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Alizarin red S marking in otoliths: A study on the growth dynamics of *Diplodus vulgaris* juveniles

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A B S T R A C T

This study investigates the age and growth rates of juvenile *Diplodus vulgaris* in the Çanakkale Strait, utilizing Alizarin Red S (ARS) marking to analyze otoliths for daily growth increments. The research aimed to validate the daily deposition of growth rings in otoliths as a reliable method for age determination in young fish. A total of 65 specimens were subjected to ARS immersion at varying concentrations to identify the optimal marking conditions that would not adversely affect the survival rates of the juveniles. Post-treatment, the fish were released into controlled environments and monitored over a period to assess the incorporation and visibility of ARS marks in the otoliths. The results demonstrated a clear correlation between fish size and age, with a consistent daily growth rate that supports the hypothesis of daily increment formation in otoliths. The mortality rate associated with ARS treatment was within acceptable limits, confirming the method's viability for non-lethal age and growth studies. This research not only underscores the effectiveness of ARS marking in studying juvenile fish growth patterns but also contributes to a better understanding of the life history of *D. vulgaris* in the Çanakkale Strait, offering valuable insights for fisheries management and conservation efforts.

INTRODUCTION

The Mediterranean Sea, with its rich biodiversity and complex ecosystems, serves as a crucial habitat for a wide range of marine species. Among these, the two-banded sea bream (*Diplodus vulgaris*) stands out due to its ecological significance and widespread presence, particularly in the shallow waters of the Çanakkale Strait. This strait, acting as a natural bridge between the Aegean Sea and the Sea of Marmara, presents unique environmental conditions that are conducive to the study of marine life, especially during its early developmental stages. The early life history of marine species, including their growth rates and patterns of survival, is fundamental to understanding their overall ecology and the dynamics of their populations (Morales-Nin, 2000).

In recent years, the analysis of otolith microstructure has emerged as a key technique in ichthyology for investigating these aspects. Otoliths, the calcified structures in the inner ears of fish, contain growth increments that can be interpreted similarly to the rings of a tree, providing a detailed chronology of an individual's age and growth history (Campana & Neilson, 1985). For species like *D. vulgaris*, understanding the formation and significance of these growth increments is essential for accurate age determination, which in turn, is crucial for assessing population dynamics, growth patterns, and survival strategies. However, despite the acknowledged importance of otolith analysis, there remain gaps in our knowledge regarding the validation of daily growth rings in the otoliths of young *D. vulgaris* within the Çanakkale Strait. This lack of information hampers our ability to accurately determine the age and growth rates of these fish, which is vital for their conservation and management (Wilson et al., 1999).

The application of fluorescent dyes, such as alizarin complexone, to mark otolith growth increments has been recognized as a powerful tool for validating age determination methods (Wilson et al., 1999). The incorporation of these chemicals into the otoliths at known time intervals allows researchers to accurately count the growth increments, thereby providing a direct validation of the age of the fish. This technique has proven to be invaluable in enhancing the accuracy of age determination in marine species, offering a more precise understanding of their growth patterns and life history traits.

Therefore, the primary objective of this study is to validate the formation of daily growth rings in the sagittal otoliths of juvenile *D. vulgaris* inhabiting the shallow waters of the Çanakkale Strait.

MATERIALS AND METHODS

Study Area and Specimen Collection

The research was carried out in the Çanakkale Strait, a vital ecological corridor linking the Aegean Sea with the Sea of Marmara. This area is recognized for its rich biodiversity and serves as an essential habitat for a variety of marine life, including the species *D. vulgaris*. Throughout the year, juvenile *D. vulgaris* specimens were systematically collected on a monthly basis. The collection utilized a beach seine net, specifically targeting shallow waters (less than 2 meters deep), where juvenile congregations are predominantly found. Upon capture, specimens were promptly placed in aerated containers and transported to the laboratory for further processing.

Acclimatization and Maintenance

Upon arrival at the laboratory, young of the year (YOY) *D. vulgaris* were acclimated to a 4500-liter closed recirculating seawater system, equipped with a biofilter and aeration system. The fish were fed granular feed suited to their size and mouth opening. The system maintained a natural photoperiod, with water temperature held steady at 20°C±1.0°C, and salinity around 37 ppm, mirroring the natural conditions of their capture site. Water quality parameters, including temperature, salinity, and pH, were monitored daily. The temperature has been kept constant with the help of air conditioning. There has been no change in salinity levels.

