Validating Daily Otolith Increment Formation in Young of the Year *Pagellus acarne* in the Çanakkale Region: Employing Alizarin Red S for Accurate Age Determination

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Abstract

Accurate age estimation is essential for population dynamics modeling and fisheries management. Otoliths serve as a critical resource for age determination in ichthyological studies, capitalizing on the embedded growth increments. The periodicity of these increments, however, requires thorough validation to confirm the accuracy of age assessments. This study validated daily increment deposition in sagittal otoliths of young-of-year *Pagellus acarne* from Çanakkale, Turkey using alizarin red S (ARS), a fluorescent calcium-binding marker. Specimens (n=107; 1.8-4.7cm TL) were exposed to ARS concentrations of 0 (control), 50, 100, 150, 200, 300mg/L for 24h, then reared 13 days. After a recovery period of 13 days, the second dyeing was performed with the aim of have a specific fluorescent mark to test the 13 rings found between the 2 fluorescent marks. Otoliths were examined under fluorescent microscopy. ARS marks were detectable in 81.57% of otoliths at ≥100mg/L, with highest visibility at 300mg/L. Survival exceeded 84.2% across treatments. Increment counts between the first and second ARS marks (13 days) and from the second mark to the edge (20 days) matched elapsed time. Ages from wild fish (n=95) ranged 39-201 days with a growth rate of 0.176mm/day. This study demonstrates ARS is an effective, low-impact tool for periodicity validation and provides the basis for accurate age determination and population modeling of this ecologically important species in the region.

Keywords: *Pagellus acarne*, otolith, daily increment, periodicity validation, fluorescence marking, alizarin red S

1. Introduction

In the realm of fisheries science, accurate age determination stands as a cornerstone for understanding population dynamics, assessing stock sustainability, and implementing effective management strategies. Among the various methods available for age estimation, otolith analysis has emerged as a particularly valuable tool. Otoliths, calcareous structures found in the inner ears of teleost fish, record growth increments throughout a fish's life. These increments can provide detailed insights into the age, growth rates, and life history strategies of fish (Campana, 2001). However, the fundamental assumption that these increments are deposited daily requires rigorous validation, particularly for young of the year fish where accurate age determination is critical for understanding early life history traits (Secor et al., 1995).

Pagellus acarne, a species of significant commercial and ecological importance in the Mediterranean Sea and adjacent Atlantic waters, including the region of Çanakkale, has been the subject of numerous studies aimed at elucidating its life history characteristics (Morales-Nin & Moranta, 1997; Morales-Nin, 2000; Pajuelo & Lorenzo,1999; Recasens et al., 2008; Tserpes & Tsimenides, 1995; Tserpes et al., 2002; Tsikliras & Stergiou, 2014).

Despite its importance, the validation of daily otolith increment formation in *Pagellus acarne*, particularly among young of the year individuals, remains a relatively unexplored area of research. This gap in knowledge hinders our ability to accurately assess the age structure of *Pagellus acarne* populations, which is essential for effective fisheries management and conservation efforts (Morales-Nin, 2000).

The validation of otolith increment periodicity has traditionally been approached through various methods, including the analysis of known-age fish reared under controlled conditions, and the application of chemical markers to in situ specimens (Beamish and McFarlane, 1983). Among these methods, the use of alizarin red S (ARS) has gained prominence due to its efficacy in binding to calcium, thereby providing a distinct mark on the otolith that can be used as a reference point for validating increment deposition rates (Brothers, 1987). This technique involves immersing fish in an ARS solution, resulting in a fluorescent mark on the otolith, which, under microscopic examination, can be used to count the number of increments formed post-exposure, thereby validating the daily deposition hypothesis (Wilson et al., 1987).

Given the ecological and economic significance of *Pagellus acarne* and the critical need for accurate age estimation methods, this study aims to validate the daily deposition of growth increments in the otoliths of young of the year *Pagellus acarne* from the Çanakkale region. Employing ARS as a chemical marker, we seek to provide a robust framework for age determination in this species, thereby contributing to the broader understanding of its life history and supporting sustainable fisheries management practices.

