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Original Article

Population structure of the European anchovy, *Engraulis encrasicolus*, in the SW Mediterranean Sea, and the Atlantic Ocean: evidence from otolith shape analysis

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Understanding the influence of oceanographic features on the structure of fish population is of basic importance to population dynamics studies and fisheries management. The European anchovy (*Engraulis encrasicolus*) exhibits a complex population structure which has produced conflicting results in previous genetic studies. This study examines the variability in the shape of the anchovy's otolith as a tool for identifying different stocks, and investigates the effects of oceanographic features on population structure. Anchovies were analysed from seven locations in the SW Mediterranean Sea and Atlantic Ocean along the northwestern African (Morocco) and Portuguese (Bay of Cadiz) coasts. A combination of otolith shape indices and elliptic Fourier descriptors were investigated by multivariate statistical procedures. Within the studied area, three distinct anchovy stocks were identified: the Algero-Provençal Basin, the southern Alboran Sea, and the Atlantic Ocean (Morocco and Gulf of Cadiz). The separation of the stocks was based on non-parametric discriminant analysis returning a classification percentage. Over 81% of the separation of the stocks could be explained by oceanographic features. Shape variability of anchovy otoliths was associated with the presence of the Almeria-Oran front, and the strait of Gibraltar. The Alboran stock was distinct from the Algero-Provençal Basin and from the closest Atlantic stocks (Gulf of Cadiz or Atlantic coast of Morocco). Results are discussed and compared with those previously obtained by genetic studies. This study supports the efficiency of otolith shape analysis for the stock identification of anchovy, and highlights the role of oceanographic features in stock separation.

Keywords: Almeria-Oran front, anchovy, Atlantic, Mediterranean, otolith, shape analysis, stock identification.

Introduction

In the marine environment, low genetic differentiation is expected across vast areas, as gene flow is assumed to occur over large geographic scales due to the lack of obvious barriers to dispersal, and to the existence of pelagic larvae in many species (Graves, 1998; Waples, 1998). However, oceanographic features such as currents, fronts, and upwelling cells have been recognized as possible factors driving population differentiation within species (Bembo *et al.*, 1996; Patarnello *et al.*, 2007; Galarza *et al.*, 2009). Determining the influence of hydrographic features and barriers to dispersal on population structure and connectivity remains a major challenge for marine ecologists and fisheries biologist. Few fish species form single homogeneous populations, and these are often composed of discrete stocks. Each of these stocks may have unique demographic properties and responses to exploitation, which should be managed separately to ensure sustainable fishery benefits and efficient conservation (Begg and Brown, 2000; Stransky *et al.*, 2008; Neves *et al.*, 2011) as recommended in the portfolio approach (Schindler *et al.*, 2010). Therefore, stock identification is of crucial importance in fishery science and management, and understanding the population dynamics of a species in an ecological sense (Cadrin *et al.*, 2005).

The study of the morphological and chemical characteristics of otoliths has been put forward as an efficient tool for fish stock identification (Campana and Neilson, 1985; Ferguson *et al.*, 2011). Otolith shape is markedly species-specific, and often varies geographically within species in relation to environmental factors (Cardinale *et al.*, 2004; Stransky *et al.*, 2008). Otolith shape analysis has been proven a useful tool for spatial and temporal discrimination of fish stocks (Campana and Casselman, 1993; Agüera and Brophy, 2011), including pelagic species (e.g. Burke *et al.*, 2008).

The European anchovy, *Engraulis encrasicolus,* is a small pelagic coastal marine fish, forming large schools, largely spread from the North Sea to central Africa, including the entire Mediterranean basin. This species represents an important fisheries and economic activity for the countries bordering the Mediterranean Sea (Lleonart and Maynou, 2002). Due to its market value, production, and wide distribution in several Mediterranean countries, anchovy is a major shared resource in the region. Attempts to differentiate populations and stocks date from the 1920s (e.g. Fage, 1920; Alexandrov, 1927), yet stock structure of the Mediterranean anchovy is not clear. Given its fragility, tagging and tracking its movement is not possible, making the study of its migration and population structure difficult. Several conflicting studies have attempted to understand the genetic structure of this species, characterized by high gene flow (e.g. Bembo *et al.*, 1996; Borsa, 2002; Bouchenak-Khelladi *et al.*, 2008; Sanz *et al.*, 2008). More recently, studies have revealed a complex population structure in the Mediterranean Sea and Northeast Atlantic Ocean (Zarraonaindia *et al.*, 2012; Viñas *et al.*, 2014).

