

Validating the growth increment periodicity in the otoliths of three small progenetic gobies

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Summary: We determined the efficacy of marking the otoliths of three small-sized progenetic gobies to validate their increment periodicity. These small gobies have high mortalities and rearing difficulties, making direct validation difficult. The otoliths were marked by immersing the fish in a bath of alizarin red S. The fishes were euthanatized and the number of increments in their otoliths laid down after the fluorescent mark were counted and compared with the number of elapsed days. The results validated the daily periodicity of *Aphia minuta* and *Pseudaphya ferreri*. The high mortality hindered the validation of *Crystallogobius linearis*.

Keywords: age; mortality; rearing; *Aphia minuta*; *Pseudaphya ferreri*; *Crystallogobius linearis*.

Validando la periodicidad de los incrementos de crecimiento en los otolitos de tres pequeños góbidos progenéticos

Resumen: Se determinó la eficacia en el marcado de los otolitos de tres góbidos progenéticos de pequeña talla con el fin de validar la periodicidad en la deposición de los incrementos de crecimiento. Estos pequeños góbidos presentan altas mortalidades y dificultades en su cría, por lo que la validación directa de su crecimiento es difícil. Los otolitos fueron marcados con rojo de alizarina S (ALR). Los peces fueron eutanasiados y el número de incrementos depositados después del marcaje con el colorante fluorescente se contó y comparó con el número de días transcurridos. Los resultados obtenidos validaron la periodicidad diaria para *Aphia minuta* y *Pseudaphya ferreri*. La elevada mortalidad impidió la validación en *Crystallogobius linearis*.

Palabras clave: edad; mortalidad; cría; *Aphia minuta*; *Pseudaphya ferreri*; *Crystallogobius linearis*.

Citation/Como citar este artículo: Pérez-Mayol S., Álvarez I., Riera-Batle I., Grau A., Morales-Nin B. 2021. Validating the growth increment periodicity in the otoliths of three small progenetic gobies. Sci. Mar. 85(3): 169-173. <https://doi.org/10.3989/scimar.05162.015>

Editor: V.M. Tuset.

Received: January 8, 2021. **Accepted:** April 9, 2021. **Published:** August 13, 2021.

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INTRODUCTION

Three small-sized pelagic neritic gobies are the target species of a valuable small-scale fishery which has developed particularly in the western and central Mediterranean. The target species are transparent goby *Aphia minuta* (Risso, 1810) and Ferrer's goby *Pseu-*

daphya ferreri (De Buen and Fage, 1908), and the by-catch species is crystal goby *Crystallogobius linearis* (von Düben, 1845). This traditional seasonal fishery uses a special purse-seine net over sand and gravel bottoms inside bays of Majorca (Balearic Islands) (Iglesias and Martorell 1992, Iglesias et al. 1994). Similar fisheries are carried out in Spain off Murcia (Martín-

ez-Baño et al. 1993), in the Ligurian Sea (Relini-Orsi and Fanciulli 1977, Relini et al. 1996), in the northern Tyrrhenian Sea (Serena et al. 1990, Bairo et al. 1996) and in the Adriatic Sea (Frogliola and Gramitto 1989, Ungaro et al. 1994). In the western Mediterranean, the commercial exploitation takes place during one fishing season each year, generally from December to March, in concomitance with coastal migration and shoaling of transparent goby in winter (Morales-Nin et al. 2017).

The three species share morphological and evolutionary traits (Kon and Yoshino 2002), being small pelagic species (<60 mm total length) which live no more than one year (De Buén 1931). *A. minuta* and *P. ferreri* gobies are distributed in the European Atlantic from Gibraltar to the coasts of Norway and the Baltic Sea, and in the Mediterranean (Miller 1986, La Mesa et al. 2005), whereas *C. linearis* is restricted to the Mediterranean (Wheeler 1992). The life history traits of *A. minuta* have been studied in the Mediterranean, including reproductive biology (Caputo et al. 2001, 2003, Giovannotti et al. 2007), age and growth (Iglesias et al. 1997, La Mesa 1999), life cycle (Iglesias and Morales-Nin 2001) and population structure (Giovannotti et al. 2009, Ruggeri et al. 2016). *C. linearis* reproduction (Caputo et al. 2003) and age and growth (La Mesa 2001) have been determined. No data are available for *P. ferreri*.