Experimental Setup and Alizarin Red S (ARS) Marking

Following acclimatization, YOY *D. vulgaris* were transferred to 80-liter ventilated tanks for a 14-day period before the commencement of the experiments. The fish were exposed to six different concentrations of Alizarin Red S: 0 (control), 50 mg/L, 100 mg/L, 150 mg/L, 200 mg/L, and 300 mg/L. During the marking period, no food was provided, and tanks were heavily aerated to maintain a pH of approximately 7. Post-dyeing, fish were held in separate aquariums for 20 days. In the control group, there are 10 samples, while in the other group, there are 11 samples. Fish that perished prior to the experiment of the study were excluded from the analysis.

Otolith Extraction and Analysis

Sagittal otoliths were carefully extracted from the sacrificed fish, embedded on a glass slide, and prepared for analysis. The preparation process involved grinding the proximal side of the otolith with abrasive papers (gradually from 12, 9 to 3 mm) and polishing with 0.3 mm paper until daily growth rings were visible from the center to the edge, following established protocols (Secor et al., 1991; Jones, 1992). The detection of ARS marks was conducted under a fluorescent microscope (Carl Zeiss Axio Scope A1), and images were captured using a digital camera (Carl Zeiss Axiocam 305).

Data Analysis

The counting of daily growth increments between the fluorescent mark and to the otolith edge was performed independently by two readers to ensure accuracy. The correlation between the number of daily growth increments within the ARS mark and the days post-treatment was assessed through linear regression analysis. Chi-square test was employed to evaluate the congruence of daily growth increment counts against the elapsed time. Statistical significance was determined at an alpha level of 0.05 (Agresti, 2002; Peck et al., 2012). All statistical analyses were performed using the PAST (version 4) program (Hammer et al., 2001).

RESULTS

In the present study, a cohort of 65 young of the year (YOY) *D. vulgaris* specimens, with total lengths ranging from 2.5 cm to 6.5 cm, were subjected to experimental conditions.

These juvenile fish were marked using an ARS solution, which resulted in visible ring under fluorescent light in the sagittal otoliths of the specimens (Figure 1). It was observed that immersion in ARS solutions, with concentrations varying between 50 mg/L to 300 mg/L for a duration of 24 hours, led to a mortality rate peaking at 7.69% during and immediately following the treatment period.

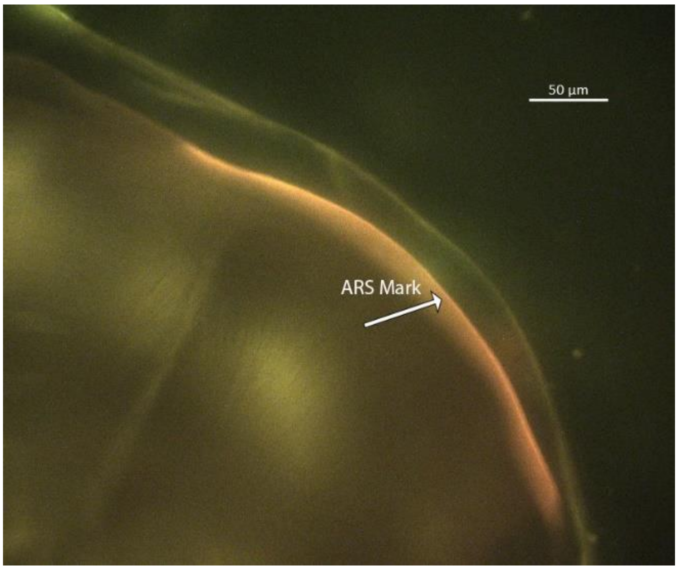

Figure 1. Appearance of ARS mark on *D. vulgaris* sagittal otolith.

Out of the initial 65 specimens, 60 successfully completed the experimental protocol, including those in the control group. The ARS marking was clearly discernible in the sagittal otoliths of 45 YOY *D. vulgaris* under a fluorescent microscope. Notably, mark was observed in five specimens from the group treated with 50 mg/L ARS solution.