In the Mediterranean Sea, European anchovy stocks are fully exploited, or overexploited (GFCM, 2012). In the context of rational management, there is a need for a better identification of stock units to prevent loss of some of them, and maintain high diversity and the overall resilience to fishery and other pressures (Hilborn *et al.*, 2003; Schindler *et al.*, 2010). While the knowledge of anchovy fisheries and population structure is relatively rich for the northern Mediterranean zones (e.g. Pertierra and Lleonart, 1996; Palomera *et al.*, 2007), very few studies have been conducted in the southern Mediterranean regions (e.g. Bouchenak-Khelladi *et al.*, 2008).

The aim of this study was to analyse the effects of oceanographic features on the population structure of European anchovy. Geographic variation in anchovy's otolith shapes were investigated in the southwestern part of Mediterranean Sea (Alboran Sea and the Algero-Provençal Basin), and in the Atlantic along the northwestern African (Morocco) and Portuguese (Bay of Cadiz) coasts, in relation to oceanographic features. The western Mediterranean Sea is an ideal study area because it is characterized from an oceanographic point of view by a complex circulation scheme (Millot, 1985; Tintore *et al.*, 1988), and the presence of the Strait of Gibraltar. The existence of the quasi-permanent Almeria-Oran front (AOF), which is the major oceanographic discontinuity in the western Mediterranean Sea, induces different environmental conditions between the Alboran Sea and the Algero-Provençal Basin (Tintore *et al.*, 1988). Because these hydrographic features may act as barriers to fish dispersal and migration, the hypothesis that the stocks may develop under different environmental conditions which in turn influence the otolith shape was tested.

Material and methods

Study area and fish sampling

Anchovy samples were obtained from the southwestern part of the Mediterranean Sea at two locations along the Algero-Provençal Basin (Béjaia and Annaba), and three in the Alboran Sea (Benisaf, Ghazaouet, and Nador), and from the Atlantic ocean along the northwestern African (Morocco) and Portuguese coasts (Bay of Cadiz; sampling details are summarized in Table 1 and Figure 1).

Table 1. Sample locations, anchovy sizes, and number of otoliths analysed.

Area code	Area name	Body size range (cm)	Mean body size	No. of otoliths used for shape analyses
1	Annaba	11 – 15.3	12.29	119
2	Béjaia	11 – 17.5	13.18	326
3	Bénisaf	11 – 16.7	14.13	131
4	Ghazaouet	11 – 16.8	14.06	230
5	Nador	11 – 15.0	12.95	74
6	Gulf of Cadiz	11 – 13.4	12.17	125
7	Casablanca	11 – 15.5	12.90	54

In the laboratory, the fish were measured (total length TL, mm) to the nearest 1 mm, sexed (when possible), and weighed (total weigh *W*, g) with a precision of 0.1 g. Sagital otoliths were then extracted through the gills. After washing and cleaning in distilled water, they were dried in the ambient air. From fish ranging from 11 to 17.5 cm TL, a total of 1059 left otoliths (not showing any clear distortion or damage) were used in this study.

Image and shape analysis

For image analysis, the otoliths were placed on a microscope slide with the sulcus facing downwards and the rostrum always pointing in the same direction (upwards). This was done to minimize distortion errors within the normalization process. The otolith outlines were digitized using an image analysis system consisting of a high-resolution Sony video camera connected to an optical microscope. High-contrast video images were obtained using transmitted light, producing dark two-dimensional objects with a bright background. The digitized image was then analysed using image analysis software (TNPC 5.0, NOESIS) to measure its area (Ao), perimeter (Po), maximum length (Lo), and maximum width (Wo). Otolith shape indices, including circularity, ellipticity, rectangularity, form-factor, roundness, and aspect ratio, were then calculated (Table 2; Tuset *et al.*, 2003).