The *A. minuta* population is composed of two cohorts, one born in summer and one in winter (Iglesias and Morales-Nin 2001), while *C. linearis* has a long spawning period of eight months (La Mesa 2001). The short life span, low fecundity and high mortality rate of these species make them particularly vulnerable to fishing pressure. The state of the stocks in Catalonia and Majorca (western Mediterranean) seems stable for *A. minuta*, shows a decline for *C. linearis* and no estimates are available for *P. ferreri* (STECF 2018). In Majorca (Balearic Islands) the captures of these species show inter-annual and seasonal fluctuations that

have consequences on the productivity of the fishery. Therefore, it is necessary to improve the knowledge of these species and contribute to the management of the fishery. Owing to their short life span, age can be determined by daily growth increments (DGI) in the otoliths (Iglesias and Morales-Nin 2001). Iglesias et al. (1997) validated the periodicity of DGI of transparent goby by marking them with SrCl₂ in captivity, a technique that requires the use of scanning electron microscopy to detect the mark. No validation has been carried out for the other species. The aim of the present study was threefold: to test the feasibility of a fluorescent marker for marking the otoliths; to validate the daily periodicity of the increments in the otoliths of the three species; and to try to rear *C. linearis* and *P. ferreri* in captivity for the first time.

MATERIALS AND METHODS

To carry out the study, fish were collected from hauls of the commercial small-scale fishery under normal fishing boat operations conducted during the winter seasons of 2014 and 2015. For transport to the Balearic Government aquaculture facilities of the Laboratori d'Investigacions Marines i Aquicultura (LIMIA), the fish were kept in a 50 L container without water renewal but equipped with aeration, maintaining the O₂ level above 7 mg L⁻¹. The duration of the transport never exceeded 2 h. The sampling, survival and experimental protocols are detailed in Table 1.

The first (*A. minuta*) fishes were captured in February 2014 at 30 m depth and a water temperature of 13.5°C. Fifty to sixty individuals were placed in a set of three 90 L aquaria with open seawater flow, constant aeration and at room temperature (ranging from 14.5°C to 18.5°C during the experimental period). On the following day, they were fed with fresh *Artemia* (nauplius and metanauplius) enriched with fatty acids and vitamins, and frozen copepoda, krill, mysidacea

Table 1. – Experimental procedures, survival after capture and marking, mean length and weight of the fishes and otolith growth relationships. Only data of surviving fishes and repeated readings with a coefficient of variation of less than 10% were considered. SD, standard deviation. SL, survival at landing; SW, survival at 1st week; S1, survival to 1st marking; S2, survival to 2nd marking; TL, fish mean total length; W, fish mean weight; N, number of otoliths examined; D1, mean distance from the 1st mark to the otolith edge; D2, mean distance from the 2nd mark to the otolith edge; R1, relationship between observed (O) and expected (E) number of daily growth increments (in days) for the 1st mark; R2, relationship between observed (O) and expected (E) number of daily growth increments (in days) for the 2nd mark.

Species	Date of capture	SL (%)	SW (%)	Date of 1 st marking	S1 (%)	Date of 2 nd marking	S2 (%)	TL (±SD) (mm)	W (±SD) (g)	N (retained)	D1 (±SD) (µm)	D2 (±SD) (µm)	R1	R2
<i>Aphia minuta</i>	07/02/2014	57.14	62	20/02/2014	100	12/03/2014	100	36.83 (±3.32)	0.261 (±0.062)	42 (35)	63.31 (±28.87)	38.47 (±18.64)	O =	O =
	12/02/2014												R ² =	R ² =
<i>Pseudaphya ferreri</i>	24/04/2015	42.81	17	28/04/2015	100	-	-	31.26 (±1.95)	0.213 (±0.045)	24 (13)	63.69 (±42.18)	-	O =	-
													R ² =	-
<i>Crystallogobius linearis</i>	24/04/2015	14.07	13	28/04/2015	90	-	-	25.97 (±1.30)	0.053 (±0.011)	14 (3)	7.371 (±2.08)	-	O =	-
													R ² =	-

