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OTOLITH SHAPE DISCRIMINATION OF *LIZA RAMADA* (ACTINOPTERYGII: MUGILIFORMES: MUGILIDAE) FROM MARINE AND ESTUARINE POPULATIONS IN TUNISIA

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Background. The thinlip grey mullet, *Liza ramada* (Risso, 1810), is widespread in Tunisia. Despite its economic importance, the stock discrimination of this fish is poorly understood. The aim of this study is to investigate, for the first time, the stock discrimination of this species for two Tunisian populations based on the otolith shape, using different statistical approaches.
Materials and methods. The specimens of *L. ramada* were collected during three months (from March to May 2013) at two sampling sites: the marine (Cap Zebib sea resort) and the estuarine (Mellegue Dam) in Tunisia. We analysed sagittal otolith shape variation for 120 individuals (60 fish of each study site comprising 30 males and 30 females) for both sexes (males and females) and two sides (left and right otolith) for each specimen.
Results. Statistical- and discriminant function analysis of the sagittal otolith shape clearly demonstrated statistically significant differences from the two populations. These results were also confirmed by highly statistically significant difference between otolith shape (left and right) for both sexes. An asymmetry was detected when comparing otoliths of the same side (RR–LL) between different sampling sites.
Conclusion. The shape variability of otolith between these two sampling sites is probably correlated with local environmental and ecological factors.

Keywords: sagitta otolith, morphology, asymmetry, fisheries management, Cap Zebib sea resort, Mellegue Dam

INTRODUCTION

The thinlip grey mullet, *Liza ramada* (Risso, 1810), is widely distributed in the eastern part of the Atlantic. The range of this fish stretches from the southern coast of Norway to Morocco, including the Mediterranean and the Black Sea (Jonsson and Jonsson 2008). This euryhaline and pelagic species occurs in different habitats (estuaries, deltas, lagoons, brackish waters, and marine areas of full salinity (Kasımoğlu and Yılmaz 2012). It's noteworthy that this fish tolerates the extreme conditions of salinity and withstands sudden changes in water quality (Papasotiropoulos et al. 2002).

In Tunisia, six species belonging to the family Mugilidae were previously described: *Mugil cephalus* Linnaeus, 1758; *Chelon labrosus* (Risso, 1827); *Liza aurata* (Risso, 1810); *Liza ramada* (Risso, 1810); *Liza saliens* (Risso, 1810); and *Oedalechilus labeo* (Cuvier, 1829) (see Blel et al. 2008). The annual production of Mugilidae in Tunisia is important and has been increasing in recent years (Fehri-Bedoui et al. 2002). Among these species, the thinlip grey mullet (grey mullet thereafter) is one of the target fish species of commercial fishing along the coast of Tunisia (Masmoudi et al. 2001).

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In view of its economic importance, the grey mullet has been investigated in different geographical regions, focusing on its biology, ecology, parasitology, genetics, biochemical composition, heavy metal concentrations, and age determination (Merella and Garippa 2001, Almeida 2003, Yacoub and Abdel Satar 2003, Nirchio et al. 2005, Boglione et al. 2006, Glamuzina et al. 2007, Bahnasawy et al. 2009, El-Ghobashy 2009, Rabeh et al. 2010, El Zaeem 2011, Dakrory et al. 2012). Additionally, age of grey mullet was determined by different methods using scales, otoliths, opercular bones, vertebrae, and dorsal fin rays from the Mersin Bay (Göçer and Ekingen 2005). Also Almeida et al. (1995) used otolith analysis for the purpose of age determination of this species in the Tagus River in Portugal.

The otolith is considered as a true biological and environmental archive with a potential to facilitate reconstruction of environmental parameters (temperature, salinity) and life history traits of fish (age, growth, reproduction, and migration) (Radhakrishan et al. 2009).

Sagittal otoliths have some phenotypic plasticity inter and/or intra-specific and inter-and/or intra-populations (Lombarte and Lleonart 1993, Annabi et al. 2013). This property has been used very often in the discrimination of populations evolved in different environmental conditions (temperature, depth, substrate) taking into account possible confounding variables (sex, size) (Volpedo and Vaz-dos-Santos 2015). Therefore, analysis of the morphology of otoliths is a very powerful tool to identify different fish species (Furlani et al. 2007). This tool helped to distinguish different stocks of fish (Jónsdóttir et al. 2006). The identification of mugilid species is important for local fisheries management (Mehanna 2006). In fact, Reichenbacher and Reichard (2014) reported the first analysis of inter- and intraspecific otolith variation within a genus belonging to the Aplocheiloidei. They analysed five species of the genus *Nothobranchius* Peters, 1868 from Tanzania and southern Mozambique, obtained from wild and wild-derived captive populations.