The method of elliptic Fourier descriptors (FDs) was used in accordance with the procedures suggested by Kuhl and Giardina (1982), and calculated with Shape software (Iwata and Ukai, 2002). The Fourier power (FP) spectrum was calculated to determine the sufficient and necessary number of harmonics for the best reconstruction of the otolith outline. As the first 13 harmonics reached 99% of the mean cumulated power, the Fourier analysis indicated that the otolith shape of *E. encrasicolus* could be summarized by these 13 harmonics, i.e. 52 ($13 \times 4 = 52$) FDs. The first harmonic represents the starting point of the outlines, and is not relevant to a shape analysis. The coefficients corresponding to this harmonic were therefore eliminated (Gonzalez-Salas and Lenfant, 2007).

Data analysis

Size correction

Most important for morphometric analysis is the correction of the data by fish size, i.e. uncoupling of otolith shape and anchovy TL. To remove the effect of the size on the morphometric data (shape indices and coefficients), standardized morphometric measurements (MS) were calculated following the method of Thorpe (1975) and Lleonart *et al.* (2000) for each fish as: $MS = MO\ (Tl/TL)^b$, where MO is the original morphometric measurement, Tl the mean total length of all fish from all regions, TL the total length,

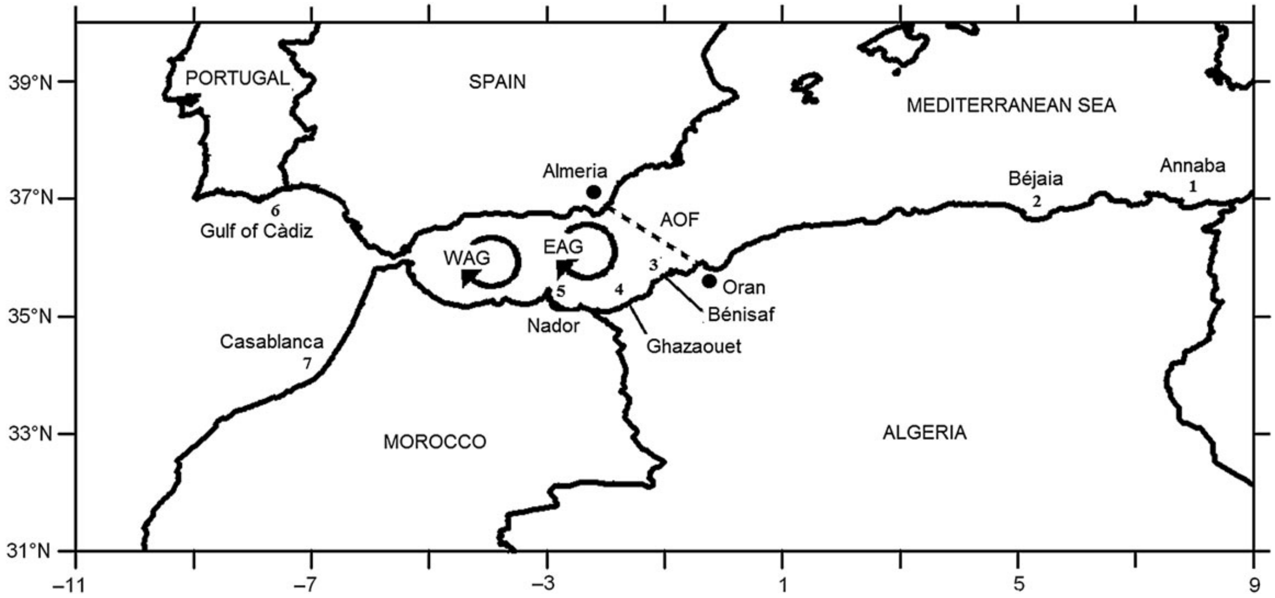

Figure 1. Map of the seven locations where *E. encrasicolus* individuals were sampled. The circulation in the Alboran Sea and location of the AOF are shown (EAG, east Alboran gyre; WAG, west Alboran gyre).

Table 2. Otolith size parameters and resulting shape indices utilized for the shape analysis.

Size parameters	Size-based shape indices
Area (A)	Circularity (Cir) $= P/A^2$
Perimeter (P)	Rectangularity (Rec) $= A/(\mathrm{OL} \times \mathrm{OW})$
Otolith length (OL)	Form-Factor (FF) $= (4\pi A)/P^2$
Otolith width (OW)	Ellipticity $= (\mathrm{OL} - \mathrm{OW})/(\mathrm{OL} + \mathrm{OW})$
	Roundness (Rnd) $= (4A)/(\pi \mathrm{OL}^2)$
	Aspect ration (AR) $= \mathrm{OL}/\mathrm{OW}$

and b was estimated for each morphometric character as the slope of the regression between log MO and log TL (Elliott *et al.*, 1995). Correlation between transformed variables and total length were calculated to inspect if the data transformation was effective in removing the effect of size in the data.