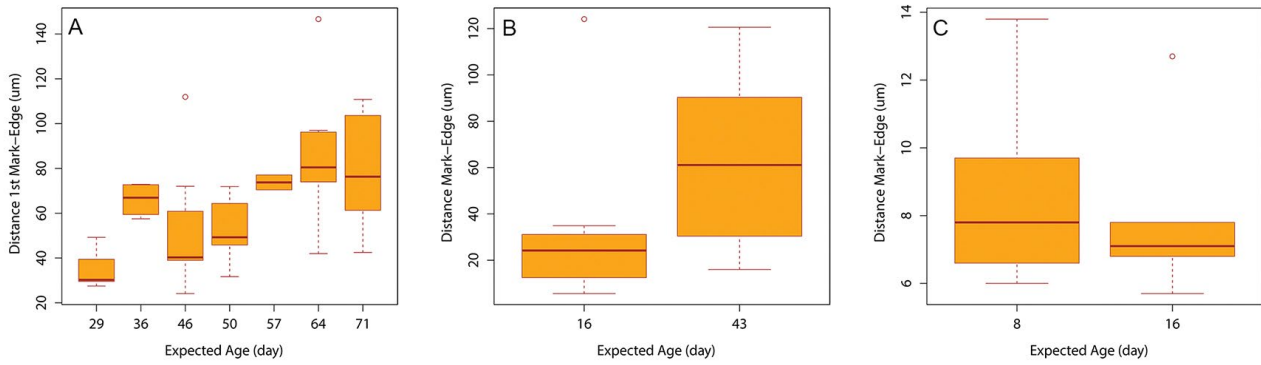


Fig. 1. – Box-plots showing the relationship between the otolith growth (as distance from the ALZ mark to the edge) and the expected age. A, *A. minuta*; B, *P. ferreri*; C, *C. linearis*.

and adult artemia. After one week, the fish were acclimatized to the environment and were responding favourably to the diet. On 20 February, a total of 159 *A. minuta* were marked by immersion in the fluorescent marker alizarin red S (ALR) at a concentration of 65 mg L⁻¹ for 23 h (Taylor et al. 2005). A second marking was performed 20 days after the first one. Beginning at the first marking, periodic sacrifices of 10 fishes were performed at ~8-day intervals until the end of the experimental period (71 days from the first marking). As the captures of both *P. ferreri* and *C. linearis* were very low in 2014, a second set of experiments was performed for these two species in 2015. Fish were captured in April 2015 at 37 m depth and a water temperature of 15.2°C. After acclimatization and rearing conditions as above, 56 *C. linearis* and 67 *P. ferreri* were marked with ALR and sequentially sacrificed 16 and 43 days from marking, respectively (18.0°C to 23.5°C during the experimental period). The end of each experiment was set in concomitance with the increase in daily mortality (Table 1). The fishes were sacrificed with an overdose of tricaine metanosulphonate according to animal welfare procedures approved by Spanish law (RD53/2013).

The sacrificed fishes were measured with an electronic profile projector (Mitutoyo PJ-A3000) and weighed to the nearest mg, and the otoliths were extracted following standard procedures. A total of 94 otoliths of *A. minuta*, 43 of *C. linearis* (corresponding to dead and sacrificed individuals) and 28 of *P. ferreri* were extracted and processed. The left otolith was sanded and polished for examination under a microscope. Digital images (200× and 400×) were obtained with both brightfield and UV lights to visualize the increments and the ALR marks, respectively. DGI laid down between marks and to the otolith edge were enumerated using the ObjectJ plug-in of the ImageJ free software. In addition, the distances between marks and to the otolith edge were determined (Fig. 1). All otoliths were read at least twice and repeated readings with a coefficient of variation over 10% were not considered. The observed (read) and expected (days) number of increments were plotted, and daily periodicity was assumed if the slope of the regression was not significantly different from 1 (Fig. 2).