Materials and Methods

Study Area and Fish Collection

This study was conducted in the Çanakkale Strait, a vital marine biodiversity hotspot located in the northeastern Mediterranean Sea, bordering the Aegean Sea. Young of the year (YOY *) Pagellus acarne* specimens were collected alive by using beach seine from the shallow waters of 0-2 m depth, and was

transferred in aerated tanks to laboratories at the Çanakkale Onsekiz Mart University Marine Fish Research Unit (COMU MFRU). The YOY fish were kept in a 4500 L closed recirculating seawater system fitted with biofilter and aeration for adaptation to the culture systems. The YOY fish started to be fed with granular feed according to their mouth opening and size. They were maintained under natural photoperiod at temperature 20°C±1.0°C, and at a salinity of ~37 ppm. The temperature and salinity were adjusted according to the temperature and salinity available in the area at that time of the year. Water quality (temperature, salinity, and pH) was checked by daily. After the adaptation process, the YOY *Pagellus acarne* was transferred in 80-liter ventilated separate tanks and kept in there for 14 days prior to experiments.

Validation of Daily Increment Formation Using Alizarin Red S

To validate the daily deposition of otolith increments, YOY *Pagellus acarne* was immersed in different dosages, at a concentration of 0 (control), 50 mg/lt, 100 mg/lt, 150 mg/lt, 200 mg/lt and 300 mg/lt. solution of Alizarin Red S (ARS) for 24 hours, a method proven effective in marking otoliths for age validation studies (Wilson et al., 1987). The natural photoperiod was applied, and no food was given throughout the marking period. The tanks are strongly aerated to keep the pH ~7. Following exposure, fish were returned to holding tanks with conditions mimicking their natural habitat for 13 days. After a recovery period of 13 days, the second dyeing was performed with the aim of have a specific fluorescent mark to test the 13 rings found between the 2 fluorescent marks. Then, for clear increment deposition post-marking, fish were euthanized, and otoliths were extracted.

Otolith Extraction and Preparation

Upon collection, fish were measured for total length to the nearest millimeter and weighed to the nearest gram. Otoliths (sagittae) were then carefully extracted under a dissecting microscope, cleaned with distilled water, and stored dry in labeled vials. For analysis, otoliths were mounted on glass slides using Crystalbond™ adhesive (Beamish and McFarlane, 1983). Prepared otoliths were polished with lapping film from 12, 9 to 3 mm and polished with 0.3 mm to enhance the visibility of growth increments.

ARS Mark Detection

The ARS mark detection was observed under a fluorescent microscope Carl Zeiss Axio Scope A1. Images of the ARS marks were taken with a digital camera mounted on a fluorescent microscope Carl Zeiss Axiocam 305. ARS mark quality was evaluated using a 0–5 ratings; 0, no mark; 1, very weak mark; 2, weak mark visible; 3, clearly visible mark; 4, the mark shining brightly; 5 was evaluated as a distinguishable clear mark fluorescent light (Taylor et al., 2005; Liu et al., 2009). Mark quality was rated twice separately, and scores were rated a third time when the two scores were not coherent. Marks with a quality of 2 or higher was a good mark because they can be easily identified in sagittal otoliths.

Increment Analysis

Otolith increments were examined using a compound microscope equipped with transmitted light at 100x magnification. The first ARS mark served as a reference point from which increments were counted towards the second ARS mark to determine the number of days elapsed since marking. This count provided a direct measure of daily increment deposition rates. To ensure accuracy, increment counts were conducted independently by two experienced readers, with discrepancies resolved through consensus.

Statistical Analysis

The agreement between the observed increment counts and the known elapsed time since ARS marking was assessed using linear regression analysis. The slopes of the linear regressions within each ARS experiment were compared with ANCOVA test. The Chi^2 test was used to compare the number of daily growth increments against the time elapsed. All statistical analyses were performed using PAST 4.03 statistical software, with significance levels set at $p < 0.05$.