Multivariate analysis

Owing to the nature of the data from the otolith shape analysis, some involved otolith shape variables did not follow a normal distribution. For this reason, instead of using a common parametric statistical approach, a non-parametric discriminant analysis (SAS version 9.3; SAS Institute, Inc., 2013) using the normal kernel density estimation was applied to investigate the usefulness of otolith shape in separating anchovy stocks. Cross validation was used to estimate the accuracy of classification rules. Results are presented as a matrix showing the numbers and percentages of fish correctly and incorrectly classified for the seven areas sampled. Comparisons between stocks were conducted using a permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001); a non-parametric statistical test that does not require the normal distribution of data which allows testing the simultaneous response of one or more variables to one or more factors in the ANOVA experimental design. The PERMANOVA was based on the Bray–Curtis dissimilarity measure (4999 random permutations). To represent graphically the major trends in shape differences between stocks, non-metric multidimensional scaling (nMDS) and cluster analysis on adjusted morphometric data (shapes indices and EFC) were used. Similarity matrices were constructed based on Bray–Curtis' similarity. MDS assign a non-dimensional location to each group and calculate the distance among groups. A non-dimensional plot is produced based on those distances, which reflects the similarities or dissimilarities among groups, so that similar groups are plotted closer to each other and dissimilar groups are further apart. Analysis and test were carried out using the SAS 9.3 (2013) and PAST3.

Results

Shape analysis

The effect of anchovy size on the otolith shape was successfully eliminated. The size-correction greatly reduced the correlation between otolith shape and fish lengths ($r^2 < 0.001$). Both otolith shape indices and EFCs were significantly different among the seven sampling sites (PERMANOVA, pseudo-F: 25.16, $p = 0.0002$). Results of non-parametric discriminant analysis are given in Table 3. The overall percentage of correct classification obtained was 78% ranging from a high of 85% (Béjaia) to a low of 73% (Bénisaf; Wilk's $\lambda = 0.12$, $p < 0.001$). High misclassification percentages were observed within areas 3 and 4 in the Alboran Sea (up to 18%), the Algero-Provençal Basin areas 1 and 2 (up to 17%), and areas 6 and 7 in Atlantic (up to 14%). Area 5 misclassified into both the Alboran area 4 (16%) and the Atlantic areas 6 closest to the Strait of Gibraltar (8%). This suggested three main area groups: "Algero-Provençal Basin" (areas 1 and 2), "Alboran" (areas 3–5), and "Atlantic" (areas 6 and 7). The PERMANOVA tests performed on combined shape (EFC) and otolith indices yielded significant differences among the three stocks identified (PERMANOVA, Table 4). Pairwise comparison detected differences between Algero-Provençal Basin (areas 1 and 2) and Alboran Sea (areas 3–5; $p < 0.0002$), between Algero-Provençal Basin and Atlantic areas ($p < 0.0002$)

Table 3. Results of non-parametric discriminant function analysis between sampling areas of *E. encrasicolus*.

	1	2	3	4	5	6	7
1	91 (**76.47**)	21 (17.65)	5 (4.2)	1 (0.84)	0 (0)	1 (0.84)	0 (0)
2	36 (11.04)	277 (**84.96**)	6 (1.84)	2 (0.61)	0 (0)	5 (1.54)	0 (0)
3	0 (0)	2 (1.53)	95 (**72.52**)	24 (18.32)	10 (7.63)	0 (0)	0 (0)
4	1 (0.43)	2(0.86)	31 (13.47)	176 (**76.52**)	20 (8.69)	0 (0)	0 (0)
5	0 (0)	1 (1.34)	5 (6.76)	12 (16.22)	50 (**75.68**)	6 (8.10)	0 (0)
6	0 (0)	0 (0)	2 (1.6)	4 (3.2)	2 (1.6)	100 (**80**)	17 (13.6)
7	0 (0)	1 (1.85)	2 (3.70)	1 (1.85)	2 (3.70)	8 (14.81)	40 (**74.07**)

The number of fish classified to category followed by percentage (in parentheses) are shown, the bold values represent the correctly classified percentage in each area [overall correct classification = 78% (829/1059); Wilk's $\lambda = 0.12$, $p < 0.001$]. See Table 1 for codes used.