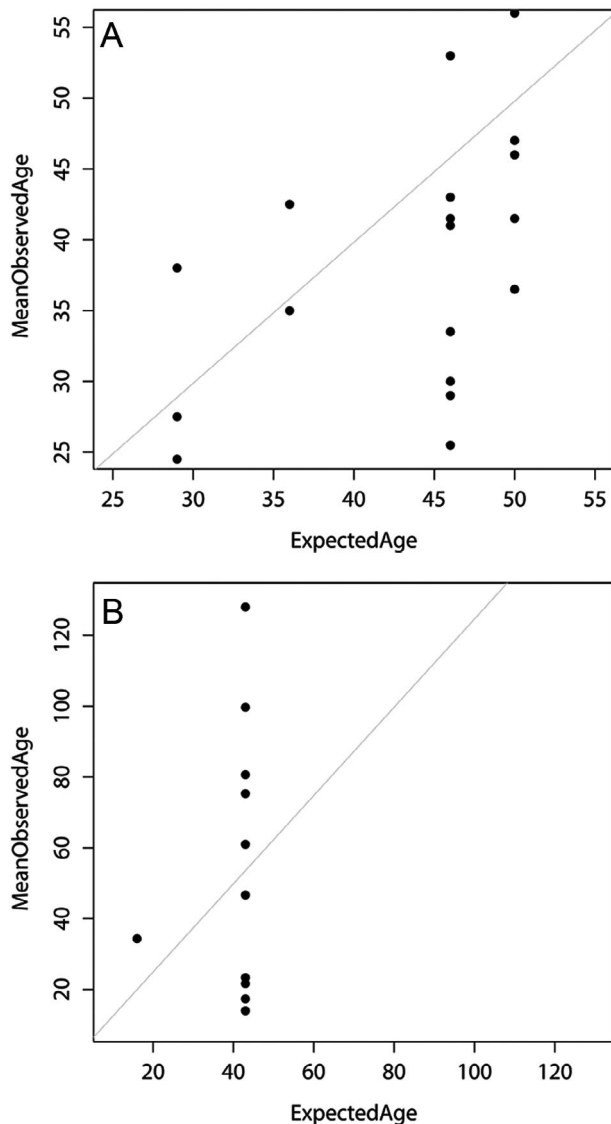


Fig. 2. – Relationship of the mean number of daily growth increments laid down after marking (two observations) and the expected (elapsed) days for *A. minuta* (A) and *P. ferreri* (B). Grey line represents slope relationship. Note that for *P. ferreri* several mean observations are coincident (in 23 and 46 observed days) and black dots are overlapped.

RESULTS AND DISCUSSION

Survival rate at landing varied between species, being higher for *A. minuta* (57.14%), medium for *P. ferreri* (42.81%) and lower for *C. linearis* (14.07%). According to our results (Table 1), these progenetic gobies are very sensitive to handling caused by fishing and experimental acclimatization, and *C. linearis* is the most sensitive. Depth and temperature at fishing seem to be the most important environmental drivers in the survival of target species of the transparent goby fishery (Gil et al. 2020). Injuries produced by changes in pressure (barotrauma) and temperature shock, especially at temperate and tropical latitudes, have been reported to affect survival of fishes subjected to fisheries activities (Giomi et al. 2008, Curtis et al. 2015, Gil et al. 2020). Survival at landing and after the first week seems to be affected by these drivers. Furthermore, gobies showed poor acclimatization to captive rearing conditions, especially *C. linearis* which exhibited the worst adaptation (Table 1). The accumulated effects of barotrauma, temperature shock, trauma related to handling and poor adaptation to diet (more typical for larvae than for adult gobies) could be factors explaining the delaying mortality observed during the maintenance period in captivity, as in other laboratory discard experiments (Davis 2002). *C. linearis* was the most sensitive to experimental conditions, with high mortality rates during the experimental period (66.07% in only 20 days), which forced its duration to be shortened. Mortality in *A. minuta* was 59.12% in 71 days and mortality in *P. ferreri* was 58.21% in 43 days. No mortalities associated with marking were detected, except very few cases of *C. linearis* (Table 1). As these species are very sensitive to water temperature, the differences in the temperature between the two experimental periods could have caused the lower survival in the 2015 experiments. Owing to the poor conditions of *C. linearis*, only the otoliths of sacrificed fishes were retained.

