can differ among the types of otoliths and among species for the same type of otolith. Due to the interspecies differences in the characteristics of each otolith, researchers need to confirm that a predictable relationship exists between otolith radius, increment size, and body size

within all otolith types of a particular species prior to using microstructure as a demographic proxy. The three-dimensional shape, clarity of deposition, cracking, opaque zones, and secondary growth patterns should influence a researcher's selection of otolith type for a species, yet sagittae are commonly used, often without validation or examination of other otolith types.

Otoliths should be validated to ensure that one increment represents a day of growth (Panfili et al., 2009). A common technique to validate the otolith is to mark the otolith with a chemical at two known time points and compare the number of visible increments with the number of days elapsed between events (Simon & Wickstrom, 2020). One such chemical, Alizarin Red S (ARS hereon), is frequently used to mark otoliths as it has low toxicity (Bensimon-Brito et al., 2016) and is cost effective compared to other stains, such as oxytetracycline (Niva et al., 2005). The red hue emitted under fluorescence is also superior for identifying faint marks relative to the green hue of other otolith stains, such as oxytetracycline.

It is important to know whether marking affects somatic growth for experiments that attempt to both validate increment deposition and measure growth rates in laboratory settings. Despite the suggestion that ARS does not impact growth (Bashey, 2004; Liu et al., 2009), some researchers have indicated that growth inhibition occurs between different manufacturers (Jurgelėnė et al., 2022). As the use of stain extends beyond validation, such as bone mineralization research, fish performance, and fish stock programs, it is important to establish whether ARS impacts growth and physiological processes that may interfere with the interpretation of growth from calcified structures, and whether a lower concentration should be used to minimize any potential effects.

Abudefduf vaigiensis (Quoy & Gaimard 1825) is frequently used in range-expansion studies as a model fish species (Beck et al., 2014; Booth et al., 2011; Feary et al., 2014; Figueira et al., 2009) due to its high reliable seasonal abundance and recruitment to temperate areas. Despite A. *vaigensis* daily increment in otoliths not being validated, numerous studies have assumed daily deposition lines in juvenile otoliths (Kingsbury, Gillanders, Booth, & Nagelkerken, 2020b; Thresher & Brothers, 1989; Wellington & Victor, 1989) and annuli deposition in adults (Nakano et al., 2004). The current study aimed to (i) assess the increment deposition rate in juvenile *A. vaigiensis* in three otolith types; (ii) compare and evaluate the chemical marking efficacy at different stain concentrations (low 150 mg/L, medium 250 mg/L, and high 350 mg/L) in three otolith types; and (iii) assess the effect of stain on otolith increment width, used as a proxy for growth.

Twenty-four juvenile *A. vaigiensis* were collected from Little Manly (33°48′25.7″S, 151°17′07.6″E) and Narrabeen (33°42′08.8″S, 151°18′05.6″E), Sydney, Australia, during February to April 2022. Fish were collected in accordance with a New South Wales Department of Primary Industries collection permit (F94/696[A]-9.0) and University of Technology Sydney (UTS) Animal Ethics requirements (ETH-6609). For each stain treatment, fish were separated into two 12-L tanks (six fish per treatment, three fish per tank) containing fish with similar body size, ranging in total length from 17 to 50 mm. Similar sample sizes have been used in previous studies to validate otoliths (Parkinson et al., 2012). Ocean temperatures ranged from 22 to 24°C at the time of collection, and fish were therefor acclimated over 1 day to 23°C in the laboratory. A 12 h light:12 h dark cycle was maintained throughout the investigation. Fish were fed 50 mL of live Artemia twice a day and water temperature (23°C), pH (8.1), dissolved oxygen (6–8 mg/L), and salinity (35) were monitored twice daily, followed by the removal of debris and a 20% water change. Fish were immersed for 24 h in ARS (A5533; Sigma-Aldrich) at days 0 and 7–9 following acclimation. Stain treatments were 150 mg/L (low, 9 days), 250 mg/L (medium, 8 days), 350 mg/L (high, 7 days) and a control group that was kept under the same conditions and timeframe of experimentation (20 days) but did not experience staining. There were two mortalities after the first stain (one each for medium and high stain amounts). Fish were euthanized using an ice bath at the conclusion of the investigation. The sagittae, lapilli, and asterisci were extracted from each fish and mounted dorsal side up on a microscope slide using Crystalbond™ (509-1A). On inspection, daily increments were clearly visible and did not require polishing. A Nikon Eclipse *Ni-E* compound microscope (400x) equipped with fluorescence (emission λ wavelength 852 nm, excitation wavelength 562 nm) was used to view the markings. The increments visible between the two marks were counted and compared to the number of days elapsed between staining events. All samples were counted by two researchers. The mark quality was assessed following a 0–5 ordinal scale, where 0 = no visible mark or fluorescence, 1 = weak fluorescence, 2 = mark easily visible under fluorescent light, 3 = shining brightly under fluorescence, 4 = mark visible under visible and fluorescent light, and 5 = mark distinct under visible and fluorescent light (Taylor et al., 2005). Then, 20% of samples were randomly selected by a second researcher and evaluated for mark quality. If the score differed by one, the sample was discussed against the criteria and a score was decided on. Increment widths were measured on the lapilli for all treatment groups, as the lapilli resulted in the clearest increment banding. Individual increments of each fish were measured to determine the total increment width for the experimental period (20 days) and were measured on the same axis (dorsal view, from the center of the primordium to the furthest edge along the anterior–posterior axis) using Nikon NIS-elements Advanced Research software (V5.02.02).