Table 4. PERMANOVA comparison of otoliths of *E. encrasicolus* from the three stocks.

Variables	Regions	*F*	*p*-value
Combined Fourier coefficients and shapes indices	All	60.79	0.0002
	Algero-provincal/ Alboran	73.97	0.0002
	Algero-provincal/ Atlantique	43.08	0.0002
	Atlantique/Alboran	60.29	0.0002

Shown are overall comparison among regions for combined otolith shape and elliptical Fourier coefficients including pairwise comparisons between regions.

and Atlantic areas and Alboran Sea ($p < 0.0002$). Indeed, most of the patterns observed in the non-parametric discriminant analysis were validated by the nMDS and cluster analysis of the average FDs and shape indices by area (Figure 2). To validate the separation of these three areas groups, a non-discriminant analysis was carried out revealing an overall correct classification of 81% (Wilk's $\lambda = 0.28$, $p < 0.001$; Table 5).

Shape visualization

The reconstruction of the shape outline from the average harmonics showed distinct differences between the three clusters of areas identified by discriminate analysis (Figure 3). The highest between-stocks variation clearly occurred in the length direction of the otolith, especially between the rostrum and the anti-rostrum part, and to a lesser degree, in the ventral part. There was a well-developed anti-rostrum for both the Atlantic and the Algero-Provençal stocks. On the other hand, the space between rostrum and post-rostrum in the Alboran stock was deeper than the other two stocks. The ventral side was deeper for the Atlantic stocks, while the dorsal side shows only minor differences between stocks.

Discussion

The results of this study showed that the combination of size-based otolith shape, and Elliptic FDs, are useful tools for discriminating between anchovies belonging to separate stocks. Within the study area, three distinct stocks were identified: the Algero-Provençal Basin, the southern Alboran Sea, and the northwestern Atlantic (Morocco and Gulf of Cadiz). The separation of the stocks seemed to be linked to the oceanographic features of the studied area.

The influence of oceanography on the population structure of marine species is now widely recognized (Galarza *et al.*, 2009; White *et al.*, 2010; Schunter *et al.*, 2011). Nevertheless, there is still limited knowledge on how oceanographic conditions may pose barriers for the population structure of a species, and especially why different species with similar life history traits show distinct responses to oceanographic discontinuities (Galarza *et al.*, 2009). Although anchovies are commonly considered highly mobile, their localized spawning behaviour or migratory patterns may result in restricted gene flow, especially among waters of contrasting hydrography. For example, Sabatés *et al.* (2013) suggested that oceanographic features, such as eddies, play a role in retaining and concentrating anchovy larvae. In the SW Mediterranean Sea, anchovy larval retention, spawning, and nursery location are influenced by oceanographic processes (Catalán *et al.*, 2013). Levi *et al.* (1994) reported growth differences between anchovies from the northern and central basins of the Adriatic Sea, pointing out that circulatory patterns in the two areas are independent, and may thus form an effective stock boundary. Such oceanographic discontinuity may play a role in maintaining a degree of genetic differentiation between anchovy in the two basins (Bembo *et al.*, 1996).