According to El-Dahhar et al. (2013) grey mullet, being collected in marine, brackish and fresh waters, makes this fish a good model for research. The species that breed only in seawater is currently under extensive rearing in some Tunisian dams from fry collected along the coast (Abdennadher et al. 2003). The stock discrimination of grey mullet species is poorly understood up to date.

The aim of this study was to evaluate by using otolith shape, the stock structure of grey mullet from two ecologically different sites in Tunisia—marine (saltwater) and estuarine (freshwater). The marine site was the Cap Zebib sea resort, while the estuarine site was the Mellegue Dam.

MATERIAL AND METHODS

A total of 120 specimens of grey mullet, *Liza ramada*, were collected between March and May 2013 from two different sampling sites differing is water salinity: the marine (Cap Zebib sea resort) and the estuarine (above the Mellegue Dam) (Fig. 1). The fishing gear was a gillnet of 50 to 70 m in length and 3 to 6 m meshes of height. A total of 60 fish for each station were examined, in equal proportion for each sex (30 males and 30 females). Common names of fishes used in this paper follow FishBase (Froese and Pauly 2016).

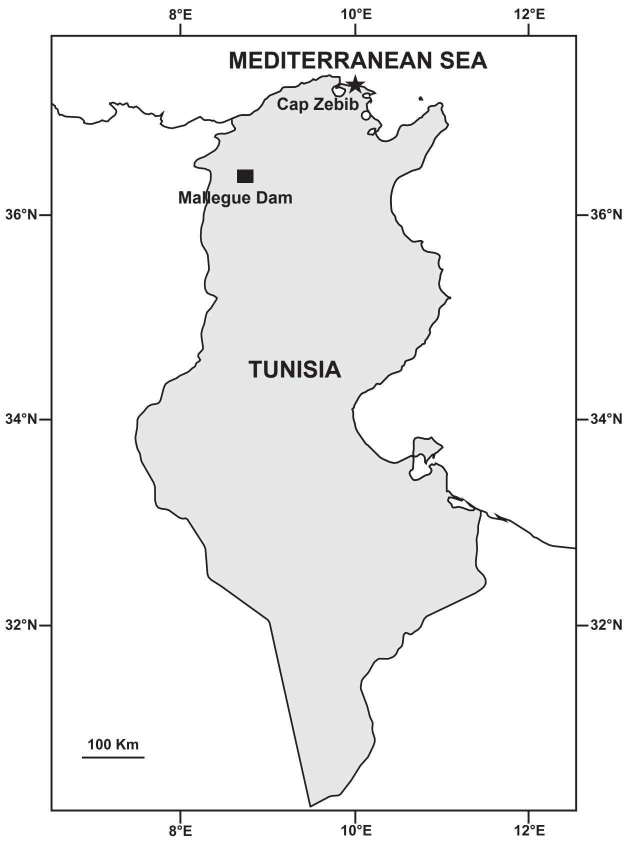

Fig. 1. Sampling sites of thinlip grey mullet, *Liza ramada*, in Tunisia (Cap Zebib and Mellegue Dam)

The Mellegue Dam (36°18′51″N, 8°42′08″E) is located about 7 km west of the city of Nebeur (Kef). The dam height and length are 65 m and 470 m, respectively and the mean annual precipitation in this area is approximately 400 mm. The waters of the river are very muddy, especially in times of flood. The salinity reported by Bahri (1993) was 2.0‰–2.5‰[*]. At this sampling site, grey mullet is abundant between January and May (Ben Mammou and Louati 2006).

Cap Zebib is located on the northern coast of Tunisia (37°15′41.40″N, 10°04′24. 86″E) and characterized by a pristine coastline, low human activity, and strong water currents (Belgacem et al. 2013). The salinity is fairly constant (36.5‰–37‰). This sea is a spawning ground for a number of teleost fishes (Turki and Ktari-Chakroun 1985).