Ethical Considerations

All procedures involving the handling and treatment of fish were conducted in accordance with ethical guidelines for the use of animals in research, approved by the [Çanakkale Onsekiz Mart University, Animal Experiments Local Ethics Committee, 2018/02-10].

Results

A total of 107 YOY *Pagellus acarne* specimens ranged in total length from 1.8 to 4.7 cm were used in the experiments. The application of Alizarin Red S (ARS) for the validation of daily increment formation in the otoliths of YOY *Pagellus acarne* from the Çanakkale region yielded definitive results (Figure 1). The experimental application of varying concentrations of Alizarin Red S (ARS) to validate daily age formation in otoliths of young of the year *Pagellus acarne* revealed differential efficacy across the concentration spectrum. The control group (0 mg/L) showed no fluorescent marking on the otoliths, serving as a baseline for comparison against treated groups.

Figure 1. Appearance of ARS marks in the sagittal otolith of YOY *Pagellus acarne*. 40X magnification was applied.

Visibility and Quality of ARS Marks Across Concentrations

The visibility and quality of the marks on the otoliths were found to be concentration dependent. At the lowest concentration of 50 mg/L, marks were observable in a limited number of otoliths, indicating a minimal efficacy threshold. Increasing the concentration to 100 mg/L resulted in an improvement in mark visibility, with a clear fluorescent ring evident in most of the otoliths examined. Concentrations of 150 mg/L and 200 mg/L yielded even more pronounced and distinct marks. At the highest tested concentration of 300 mg/L, the marks were highly visible (Figure 2).