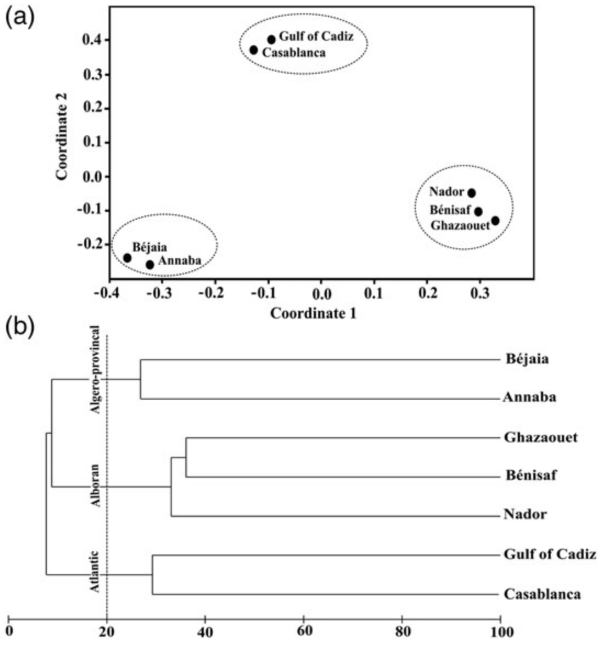

Figure 2. (a) nMDS and (b) cluster analysis output depicting the linkage dendrogram (Bray–Curtis' similarity) computed on otolith shapes indices and EFC of *E. encrasicolus* from the seven areas. Circles represent groupings stocks (stress: 0.08).

In the western Mediterranean, the entrance of the Atlantic Jet (AJ) is the main forcing agent modulating hydrological processes (García-Lafuente *et al.*, 1998). The AJ provokes high mesoscale

Table 5. Results of non-parametric discriminant function analysis between the three stocks of *E. encrasicolus*.

Stocks	Alboran	Algero-provincal	Atlantic
Alboran	371 (**86**)	54 (12.47)	8 (1.85)
Algero-provincal	53 (11.91)	387 (**87**)	5 (1.12)
Atlantic	37 (20.67)	17 (9.50)	125 (**70**)

The number of fish classified to category followed by percentage (in parentheses) are shown, the bold values represent the correctly classified percentage in each area [overall correct classification=81%, Wilk's $\lambda = 0.28$, $p < 0.001$)].

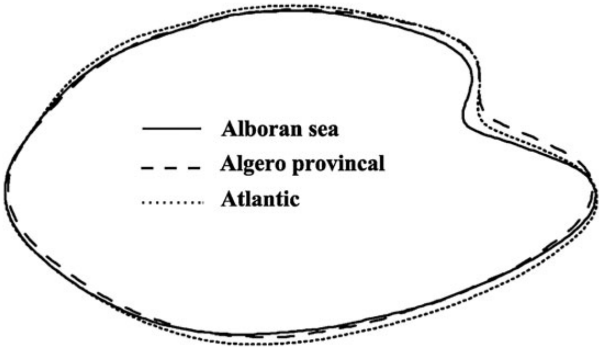

Figure 3. Average shapes of the otoliths of *E. encrasicolus* in the three stocks identified, based on the mean FDs.

activity (Tintoré *et al.*, 1991; Viúdez *et al.*, 1998), which is observable in structures such as eddies and quasi-permanent upwelling. The interaction of this AJ with the denser, more-saline Mediterranean water, results in the formation of a quasi-continuous front, known as the AOF. Although most genetic studies have been conducted in the northern Mediterranean region, the AOF has been suggested as the point of genetic break between the Atlantic Ocean and the Mediterranean Sea for many species of different taxa, including seaweeds, sponges, molluscs, crustaceans, fish, and mammals (see the review in Patarnello *et al.*, 2007). This genetic geographic isolation from either side of the AOF is particularly well documented for different fish species including pelagic species such as the European anchovy (Bouchenak-Khelladi *et al.*, 2008), and European sardine, *Sardina pilchardus* (Ramon and Castro, 1997). The AOF is an oceanographic front exhibiting a pronounced step temperature (1.4°C) and salinity (2 ppt) gradient over a distance of 2 km (Tintore *et al.*, 1988), and is characterized by different planktonic species communities on either side (Estrada *et al.* 1985; Ruiz *et al.*, 2013). Many genetic studies have reported the isolation of the Alboran Sea anchovy from the rest of the Mediterranean, suggesting that the AOF is acting as a dispersal barrier for anchovies (Bembo *et al.*, 1996; Magoulas *et al.*, 2006; Bouchenak-Khelladi *et al.*, 2008; Sanz *et al.*, 2008). This paper's results confirm the results of studies on genetic differentiation in anchovies, and have shown for the first time that the morphological variability of anchovy otoliths is linked to the presence of the AOF. Based on meristic and body shape criteria, Bacha (2009) also found a clear differentiation between anchovy from the Algero-Provençal Basin and those from the Alboran Sea.