The analysis of daily growth increments, between the fluorescent mark and extending to the edge of the otoliths, was conducted. This analysis was performed on 45 specimens, which ranged in total length from 2.5 cm to 6.5 cm. The age determination based on daily growth increments revealed the youngest individual to be 89 days old, while the oldest was found to be 168 days old.

A length-age regression analysis was employed to ascertain the average daily growth rates of the YOY *D. vulgaris* individuals. The calculated daily growth rate stood at 0.031 cm per day. The linear relationship between total length (TL) and age (days) was established as follows:

$$TL = 0.248 + 0.031\,(days), \qquad N = 45\ R^2 = 0.81$$

Chi-square tests conducted to assess the linear regressions of the number of daily growth increments against elapsed time yielded a result that was not statistically significant (Chi-square = 0.297, df = 45, P = 0.91). This outcome indicates that the formation of daily growth increments in YOY *D. vulgaris* specimens, collected from the shallow waters of Çanakkale, occurs on a daily basis, corroborating the hypothesis that daily increment formation is a consistent process in these juvenile fish.

DISCUSSION

The successful marking of YOY *D. vulgaris* with Alizarin Red S (ARS) and the subsequent analysis of daily growth increments in this study contribute valuable insights into the age and growth patterns of this species in the Çanakkale Strait. Our findings align with the broader understanding of otolith marking techniques as reliable tools for studying fish growth and age determination, a concept well supported in the literature (Campana & Thorrold, 2001; Morales-Nin, 2000).

The mortality rate observed during and after the ARS treatment, peaking at 7.69%, falls within the acceptable range reported in previous studies that employed similar marking techniques (Secor et al., 1991; Jones, 1992). This suggests that the concentrations of ARS used (50 mg/L to 300 mg/L) are not unduly harmful to YOY *D. vulgaris*, corroborating findings from studies on other species where similar or higher concentrations were deemed safe (Amos et al., 1998; Green & McCormick, 2001).

The absence of ARS mark in a small portion of the 50 mg/L treatment group underscores the importance of concentration in the marking process. This observation is consistent with the dose-dependent nature of chemical marking techniques described by Tsukamoto et al. (1989), who emphasized that sufficient concentration and exposure time are critical for successful mark deposition in otoliths.

The linear relationship between total length and age established in this study provides a clear indication of the growth rate of YOY *D. vulgaris* in the Çanakkale Strait. The daily growth rate of 0.031 cm/day is slightly higher than rates reported for other *Diplodus* species in different environments (Fowler, 1990; Morales-Nin & Aldebert, 1997), suggesting that local conditions in the Çanakkale Strait may be particularly conducive to the growth of *D. vulgaris* juveniles. The relatively high (R^2) value (0.81) indicates a strong correlation between age and total length, reinforcing the reliability of otolith analysis for growth studies.

According to the results of the Chi-square test, the hypothesis that daily growth increase in YOY *D. vulgaris* is a consistent process is supported. This finding is crucial for validating the use of otolith increment analysis as a method for age determination in fish, as suggested by Fowler (1995) and Secor et al. (1991).

CONCLUSION

In conclusion, this study not only demonstrates the efficacy of ARS marking for studying the growth and daily age of *D. vulgaris* but also highlights the potential environmental and biological factors influencing growth rates in the Çanakkale Strait. Further research is needed to explore the impact of these factors in more detail and to verify the applicability of these findings to other marine species. Additionally, expanding the scope of research to include different seasons and a broader size range of specimens could provide a more comprehensive understanding of the growth dynamics of *D. vulgaris* in this ecologically significant region.

COMPLIANCE WITH ETHICAL STANDARDS

Authors' Contributions

HA: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing

EK: Data curation, Investigation, Resources, Validation

PÇ: Investigation, Visualization

AA: Data curation, Investigation, Writing – review & editing

All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

The care and use of experimental animals complied with Çanakkale Onsekiz Mart University, Animal Experiments Local Ethics Committee animal welfare laws, guidelines and policies as approved by 2018/02-10.

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Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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