For aim (i), a one-sample *t*-test was used to test the hypothesis that increments would equal the number of days between marks in each otolith type and treatment. For aim (ii), two Kruskal–Wallis tests were conducted to compare the effect of stain concentration and otolith type. The post hoc Dunn's test was performed to conduct a pairwise comparison between the concentrations for the three stain treatments and otolith types. For aim (iii), the average increment width across the experimental period was compared among stain treatments and the control with a Kruskal–Wallis test, as the assumptions of the parametric ANOVA test were not met. A pairwise comparison was used to identify which pairs were significantly different. All data were statistically analyzed using a significance level (α) of 0.05.

The number of increments between marking events was as expected for all treatments: the increment count equated to the days

between marks in all otolith types ($p > 0.05$). Our findings thus support the hypothesis that increments were deposited daily in all otolith types.

Although our findings support the usefulness and validity of using all otoliths in aging and daily growth studies of A. *vaigiensis*, care should be taken when selecting which type of otolith to use. Few studies explore the validity of otolith type (David et al., 1994), with a bias in the literature towards using the sagittae as they are generally larger, easier to find, and have larger increment widths (Panfili et al., 2009). Indeed, the larger size of the sagittae and increment widths therein was seen for A. *vaigiensis*, but we noted clearer increments in the lapillus, which was also observed by Kingsbury, Gillanders, Booth, Coni, and Nagelkerken (2020) and Soeparno et al. (2012). Although not used previously for A. *vaigiensis*, the asterisci often forms at settlement in demersal fish, which would underestimate pelagic larval duration. In many fishes, the complex three-dimensional shape of the sagittae gives rise to secondary growth patterns that make readings along a single axis difficult (Morioka et al., 2006); this was observed for juvenile A. *vaigiensis*. Given these observations and constraints the lapillus is recommended in future aging studies for A. *vaigiensis*.

Marking scores differed across otolith types (Kruskal–Wallis, $H(3) = 9.87$, $p = 0.007$; Figure 1) and across staining treatments (Kruskal–Wallis, $H(3) = 9.44$, $p = 0.009$; Figure 1). Dunn's post hoc comparisons for otolith type revealed significant differences in marking scores between the asterisci and lapilli ($p = 0.005$) and asterisci and sagittae ($p = 0.009$), but not the lapilli and sagittae ($p = 0.863$). For stain concentration, Dunn's post hoc comparisons showed a significant difference between low and high concentrations ($p = 0.008$), but not for medium and high ($p = 0.273$) and low and medium ($p = 0.061$). In many fishes, the asterisci have been shown to have fainter and smaller daily increments (Tsukamoto & Kajihara, 1987) and more irregular concentric increments (Espino-Barr, 2019). Although daily increment deposition in the asterisci of A. *vaigiensis* was validated, we hypothesize that the thinner depositions (1–2 μm, as opposed to 3–5 μm for the lapillus and sagittae) provide less opportunity for the absorption of stain, or less visibility of the mark, resulting in lower marking scores. The lapilli were laterally flatter than the sagittae, resulting in clearer concentric markings. To determine the optimal concentration of stain required often includes making a compromise between research aims and fish health, immersion time, type of otolith, and water conditions (Taylor et al., 2005). The results demonstrate that a concentration of 250 mg/L ARS for 24 h results in markings in the sagittae and lapilli that are at least as clear and reliable as with 350 mg/L concentration ($p = 0.818$), whilst minimizing wastage of product and avoiding fish exposure to higher concentrations of stain.