All the otoliths had a fluorescent mark that could be observed under the microscope and they all showed growth after the mark was laid down (Table 1), but growth rate showed high intra-individual variability in all species (Fig. 1). In the case of *A. minuta*, the ALR mark was observed with more clarity on the otolith's ventral edge. *A. minuta* showed a better otolith growth and definition in the growth increments, with 83.3% of the readings under the coefficient of variation limit (Table 1, retained otoliths). *P. ferreri* showed good otolith growth in distance between mark and edge and a multi-ring pattern per day. The presence of sub-units caused 45.8% of the readings to be rejected. *C. linearis* individuals that reached the end of the experiment showed much lower otolith growth (Fig. 1C) and increment clarity; only 21.4% of the repeated otolith readings had a coefficient of variation lower than 10% and just three otoliths were retained.

The mean increment width determined from the growth after the mark and the number of elapsed days

showed a mean increment thickness beyond the limit of the microscope resolution (0.7-1.2 μm). Therefore, the relatively low reproducibility of the readings might be due to low increment definition and the presence of sub-daily increments caused by unfavourable rearing conditions. Iglesias et al. (1997) also found a low clarity of the increments in the case of *A. minuta*, with only 18% of the marked otoliths showing a clear increment pattern. For all the species, the relationship between the observed and expected days that elapsed between the marking events and the sacrifices was validated, showing correlations of between 0.93 and 0.95 when the intercept was forced to 0 (Table 1). Regression slopes were 0.94 and 1.25 for *A. minuta* and *P. ferreri*, respectively (Fig. 2). However, *C. linearis* adjustment was poorer, with a regression slope distant from 1 (i.e. 0.71). The rearing conditions had probably affected the recent otolith growth after marking considering the elapsed number of days (Fig. 1). The variability increased during the experimental period except in *C. linearis*, probably due to the high mortality it experienced.

Direct validation of the increment periodicity in the otoliths requires for instance keeping in captivity and marking the fish (Panfili and Morales-Nin 2002), though this procedure is not always feasible. We can conclude that these delicate small gobies could be kept in captivity for discrete periods of time under low temperatures and that ALR at the reported concentration was successful in marking 100% of the otoliths. However, the daily periodicity of the observed increments was only validated for *A. minuta* and *P. ferreri*. Several authors have reported that growth increments are less clear in fishes in captivity than in wild specimens, and in our case the main difficulty was identifying the daily increments. Here we provide an alternative marking technique and directly validate the periodicity of the two main species of the fishery.

ACKNOWLEDGEMENTS

We thank E. Massuti for his technical assistance and the *Laboratori d'investigacions Marines i Aquicultura* (LIMIA) staff for their help in the experimental procedures. We also thank G. Morey and the fishermen who assisted in providing samples. SP's salary was cofunded by the DGPUR of the Balearic Government and the CSIC. This contribution is a result of the "Unidad Asociada IMEDEA-LIMIA".

ETHICS STATEMENT

The study was authorized by the Fisheries Department of the Balearic Government. All samplings, experimentation and euthanasia of individuals were carried out in strict accordance with the recommendations of Directive 2010/63/EU, transposed to Spanish law (RD 53/2013, BOE n°34 of 8 February 2013). All efforts were made to minimize suffering.

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