A large number of studies have challenged the hypothesis of the Strait of Gibraltar representing a phylogeographical break (see Patarnello *et al.*, 2007 for review). The hydrological characteristics of the Alboran Sea's surface waters are much closer to those of the northeastern Atlantic than the Western Mediterranean, from which they are separated by the AOF (Tintore *et al.*, 1988). A close relationship between the Alboran Sea and northeastern Atlantic anchovy has been reported in genetic studies. Different genetic markers studies (nuclear-DNA, multiple SNP Markers, or allozymes) showed that the anchovy in the Alboran Sea are closely related to populations in the adjacent Gulf of Cadiz (Sanz *et al.*, 2008; Zarraonaindia *et al.*, 2012) or Canary archipelago (Bouchenak-Khelladi *et al.*, 2008). In this study, otolith shape analysis suggests that the Alboran population is distinct from their closest Atlantic population (Gulf of Cadiz or Atlantic coast of Morocco), and that the constriction of the Strait of Gibraltar has isolated the two populations. These results confirm the recent genetic studies of Chairi *et al.* (2007) and Viñas *et al.* (2014), which showed that the Alboran Sea anchovy population is genetically distinct from the Northeast Atlantic populations, including neighbouring populations (e.g. Gulf of Cadiz). The fish in the Alboran Sea have most likely adapted to the prevailing hydrodynamic regime and narrow shelf, which has resulted in local coastal fish population isolation. European anchovy are typically coastal schooling planktivores with batch spawning, inhabiting spatially complex coastal areas, isolated from each other by peninsulas and narrow straits; and this complexity tends to isolate populations by reducing levels of gene flow between regions (Bembo *et al.*, 1996; Magoulas *et al.*, 2006). This is particularly true in the studied area where the narrow continental shelf may have resulted in restricted migration and discrete group of individuals with varying degrees of temporal and spatial integrity.

Otolith shape analysis has been used widely with success in stock identification studies of various marine fish species including small pelagic fish like anchovy (Gonzalez-Salas and Lenfant, 2007), Atlantic herring (Burke *et al.*, 2008), and horse mackerel (Stransky *et al.*, 2008). Although otolith shape provides a phenotypic basis for stock separation, factors affecting otolith shape are not fully understood. The otolith shape, among other morphometric traits like the body shape, is a characteristic that reflects a combined effect of genetic variation and local environmental factors (Tudela, 1999; Cardinale *et al.*, 2004; Vignon and Morat, 2010). In a recent study (Vignon, 2012), it was established that habitat environmental conditions induce an important change in otolith shape. Differences in environmental conditions can have a considerable influence on how otolith growth, and consequently otolith shapes are formed (Campana and Neilson, 1985). Differences in anchovy diet and growth associated with sea temperature and food availability were previously found in the SW Mediterranean Sea (Bacha and Amara, 2009; Bacha *et al.*, 2010; Bacha and Amara, 2012). Therefore, changes in growth rates and otolith deposition pattern during part of or all of anchovy life may have contributed to the observed variation in otolith shape between the three stocks identified.

Complex spatial structure has been prevalent among marine populations, and our understanding of its nature and consequences needs to continue growing. The results from the present study support the efficiency of otolith shape analysis for stock identification of anchovy, and highlight the role of oceanographic features and physical barriers, such as fronts and straits, in stock separation. They also suggest that environmental conditions, and not only genetic factors, influence the shape of fish otoliths. The Scientific Advisory Committee of the GFCM (General Fisheries Commission for the Mediterranean) has established management areas based on political and statistical considerations rather than biological one (GFCM, 2012). As previously shown by genetic studies (Bembo *et al.*, 1996; Magoulas *et al.*, 2006; Bouchenak-Khelladi *et al.*, 2008;

Sanz *et al.*, 2008; Viñas *et al.*, 2014), population structures observed among Mediterranean and Atlantic populations invalidate current management policies. Indeed, one prerequisite for sustainable management is the matching of biological processes and management action (Reiss *et al.*, 2009). The results from the present study clearly indicate that the SW Mediterranean Sea and neighbouring Atlantic anchovy populations are not homogenous. Disregard of stock structure and ineffective fisheries management can lead to dramatic changes in the biological attributes and productivity rates of anchovy, as well as the genetic diversity of the species. In view of this, and given the considerable commercial value of Mediterranean anchovy fisheries, studies into the effects of oceanographic features on anchovy distribution and their population structure need to be continued. They also need to be taken into account in any future anchovy management strategy.

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