We used a fish measuring board to measure the total length (TL) and standard length (SL) and a precision balance (Sartorius) to determine the total weight (TW) of each fish specimen (see Table 1).

[*] In the wake of the growing criticism of the Practical Salinity Scale concept (and especially PSU as a "unit"), Acta Ichthyologica et Piscatoria is in favour of expressing salinity in parts per thousand (‰), regardless if a direct or indirect method was employed to determine the water salinity.

Table 1

Length and weight of specimens of thinlip grey mullet, *Liza ramada*, from Tunisia

Sampling site and sex	*n*	Standard length [mm]		Total weight [g]	
		Mean ± SD	Range	Mean ± SD	Range
Cap Zebib M	30	194.73 ± 12.47	175–231	145.54 ± 29.52	110.02–250.32
Cap Zebib F	30	194.83 ± 10.00	180–228	142.29 ± 21.61	107.53–217.87
Mellegue Dam M	30	232.93 ± 21.67	190–274	221.47 ± 57.87	125.06–331.73
Mellegue Dam F	30	250.63 ± 19.75	204–280	283.12 ± 64.03	163.74–407.52

n = number of fish SD = standard deviation, M = males, F = females.

The otolith extraction protocol was proposed by Panfili et al. (2003). The otoliths were photographed by a digital camera (Samsung PL210 with the resolution of 14.2 megapixels and optical zoom 10×) under a microscope. The image analysis on a computer screen allowed us to evaluate each image and store it in a database. The photographs of all otoliths were processed by Adobe Photoshop CS6 (Fig. 2). Subsequently, the images were processed by the Shape software version 1.3 which created 20 harmonies for each otolith (each otolith represents an individual). Each harmony was composed of four coefficients, the so called Fourier coefficients (*A*, *B*, *C*, and *D*) corresponding to the values of the projection of the binary image on the axes *X* and *Y* (Kuhl and Giardina 1982), resulting in 80 coefficients per individual.

The morphological variation in individuals was based on statistical analysis of the parameters from the developments in Fourier series. Elliptic Fourier analysis (EFA) was used to perform mathematical analyses of the otolith shape. This technique describes the silhouette called harmonic. Each harmonic is characterized by four Fourier coefficients (*A*, *B*, *C*, and *D*) which calculates the Fourier power (FP), the percentage of Fourier power (FP%), and the cumulative percentage of the Fourier power (FP_n% cumulative). The respective formulas are provided below (Crampton 1995, Trojette et al. 2015):

$$FP = (A_n^2 + B_n^2 + C_n^2 + D_n^2)^{-2}$$

$$FP\% = 100FP_n\,(\textstyle\sum_1^n FP_n)^{-1}$$

$$FP_n\%_c = \textstyle\sum_1^n FP_n\%$$

The statistical analysis of the otolith shape was performed using EFA describing the shape of the otolith. To have the most accurate otolith shape, the percentage of Fourier power was calculated to determine the number of necessary and sufficient harmonics. Therefore, this number was fixed at 20 and a total of 180 replicates were obtained (60 observations for each study site).

Multivariate analysis (Wilks's Lambda test) was performed for the treatment of all otolith synchronous parameters.

To assess the differences between our different batches of otoliths, we performed multivariate analysis that enables the processing of all the otolith parameters at the same time. In addition, discriminant function analysis (DFA) was performed on shapes indices in order to illustrate the differences and similarities between the observed groups and optimize the existing variability. For this analysis, the factorial graphic designs allow visualizing individuals and variables. Various indicators and tests were also used in order to estimate the reliability of our results. All statistical analyses were performed using XLSTAT (2010) software.

RESULTS

The Wilks's Lambda test (Rao approximation) revealed the presence of statistically significant differences between the two sampling sites ($P < 0.\ 0001$) (Table 2). The matrix of Mahalanobis distances between the left and right otolith for the populations of Cap Zebib and Mellegue Dam as well as between the left and right otolith of both populations is given in Table 3.