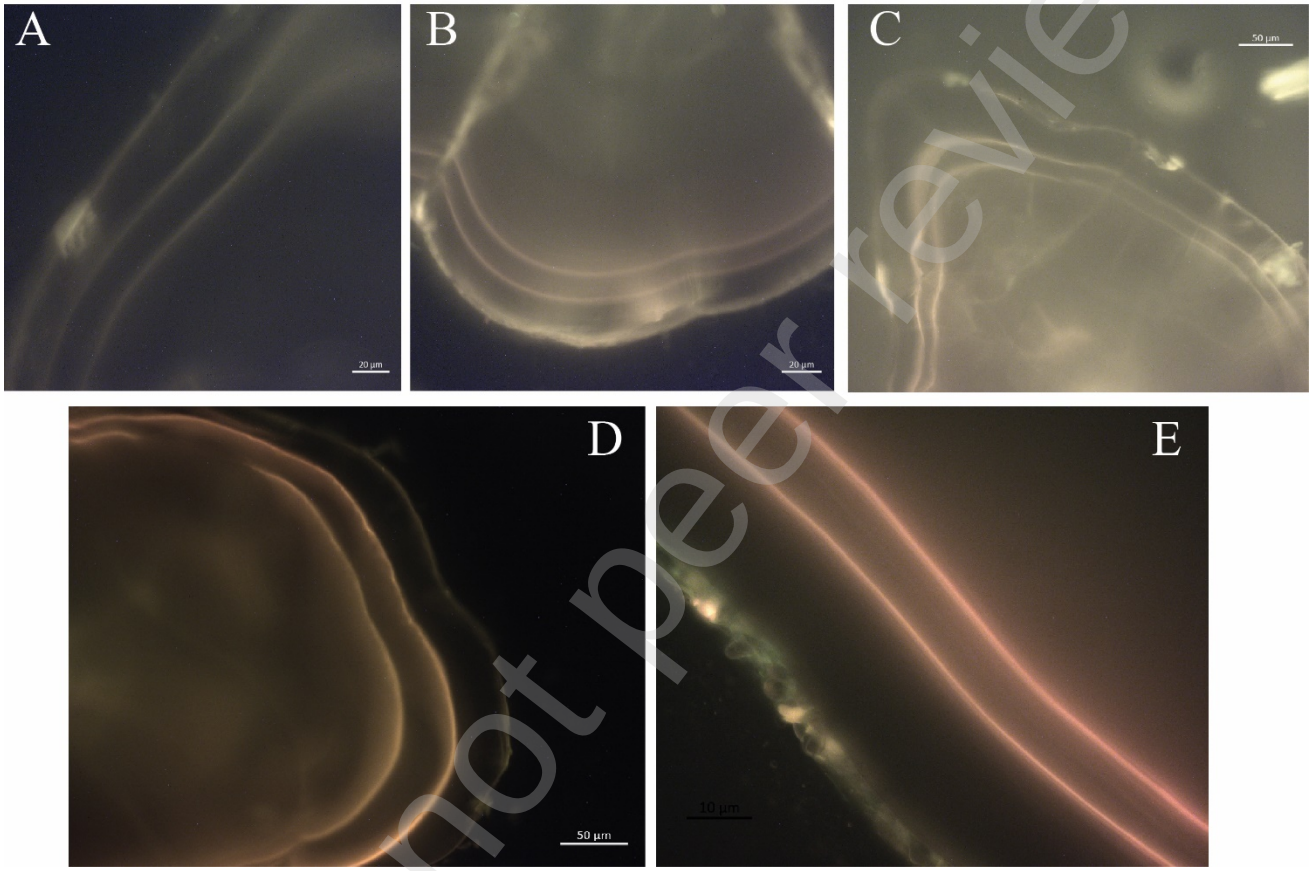

Figure 2. Images of ARS markings on the sagittal otoliths of *Pagellus acarne* individuals. A = 50 mg/L, B = 100 mg/L, C = 150 mg/L, D = 200 mg/L and E = 300 mg/L.

The quality of mark visibility has been analyzed in five categories. A mark quality of 2 and above is considered acceptable in terms of visibility. In our study, ARS marking was successfully observed in 81.57% of the otoliths from wild-caught *Pagellus acarne* specimens. This high detection rate of ARS marks in a field environment underscores the technique's applicability for age and growth studies in natural fish populations.

Survival Rates and ARS Concentration Impact

Throughout the experiment, which included 107 *Pagellus acarne* specimens, there were 12 mortalities, translating to an overall survival rate of at least 84.2%. Notably, two deaths occurred in the control group, indicating that the mortality observed could not be solely attributed to the ARS treatment. The survival rates of fish across the various ARS concentrations remained high, with a minimum rate of 84.2%, demonstrating the non-lethal nature of ARS marking over the 24-hour exposure period (Table 1).

Table 1. Survival and mortality rates of *Pagellus acarne* individuals in ARS trials obtained in the shallow waters of Çanakkale.

Alizarin Red S (ARS) Concentrations (mg/l)	Total	Death	Mortality Rate (%)	Survival Rate (%)
Control (0)	17	2	11,8	88,2
50	20	2	10,0	90,0
100	15	1	6,7	93,3
150	19	3	15,8	84,2
200	15	2	13,3	86,7
300	21	2	9,5	90,5

Daily Age Ring Formation and Validation

The otolith examination revealed 13 daily age rings between the first and second ARS marks and an additional 20 age rings from the second ARS mark to the otolith edge (OE), consistent with the 20-day duration between the second ARS application and the experiment's conclusion (Figure 3). This result was further validated by a Chi-square test comparing the number of daily growth rings formed with the elapsed time. The Chi-square test showed that there is no significant difference between the number of daily growth rings formed in the otoliths of *Pagellus acarne* and the elapsed time (Chi-square = 0.351, df = 94, P = 0.98). This indicates that the formation of growth rings occurs daily in the sagittal otoliths of *Pagellus acarne* obtained from the shallow waters of Çanakkale.