A Kruskal–Wallis test showed that higher ARS concentration results in smaller increment width ($H(3) = 13.6$, $p = 0.004$; Figure 2) in the lapillus. Post hoc tests revealed that the difference between low and medium treatments ($H(3) = 10$, $p = 0.002$) and low and high treatments ($H(3) = 10$, $p = 0.002$) was significant, whilst the differences between the control and low, medium, and high treatments were not significant (all comparisons, $H(3) = 3.6$, $p = 0.058$), suggesting higher stain concentrations may inhibit growth. These findings demonstrate that studies using ARS, such as fish-stocking research, could introduce a confounding effect when using otoliths to analyze growth–age relationships. Although we did not investigate the mechanism that caused the smaller increment widths, it may be explained by one of three different pathways: (1) inhibition of bone growth; (2) inhibiting of enzymatic pathways; and/or (3) causing a physiological and/or behavioral stress response. Although not fully explored in fish, ARS has been shown to inhibit growth and mineralization in guinea pigs, rats, and rabbits (Hoyte, 1960). Given otoliths are a calcified bone structure, at medium and high concentrations ARS could temporarily stunt growth. At the molecular level, ARS has been shown to bind to the active site of catalase (Hu et al., 2019), an enzyme involved in regulating oxidative damage and a fish's immune defense

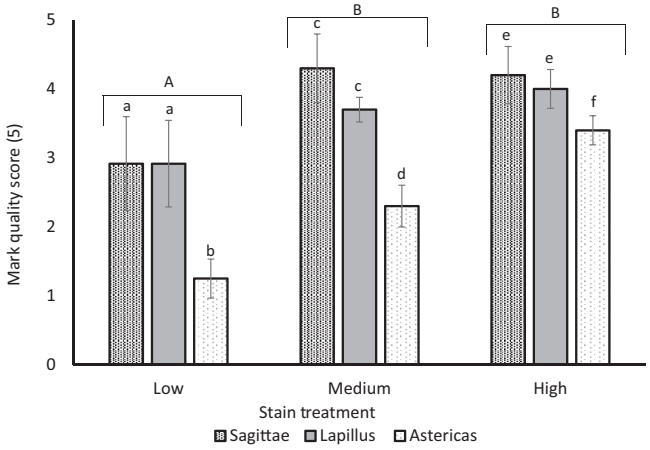

FIGURE 1 Mark quality in different concentrations of stain in the sagittae, lapilli, and asterisci. Error bars represent 95% confidence intervals and letters represent significant differences. Capitalized letters compare across treatments and lower-case letters compare within a treatment.

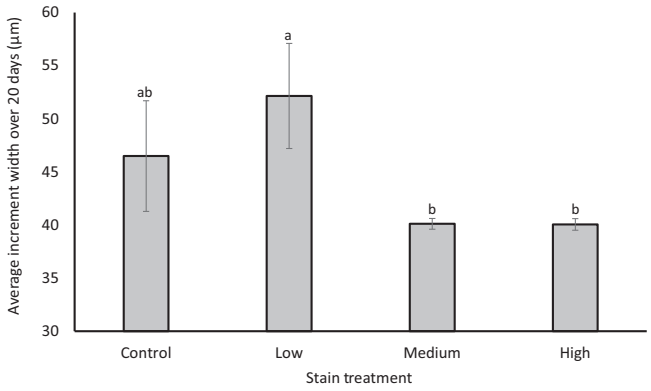

FIGURE 2 Average increment width (μm) during the experimental period in the lapillus at low, medium, and high stain concentrations and the control. Error bars represent 95% confidence intervals and letters represent significant differences. Lower-case letters represent significant relationships between concentration.

system (Kumari et al., 2014). These toxicants have been shown to result in lethargic movement, disrupt startle response, and impair swimming performance, indicating a possible mechanism for stunting growth at high concentration (ibid). Given the high concentrations of contaminates found in ARS by Jurgelėnė et al. (2022), the downstream effect on xenobiotic processes at high and medium concentrations should not be discounted. Physiological stress, such as chemical and physical changes, is well documented to have negative effects on fish growth and wellbeing (Barton, 2002). Despite no observations of changes in fish behavior in the current study, it is possible that the 24-h immersion at higher concentrations (involving less light and changes to water chemistry) could cause physiological stress to the fish, resulting in temporary decreases in growth.

Overall daily otolith increments were verified in all three otolith types, but care needs to be taken when choosing the stain concentration, as higher concentrations may affect somatic growth in fishes.

AUTHOR CONTRIBUTIONS

All the authors contributed towards data generation, data analysis, manuscript preparation, and the overarching concepts and structure of the paper.

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