Fig. 2. Otolith images of thinlip grey mullet, *Liza ramada*, from Tunisia, processed using Photoshop software; **A** = Cap Zebib, **B** = Mellegue Dam

Table 2

Wilks's Lambda test of the distance approximation between populations of the thinlip grey mullet, *Liza ramada*, from Tunisia (Rao approximation)

Parameter	Value
Lambda	0.011
F (Observed value)	1.874
F (Critical value)	1.128
DDL1	539
DDL2	11 022
P-value	< 0.0001
Alpha	0.05

The respective distances between the female and male otoliths (left–right) for Cap Zebib were: 12.205 and 11.570, and those for Mellegue Dam were 16.433 and 11.279. This result showed that the Mahalanobis distance between the individuals representing the two populations was similar except for the females of Mellegue Dam. Moreover, Mahalanobis distance between the otoliths of the two populations: Mellegue Dam–right–female and Cap Zebib–right–female was 22.415 and between Mellegue Dam–right–male and Cap Zebib–right–male was 22.152. In addition, this distance between Mellegue Dam–left–female and Cap Zebib–Left–female was 14.084 and between Mellegue Dam–left–male and Cap Zebib–left–male) was 14.873, respectively (Table 3). The values quoted for the distances between the two populations were high and clearly differentiated them.

The Mahalanobis and Fisher distances provided the same results. In the same population, the respective Fisher distance values between the female and male otoliths (L–R) for Cap Zebib were 0.830 and 0.858, while for Mellegue Dam they amounted to 1.844 and 1.009. The highest distances were revealed for the females of Mellegue Dam, while the lowest were recorded for females of Cap Zebib and for males and females of Mellegue Dam. Those between the otoliths of the two populations: Mellegue Dam–right–female, Cap Zebib–right–female and Mellegue Dam–right–male, Cap Zebib–right–male were: 2.936 and 2.902, respectively. Thus the distance between Mellegue Dam–left–female, Cap Zebib–left–female and Mellegue Dam–left–male, Cap Zebib–left–male were 1.845 and 1.948, respectively (Table 4). The values provided for the distances between the two populations (right–right/left–left) were close to each other and showed clear difference between them.

In the same population, *P*-value of Fisher distances (left–right) was highly statistically significant ($P < 0.0001$) for the females originating in Mellegue Dam only (case of left–right asymmetry). The *P* values were also significant ($P < 0.05$) for the females of Cap Zebib ($P = 0.007$), the males ($P = 0.015$), and the males of Mellegue

Table 3

Pairwise of Mahalanobis distances between side and sex of otolith of thinlip grey mullet, *Liza ramada*, from Tunisia

	BFD	BFG	BMD	BMG	MFD	MFG	MMD	MMG
BFD	0	16.433	14.078	16.074	22.415	25.215	25.942	34.737
BFG		0	13.647	7.706	24.248	14.084	27.341	19.577
BMD			0	11.279	19.482	19.586	22.152	22.938
BMG				0	20.965	12.435	22.810	14.873
MFD					0	12.205	6.334	15.047
MFG						0	11.390	6.548
MMD							0	11.570
MMG								0

BFD = dam female right, BFG = dam female left, BMD = dam male right, BMG = dam male left, MFD = sea female right, MFG = sea female left, MMD = sea male right, MMG = sea male left.

Table 4

Fisher's distances between two sides and sexes of thinlip grey mullet, *Liza ramada*, specimens from Tunisia

	BFD	BFG	BMD	BMG	MFD	MFG	MMD	MMG
BFD	0	2.153	1.844	2.105	2.936	3.303	3.398	4.550
BFG		0	1.788	1.009	3.176	1.845	3.581	2.564
BMD			0	1.477	2.552	2.566	2.902	3.005
BMG				0	2.746	1.629	2.988	1.948
MFD					0	1.599	0.830	1.971
MFG						0	1.492	0.858
MMD							0	1.516
MMG								0

BFD = dam female right, BFG = dam female left, BMD = dam male right, BMG = dam male left, MFD = sea female right, MFG = sea female left, MMD = sea male right, MMG = sea male left.

Dam ($P = 0.021$) (case of left–right asymmetry) (see Table 5). Moreover, the sexual dimorphism was recorded only in right side in Mellegue Dam population (for females and males right, $P = 0.001$). However, no differences were detected between males and females of Cap Zebib population: for females and males left, $P = 0.773$; for females and males right, $P = 0.819$.

The results of the comparison between both samples of fish (Cap Zebib and Mellegue Dam) revealed a clear difference between the otoliths (left–right) of males and females ($P < 0.05$). The asymmetry (left–right) of otoliths revealed in this study indicates that the two populations of fish (Cap Zebib and Mellegue Dam) have different morphology of otoliths and belongs to different fish stocks.