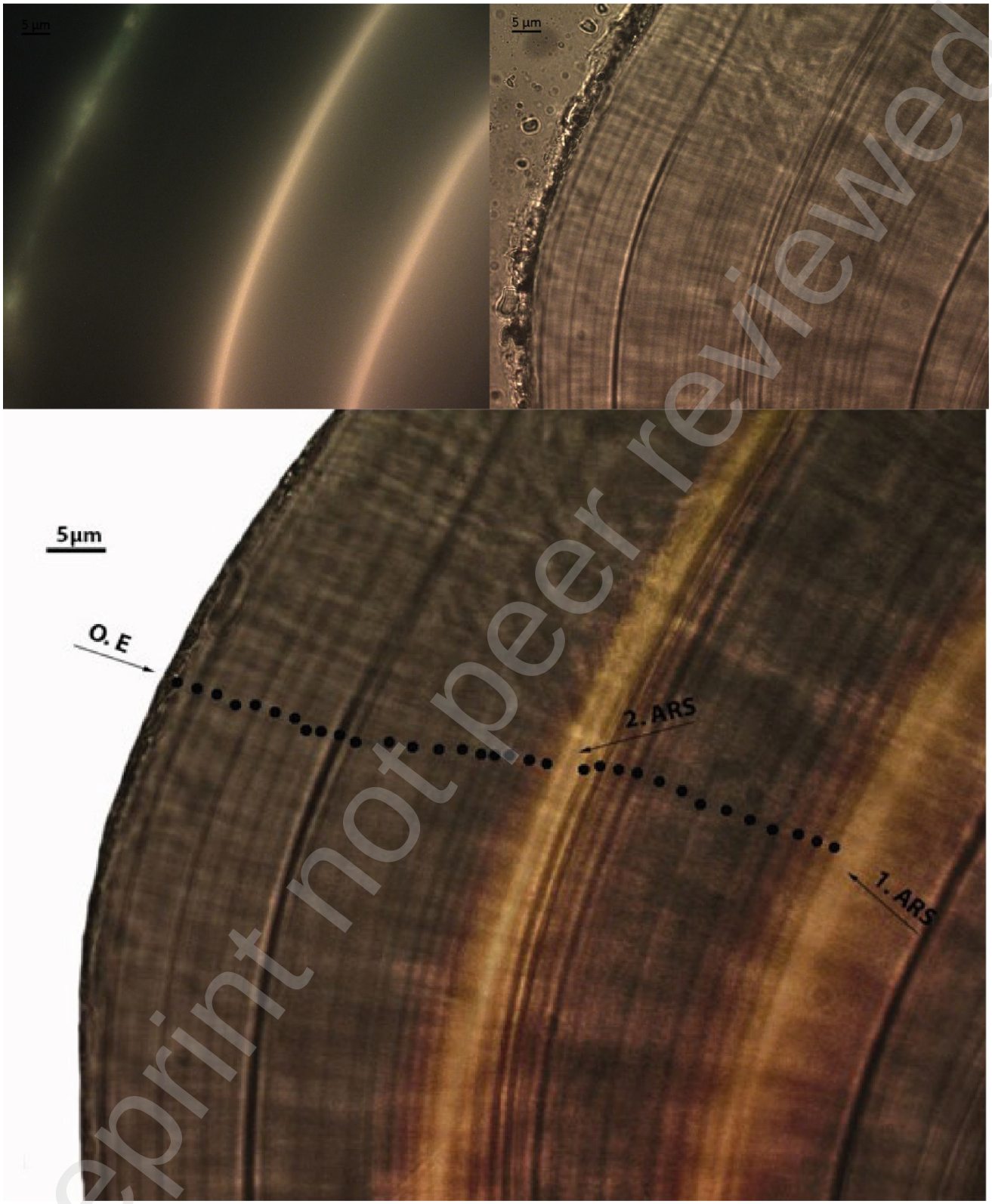

Figure 3. The appearance of the number of the daily rings between the alizarin red S marks and otolith edge. ARS: Alizarin Red S mark; O.E: Otolith edge

