



# A semi-automated method for daily age estimation in larval populations by discriminant function models



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## ABSTRACT

Discriminant analysis including the best age-correlated variables selected by SMLR's to construct a predictive model based on somatic and otolith biometry was applied. Age of anchovy larvae ranging from 10 to 12 mm collected along Western Mediterranean coasts during MEDIAS209 survey were estimated by otolith microstructure analysis and compared with the number of daily increments estimated by the model. The model compound by  $\text{Perimeter}^2 + \text{Area}^2$  was able to estimate correctly the age of the otoliths in 75% of the cases assuming  $\pm 1$  day of error increasing to 90% assuming  $\pm 2$  days of error with mean values of APE (3.33%) and CV (4.71%) systematically low. The results indicate the precision of the increment estimates. Moreover, no differences between the estimated ages from direct readings and those estimated by the model were observed. The proposed method implies a reduction in the subjectivity factor and the cost/benefit ratio for ageing studies in fish larvae.

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## 1. Introduction

Survival at early life stages of fish is crucial for future recruitment success (May, 1974) in which larval growth has a determinant role. Thus, age determination in the early life stages of fish is amongst the most important biological variables because it is essential to estimate larval growth rates and mortality (Campana, 2001). Small variations in growth at early life stages affect larval mortality rates (Houde, 1987). Greater larval mortality may be due to greater prolongation of time spans within vulnerable larval sizes and greater ontogenic stage duration (Chambers and Leggett, 1987; Folkvord and Hunter, 1986; Hare and Cowen, 1995). Low growth rates may be the cause of greater mortalities recorded in field studies (Campana, 1996; Hovenkamp, 1992; Miller et al., 1988; Rice et al., 1993; Wilson and Meekan, 2002). Thus, greater survival probability can occur with faster growth rates by decreasing inanition and predation exposure times as postulated by the growth–mortality hypothesis (Anderson, 1988). In conclusion, it may be assumed that there is a strong relationship between larval growth and recruitment success (Bergenius et al., 2002; García et al., 2003).

Age estimation by otoliths is paramount to fisheries science. Ages of around a million fish are estimated yearly by the

interpretation of otoliths (Campana and Thorrold, 2001). Otoliths are the most reliable bony part in fish to estimate age, and particularly in larvae by the analysis of their microstructure (Campana, 1999; Campana and Neilson, 1985; Secor et al., 1995). This implies a meticulous process of extraction and mounting of otoliths. The complexity of the process consumes time and the interpretation of daily increments requires expert qualifications (Megalofonou, 2006). As a result, age determination in larval fish implies a high cost/benefit analysis per otolith (Bedford, 1983; Cardinale and Arrhenius, 2004; Francis and Campana, 2004).

One of the most common sources of error relies on the subjective criteria that an age reader may have. Subjectivity, together with the differences in preparation process of otoliths and the variability of the interpretation of the periodic changes shown in calcified structures are among the major sources of between different age readers' age estimates (Boehlert, 1985; Campana and Moksness, 1991; Cardinale and Arrhenius, 2004). However, otolith measurements show better correlations with age than the somatic measurement variables (Boehlert, 1985). The inclusion of otolith morphometry in age determination can produce more objective and precise age estimates (Doering-Arjes et al., 2008). Although different authors have proposed models for estimating age from otolith morphometry (Fletcher, 1995; Pawson, 1990; Stuart and Ord, 1991; Worthington et al., 1995), the main objection relied on the low proportion of correct age estimates (Francis and Campana, 2004).

Discriminant function analysis allows classifying individuals of unknown origin into groups by using discriminant functions

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generated from a database of information of individuals of known origin (McGarigal et al., 2000). It was first used by Fletcher and Blight (1996) to determine age on the basis of somatic and otolith biometric measurements.

In this study, a database has been assembled to store somatic and biometric information of larvae of determined ages estimated by an expert reader (training sample) which was the source for acquiring discriminant functions that assigned ages to larvae of unknown age (test sample) (Francis and Campana, 2004). This technique allowed us to easily include several variables to attain the discriminant functions, which undoubtedly ameliorated the quality of age estimates (Brander, 1974).

The specific objectives of this study are: (i) to determine which are the best age-correlated variables in anchovy larvae; (ii) to construct a predictive model that allows estimating age with a high percentage of correct assignments based on the somatic and otolith biometry of larvae bringing about an important reduction of subjectivity and increasing the repeatability of age readings.

## 2. Materials and methods

The anchovy larvae used for this study were collected on board the R/V Cornide de Saavedra during May 24 to 26 June, 2009 in the yearly MEDIAS (Mediterranean Acoustic Survey). Oblique plankton tows were undertaken with Bongo 60 and Bongo 90 ichthyoplankton sampling frames. The area covered by the survey encompassed the whole NW Spanish Mediterranean and part of SW Mediterranean (Alborán Sea).

Anchovy larvae were sorted on board and conserved in liquid nitrogen. The use of different plankton gear towed at different speeds for the Bongo 60 and Bongo 90 nets (2 and 3.5 knots, respectively) and different mesh size (200 and 1000  $\mu\text{m}$ , respectively) assured a wide larval size range.