The discriminant function analysis showed the projection of individuals on the two first axes (F1 and F2) (Fig. 3). In fact, these two discriminant axes explained 43.820% and 25.640% of total variation, respectively, and accounted for 69.460% of the total variance (Table 6).

The otoliths (R–L of each pair) of specimens from Cap Zebib were separated by F1 (male) and F2 (females) axis, while the individuals of Mellegue Dam were differentiated by the F2 (males) and F5 (females). This finding was consistent with the results revealed by Fisher- and Mahalanobis distance testes and confirms the symmetry (R–L) detected for the two sexes of the sampling sites except the males of Cap Zebib which were represented an asymmetry. The otoliths (right–right) and (left–left) for Cap Zebib and Mellegue Dam are separated by the axis F1. This result showed the asymmetry between the otoliths of the two populations, which present different morphologies.

DISCUSSION

The otolith shape variability was analysed for grey mullet in two stations: Cap Zebib Sea Resort and Mellegue Dam. In the presently reported study, the elliptic Fourier analysis based on otolith shape, revealed

Table 5

P-value for Fisher distances of thinlip grey mullet, *Liza ramada*, specimens from Tunisia

	BFD	BFG	BMD	BMG	MFD	MFG	MMD	MMG
BFD	1	< 0.0001	0.001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
BFG		1	0.001	0.472	< 0.0001	0.001	< 0.0001	< 0.0001
BMD			1	0.021	< 0.0001	< 0.0001	< 0.0001	< 0.0001
BMG				1	< 0.0001	0.005	< 0.0001	0.000
MFD					1	0.007	0.819	0.000
MFG						1	0.018	0.773
MMD							1	0.015
MMG								1

BFD = dam female right, BFG = dam female left, BMD = dam male right, BMG = dam male left, MFD = sea female right, MFG = sea female left, MMD = sea male right, MMG = sea male left.

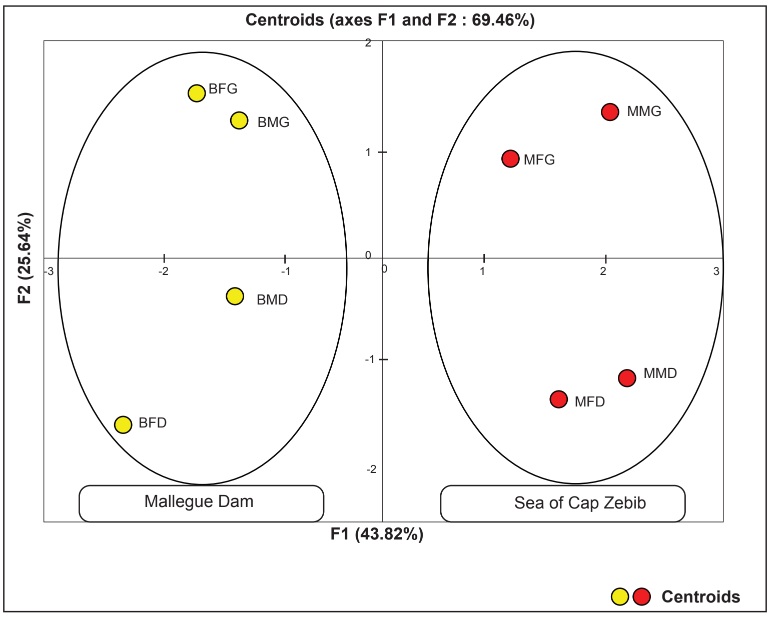

Fig. 3. Discriminant function analysis for two sexes and two sides of otoliths of thinlip grey mullet, *Liza ramada*, from Tunisia; Abbreviations: BFD = dam female right, BFG = dam female left, BMD = dam male right, BMG = dam male left, MFD = sea female right, MFG = sea female left, MMD = sea male right, MMG = sea male left

Table 6
Eigen values of the discrimination rate for the populations of thinlip grey mullet, *Liza ramada*, from Tunisia

	Eigen value	Discrimination [%]	% cumuli
F1	3.478	43.816	43.816
F2	2.036	25.642	69.459
F3	0.874	11.006	80.464
F4	0.523	6.583	87.047
F5	0.420	5.294	92.341
F6	0.355	4.471	96.813
F7	0.253	3.187	100.000

the presence of two well-differentiated and distinct populations (marine and estuarine).

