Supplementary Material on the Methodology Part XI

Microchemistry analysis of blackspot seabream otoliths

The microchemistry of the core and the edge of blackspot seabream otoliths was analysed using Laser ablation ICPMS. The samples were collected from the Atlantic and Mediterranean. This analysis was realized in order to define the stock structure of *Pagellus bogaraveo*, following habitat use and to assess the plausible difference between individuals' spawning/nursery areas.

Data analysis

The element concentration in otoliths core and edge were determined by LA-ICPMS. Four chemical elements were measured for each otolith. Values of elements that were consistently measurable above the LOD were retained for the statistical analysis.

For the core and the edge otolith element composition, we run the same series of analyses; Firstly, we conducted a MANOVA and an univariate analysis of variance (ANOVA) to assess whether individual element:Ca ratios differed significantly among areas. Post-hoc Fisher LSD tests were used to determine the nature of significant differences among areas.

A discriminant analysis was built for core and edge data to classify each individual to one of the sites from which they were collected. Classification accuracies for each species and environment were evaluated through the percentage of correctly classified individuals using jackknifed classification.

Results

Laser ablation of the otolith blackspot seabream displayed different levels of Sr/Ca, Ba/ca ratios (higher in the edge), Mn/Ca (higher in the core) and Mg/Ca between the core and the edge of otoliths sampled from the different areas. the main pattern of element concentration was different between core end edge