Daily Age Determinations in Wild Caught Individuals

In addition to the ARS marking trials, daily age determinations were carried out on 95 young of the year *Pagellus acarne* individuals, with total lengths ranging between 1.8 cm and 4.7 cm. These determinations revealed ages ranging from 39 days to 201 days. Daily growth rates of *Pagellus acarne* individuals (0 years old) were calculated as 0.176 mm/day as a result of the length-age regression analysis (Figure 4). A covariance analysis (ANCOVA) was applied to test if the daily growth rates varied of *Pagellus acarne* individuals exposed to different concentrations of ARS. Results of the covariance analysis showed that the daily growth rates of *Pagellus acarne* individuals did not differ significantly between different concentration groups of ARS ($F = 1.182$, $df = 4$, $P = 0.326$).

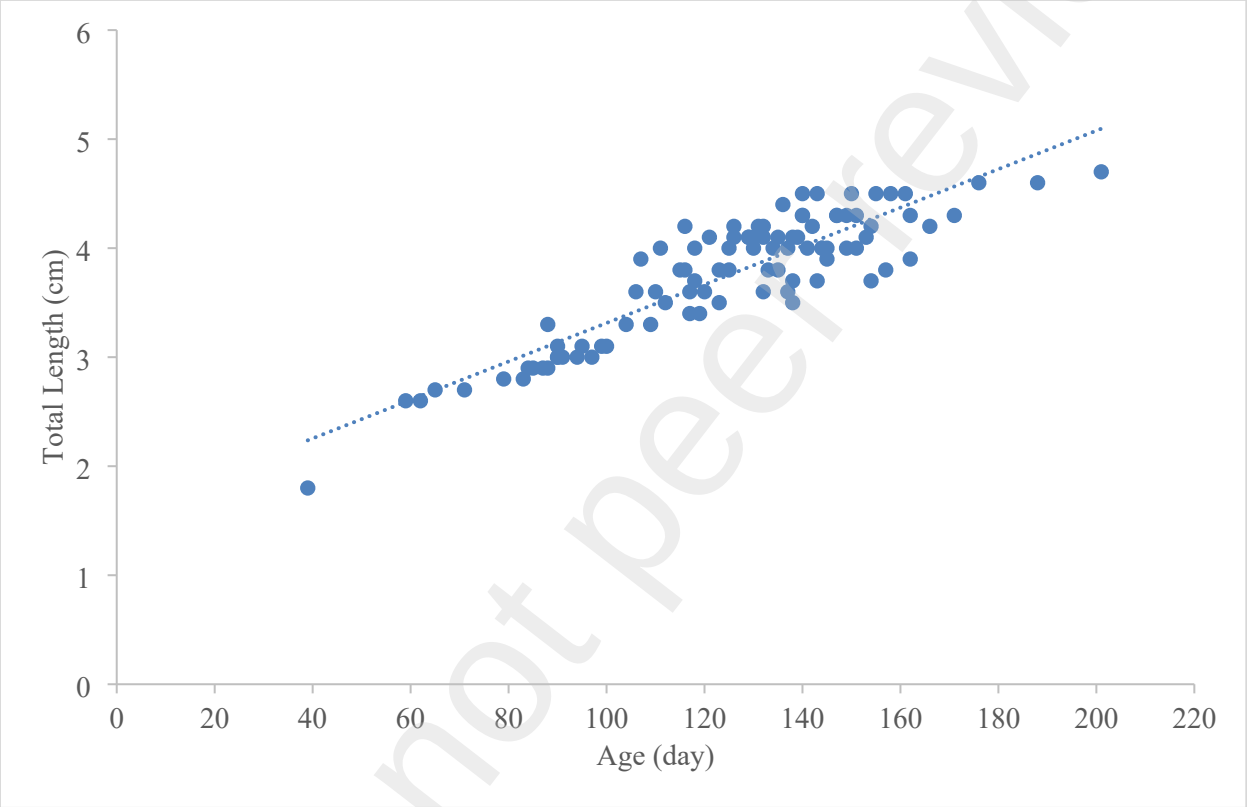

Figure 4. Total length-age relationship of YOY *Pagellus acarne* individuals found in shallow waters of Çanakkale