The discriminant function analysis showed significant differences in otolith shape between marine and estuarine populations, and significant differences in the same environment. The morphological analysis also showed a clear asymmetry between the left and right otoliths in females and males of the Mellegue Dam and Cap Zebib

The discriminant function analysis confirmed this result and revealed the presence of eight groups (BFD = dam female right, BFG = dam female left, BMD = dam male right, BMG = dam male left, MFD = sea female right, MFG = sea female left, MMD = sea male right, MMG = sea male left) with highly significant distances.

Otoliths are natural data loggers that record information at different temporal scales related to their environment (Neves et al. 2015). Hence, the differences in the morphology of otolith shape between the two populations, demonstrated in this study, were probably related to the properties of the dam reservoir environment such as food and reproductive strategies that are different from those in the marine environment (Limburg et al. 2015).

In this case, the same result was reported by Rebaya et al. (2016), which revealed the presence of two different populations of the grey mullet between two lagoon stations located in the North of Tunisia (Ghar El Melh Lagoon and Bizerte Lagoon). In addition to that, an asymmetry was detected between the otoliths of the same sex from both stations.

In this context, Avigliano et al. (2015) evaluated the potential use of otolith microchemistry (Sr ÷ Ca and Ba ÷ Ca ratios) to identify the silver mullet, *Mugil curema* Valenciennes, 1836, populations in the south-eastern Caribbean Sea. In fact, the authors mentioned above, observed the existence of different groups of in this geographic area.

However, the presence of two differentiated populations of grey mullet, in the presently reported study, is probably related to differences in physicochemical parameters of the two stations. In this way, by comparing habitat from two different sampling sites, we can assume that the distribution of grey mullet was strongly affected by the salinity. It is well documented that grey mullet well tolerates different salinities, and is capable of maintaining a stable internal osmolality in a wide range of external salinity levels, including fresh water and estuarine waters (Cardona 2006). In this case, grey mullet, is a species found over a wide range of salinity levels (Lafaille et al. 2002). We postulate herewith that the temperature and the turbidity were the parameters that primarily affected the distribution of grey mullet in Mellegue Dam and Cap Zebib sampling sites with relevant role for the salinity. Also Chang et al. (2004) reported that the otolith elemental composition of flathead grey mullet, *Mugil cephalus*, can be affected by the salinity. Campana (1999) claimed that the temperature, the major life history transitions, the somatic growth rate, the rate of crystal formation, and the water chemistry might affect the otolith variation in heterogeneous environment.

According to Fortunato et al. (2014), a clear relation between otolith size and communication strategies were described for eight mugilid species originating from the north-eastern Atlantic and the Mediterranean Sea. In the same way, the morphology of sagittal otoliths represents an adequate tool to identify the species belonging to the family Mugilidae in various geographical areas. According to the literature, the difference in the length–weight relations of grey mullet can be explained by the numerous biotic and abiotic factors such as adequacy of the food, density of the population, and peculiarity of physical and chemical parameters of the water (Kalay et al. 2008).

In this case, the otolith morphological difference between the Cap Zebib Sea Resort and the Mellegue Dam stocks may be due to environmental differences that correspond with marine and estuarine habitats, respectively. Besides, the otolith shape differences in a same stock (left–right otoliths) in grey mullet can be attributed to environmental influences (temperature, movements of water masses, depth) (Midway et al. 2014) without excluding the genetic mutations (Trojette et al. 2014).

In this study, analysis of otolith shape revealed the presence of two differentiated populations of grey mullet from marine and estuarine environments. The comparison of the otolith morphology between the two populations showed a clear difference in shape (a left–right asymmetry) of otoliths. Based on the presently reported results, it seems likely that the differences in otolith shape of grey mullet, could be explained by environmental and physicochemical factors (temperature, salinity, water composition, etc.). This work contributes to the knowledge on the stock discrimination for grey mullet and provides useful information for studying fisheries management of this species in Tunisia.

In the future, otolith biochemical analysis will be an adequate tool for better understanding the environment physiochemical parameters affecting the otolith morphology of the two populations. Genetic investigations including mitochondrial DNA such D-loop region will also be necessary to more understand our results.

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