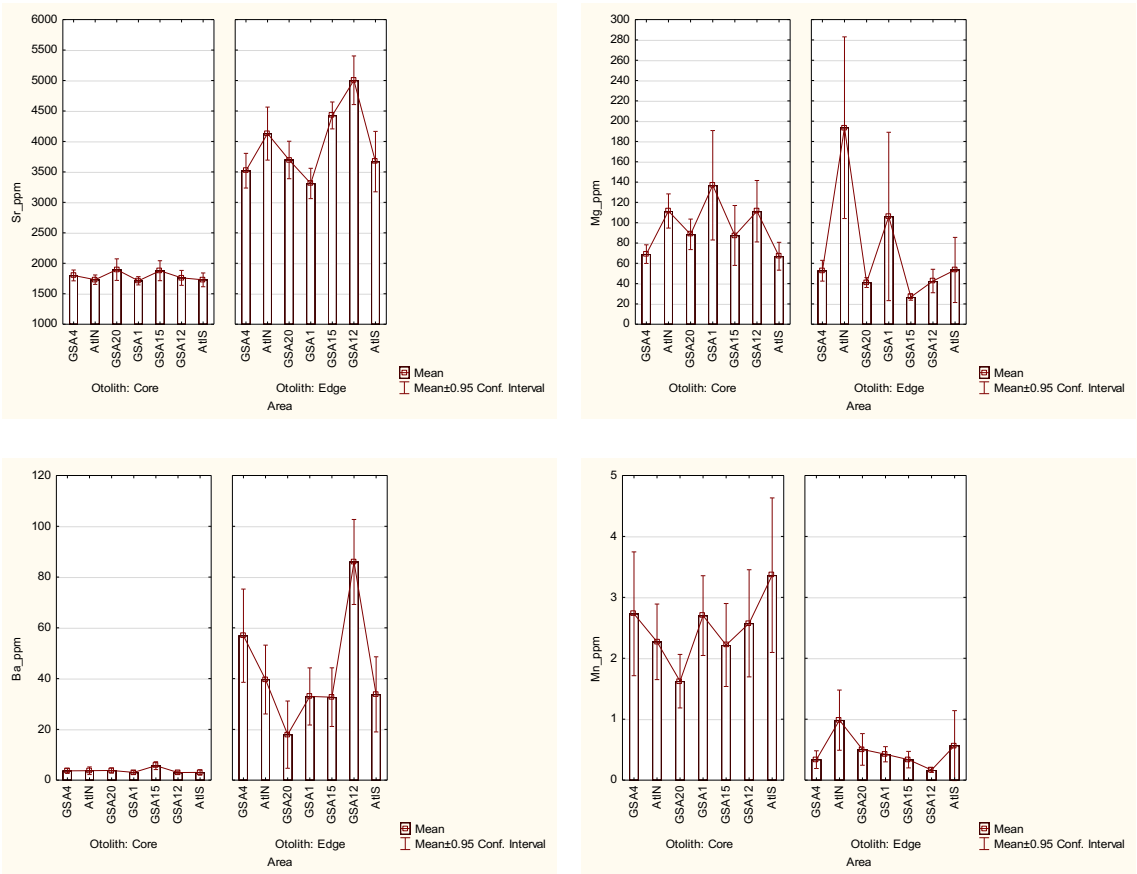

Figure 1. Comparison of individual element:Ca ratio in otolith core and edge of blackspot seabream collected from GSAs 1, 4, 12, 15 and 20 and AtlN (Atlantic North) and AtlS (Atlantic South) for the following elements Sr(a), Mg(b), Ba (c) and Mn(d)

Analysis of blackspot seabream otolith core microchemistry

MANOVA of ratio Mg:Ca, Sr:Ca, Ba:Ca and Mn:Ca was highly significant ($F=2.487$, $p<0.000$**). Microchemistry analysis of the otolith core of blackspot seabream was found to be effective in detecting differences in elemental concentration across the different areas using ANOVA (Table 1). Univariate ANOVA indicated significant differences in Mg:Ca ratio of otolith cores among the sampling areas ($p<0.05$) with the highest Mg:Ca ratio in the core observed in GSA 1. Post-hoc Fisher LSD test showed no difference in Mg:Ca between GSAs 1, 15 and 20. For manganese and strontium in the core, no-significant differences were observed between the different areas in the Mediterranean and in Atlantic. The Ba:Ca ratios, were the highest in GSA 15. The lowest concentration was observed in GSA 1. Post-hoc Fisher LSD test showed no difference in Ba:Ca between GSAs 15 and 20 (Table 1 and Figure 1).

Table 1. Results of univariate ANOVA and Post-hoc Fisher LSD tests comparing individual element ratio in otolith cores of blackspot seabream collected from GSAs 1, 4, 12, 15 and 20 and AtlN, AtlS.

Source	df	F	P	Post-hoc Fisher LSD test differences by GSA
Mg:Ca Area	6	3.945	0.001**	GSA1> GSA4*, AtlN>GSA4*, GSA20> GSA4* GSA12>GSA4* GSA15<AtlN* AtlS<AtlN* GSA12> GSA15*AtlS<GSA12*
Mn:Ca Area	6	1.78	0.1	
Sr:Ca Area	6	1.87	0.09	
Ba:Ca Area	6	3.26	0.005**	GSA15>GSA4*, GSA15>AtlN*, GSA20>GSA1*, GSA15>GSA1*, GSA15>GSA12*, GSA15>AtlS*

The discriminant analysis plot indicates a significant overlap especially among samples from GSAs 1 and 12 and AtlN and to a lesser degree with GSA 4 and AtlS. However, the samples from GSAs 15 and 20 were roughly separated from the other areas along the canonical variate 1 due to the trend for higher Ba:Ca and Sr:Ca in the otolith core for samples from GSAs 15 and 20 (Figure 2).

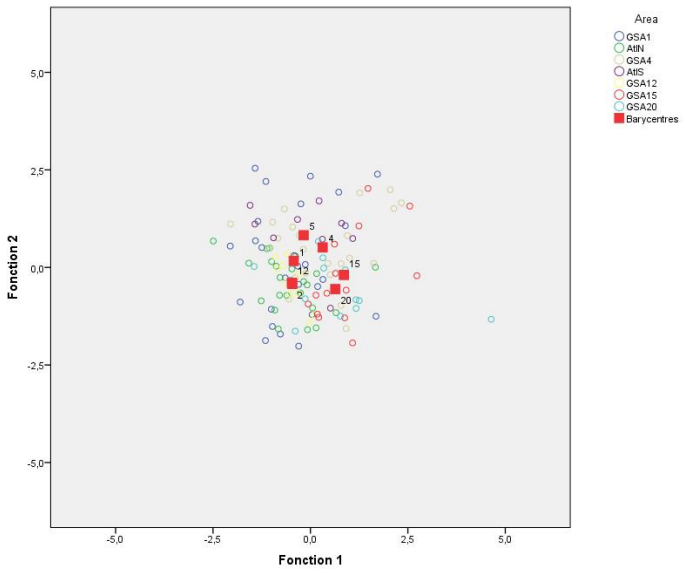

Figure 2. Scatter plot of scores obtained by DA of multi-element chemistry of otolith cores of blackspot seabream collected from GSAs 1, 4, 12, 15 and 20 and AtlN and AtlS.