In the laboratory, the larvae were defrosted at ambient temperature. Standard length (SL) of each larva was measured by means of a calibrated image using the freeware software ImageJ 1.44a (USA National Institute of Health). From each sampling area, a subsample of larval size ranges from 10 to 20 mm was taken. The NW and SW Mediterranean accounted for a total of 91 and 76 anchovy larvae, respectively. In the Catalanian coasts, the selected larvae originate from different sampling stations to avoid age estimation bias of environmental nature. After measuring SL, larvae were freeze-dried for 24 h for posterior weighing with a precision balance (mg).

Shortly afterwards, the larvae were put on a slide where these were rehydrated with distilled water. Otoliths were extracted by means of fine tungsten needles while viewed with an estereoscopic binocular (Nikon SMZ 1500). All impurities originating from extraction were cleaned to obtain a clear vision of the otolith. When the distilled water dried, the otoliths were fixed under a cover of nail lacquer.

The radius, increment widths and increment counts were determined from the analysis of the microstructures observed at 1000 $\times$  magnification using the Nikon software ACT-U2. Furthermore, a calibrated image of each otolith was taken using the Image-Pro Plus 6.2.0424 software (Media Cybernetics, Inc.) from which all the following otolith biometry was estimated: Area ( $\mu\text{m}^2$ ), Perimeter ( $\mu\text{m}$ ), SizeL ( $\mu\text{m}$ ) (Feret diameter through the major axis of the otolith) and SizeW ( $\mu\text{m}$ ) (Feret diameter through the minor axis of the otolith).

Larvae from NW Mediterranean (NWM) were then divided into two groups with the sole condition of maintaining in both groups the size distribution of the original population:

NW Mediterranean Training Sample (NWMTR)

$$\times (n_{\text{LARVAE}} = 50; n_{\text{OTOLITHS}} = 75)$$

NW Mediterranean Test Sample (NWMTS)

$$(n_{\text{LARVAE}} = 41; n_{\text{OTOLITHS}} = 71)$$

The group NWMTR was used to calculate the discriminant function that predicts otolith increment counts. On the other hand, NWMTS was used to test the model's reliability by analyzing the differences between the predicted increment counts of the model with the increment counts from direct otolith readings.

The SW Mediterranean population (SWM,  $n_{\text{LARVAE}} = 76$ ;  $n_{\text{OTOLITHS}} = 130$ ) was used to examine the applicability of our model to other populations and the influence of the training sample size in the goodness of our results.

Prior to calculating the discriminant functions, the somatic and biometric variables of the otoliths were analyzed to find which variables correlate best with the larval increment counts. To this purpose, a series of stepwise multiple linear regression (SMLR's) was applied after collinearity of the variables included were tested.

The SMLR's were applied to the full model that included all the variables (Size, Weight, Radius, Mean Increment Width, Otolith Area, Otolith Perimeter, SizeL and SizeW) and to a filtered model (Size, Weight, Otolith Area, Otolith Perimeter, SizeL and SizeW) in both directions (forward and backward). The application of the full model allows verifying the goodness of fit of the model predicting increment counts (age). On the other hand, the filtered model provides information on the true predictive capacity of the model to predict increment counts. Although the collinearity assumptions were accounted for to analyze the best increment counts determinations, the SMLR's were repeated for both models with (i) larval weight and (ii) with all the independent variables log linearized (Table 1).

Discriminant functions with the selected variables from the SMLR's, for the different models obtained were calculated. In addition, in the case of the filtered model, selected variables were squared to maximize differences between them in order to improve the assignment capacity by discriminant functions and to obtain better determinations of increment counts.

To determine the model's precision estimating increment counts, the proportion of correct increment counts assignments, the average percentage error (APE), the coefficients of variation means (CV) and the mean error in increment counts were calculated.

All statistical analyses have been done using the Statistica 7.1 Statsoft software package at the significance level  $p < 0.05$ .

## 3. Results

Four models of high explicative capacity were obtained from the application of SMLR's to the full model ( $R^2 > 96\%$ ;  $p < 0.01$ ) (Table 1). The range of coincident reader estimated increment counts varied from 38 to 54% and increases to 94% (even up to 99%) allowing an error estimate of  $\pm 1$  increment. Less than 1% of the larvae are estimated with an error of  $\pm 2$  increment counts, showing APE's lower than 2.1%, with error means less than 1 increment count and mean CV's less than 2.9% (Table 2).

With respect to the filtered models, three models were obtained ( $R^2 > 80\%$ ;  $p < 0.01$ ) (Table 1). Discriminant functions were applied which provided a percentage of error free assignments of increment counts over 21%. Assuming an error of  $\pm 1$  increment count, the accuracy of assignment increases to a minimum of 62% and over 79% for  $\pm 2$  days, registering APE's less than 4.1%, mean increment counts around 1.5 days, and mean CV's less than 5.8%. Nonetheless, less than 6% of the larvae were estimated with an error  $\pm 3$  increment counts (Table 2).

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