2. Discussion

The application of Alizarin Red S (ARS) for the validation of daily age formation in young of the year *Pagellus acarne* marks a significant contribution studying early life stages of fishes. The success of otolith marking observed in a substantial proportion (81.57%) of specimens from the shallow waters of Çanakkale not only attests to the efficacy of ARS as a chemical marker but also aligns with the broader body of research advocating for its use in ichthyological studies (Campana & Neilson, 1985; Secor et al., 1991). This high rate of marking success is particularly noteworthy as it suggests that ARS can be reliably used in diverse marine settings, providing a versatile tool for researchers. However, to fully appreciate the utility and implications of these findings, it's essential to contextualize them within the broader spectrum of otolith marking techniques, particularly focusing on other fluorescence chemicals like Oxytetracycline (OTC) and Calcein.

Oxytetracycline (OTC) has been widely used in fish age and growth studies due to its strong affinity for calcium, which makes it an effective marker for calcified structures such as otoliths (Secor et al., 1995). A study by Tsukamoto and Kajihara (1997) on the Japanese eel demonstrated the efficacy of OTC in marking otoliths, providing clear, distinguishable marks that facilitated accurate age determination. However, the use of OTC is not without drawbacks. Concerns have been raised regarding its potential to cause physiological stress and its residual effects, which may linger in the tissues of marked fish (Molony, 2001). In contrast, ARS, as demonstrated in this study for *Pagellus acarne*, shows minimal impact on survival rates, making it a potentially less invasive alternative.

Calcein, another fluorescent marker, has gained popularity for its high visibility in otoliths and minimal invasiveness. Studies such as the one conducted by Thorrold et al. (1997) on Atlantic cod larvae have shown that Calcein produces bright, easily identifiable marks without adversely affecting fish growth or survival. While Calcein and ARS share these advantageous properties, the choice between them may depend on factors such as the specific research objectives, species under study, and available resources. For instance, the study on *Pagellus acarne* highlights the effectiveness of ARS in environments where its application can be precisely controlled and monitored.

Integrating the findings from this study with those involving OTC and Calcein provides a comprehensive view of the potential and limitations of these markers. For example, while OTC has been instrumental in long-term growth studies, its potential side effects necessitate careful consideration. Similarly, while Calcein's minimal invasiveness is a significant advantage, its cost may be a limiting factor for large-scale studies. The successful application of ARS in *Pagellus acarne* research not only adds to this diverse toolkit but also underscores the need for species-specific and context-dependent approaches to otolith marking.

The minimal impact of ARS on survival rates, with over 84.2% of *Pagellus acarne* specimens surviving across all concentrations, underscores the method's safety and minimal invasiveness. The fact that survival rates did not significantly differ from those in the control group indicates that ARS, when used within the tested concentrations, does not impose stress or harm that would otherwise skew the results of growth and development studies. This finding resonates with Morales-Nin & Panfili (2005), who highlighted the importance of non-lethal marking techniques in the study of fish growth and survival.

In conclusion, the study of *Pagellus acarne* using ARS provides a valuable addition to the field of otolith research, complementing existing knowledge derived from studies using OTC and Calcein. By carefully considering the advantages and limitations of each chemical marker, researchers can optimize their methodologies to obtain accurate, reliable data on fish growth and development, ultimately contributing to the sustainable management of marine resources.

Competing interests

not applicable

Authors' contributions

Ayyildiz, H. wrote the main manuscript text and prepared figures. Kurtkaya, E and Altin, A conducted field and laboratory studies. Çelik, P. conduct aquaculture process and all authors reviewed the manuscript.

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Availability of data and materials

not applicable

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