The jacknifed classification involving all the areas was very poor in assigning individuals to their area of origin. The relatively highest classification accuracy was observed for GSA 20 at 30.8% followed by GSA 15 at 26.7%. Classification error for GSA 20 individuals were mostly related to individuals being misclassified in GSA 4, and for GSA 15 classification errors were mostly due to misclassifications in GSA 20 (Table 2).

Table 2. Results of jackknife classification of individuals based on multi-element chemistry of otolith cores

	Area classified to (% sample)						
Jacknife classification	GSA1	AtlN	GSA4	AtlS	GSA12	GSA15	GSA20
GSA1	**20**	20	8	24	12	8	8
AtlN	37.5	**16.7**	0	0	29.2	17.4	17.4
GSA 4	13	13	**13**	26.1	0	17.4	17.4
AtlS	30	0	20	**20**	10	10	10
GSA12	20	40	0	0	**20**	0	20
GSA15	0	6.7	13.3	13.3	13.3	**26.7**	26.7
GSA20	7.7	15.4	30.8	0	**7.7**	**7.7**	**30.8**

Analysis of blackspot seabream Otolith edge microchemistry

Otolith multi-element fingerprints of the edge in sampled areas showed high significant differences with MANOVA ($F = 7.946.57$, $p < 0.001$). Analysis of the otolith edge of blackspot seabream was also found to be effective in detecting differences inelemental concentration across the different areas sampled (ANOVA, Table 2). While the main pattern observed were partially different to those in the edge.

For magnesium, the Highest Mg:Ca ratios were observed in AtlN followed by GSA 1, and the lowest concentration was observed in GSA 15. Post-hoc Fisher LSD test showed no differences in concentration of Mg between GSAs 12 and 15. For manganese, Mn:Ca ratio was higher in AtlN, and the lowest values were observed in GSAs 12 and 15.

Sr:Ca ratio in the blackspot seabream otolith edge was the highest in GSA 12 followed by GSA 15 and the lowest in GSA 1. The post-hoc Fisher LSD test showed no differences in concentration of Sr:Ca ratio between AtlN and AtlS. For barium, the highest Ba:Ca

concentration was found in GSA 12 followed by GSA 4 and AtlN and the lowest concentrations were observed in GSAs 20, 15 and 1 (Table 3 and Figure 1).

Table 3. Results of univariate ANOVA and Post-hoc Fisher LSD tests comparing individual element ratioa in otolith edges of blackspot seabream collected from GSAs 1, 4, 12, 15 and 20 and AtlN and AtlS

Source	df	F	P	Post-hoc Fisher LSD test differences by GSA
Mg:Ca Area	6	12.99	0.00000**	AtlN>GSA4*,AtlN>GSA20*, AtlN>GSA1*, AtlN>GSA15*, AtlN>GSA12*, AtlS<AtlN* GSA4>GSA15* GSA1>GSA20*,GSA20>GSA15* GSA1>GSA15*, GSA1>GSA12*, GSA15<AtlS*
Mn:Ca Area	6	4.33	0.0080**	AtlN>GSA4*,AtlN>GSA20*, AtlN>GSA1*, AtlN>GSA15*, AtlN>GSA12*,
Sr:Ca Area	6	7.89	0.000001**	GSA12>GSA4*,GSA15>GSA4*, AtlN>GSA4*, GSA12>AtlN*,AtlN>GSA1*,GSA20>GSA1*,GSA12>GSA20*,GSA15>GSA20*, GSA12>GSA1*, GSA15>GSA1*,GSA1<AtlS*, GSA15>AtlS*, GSA12>AtlS*
Ba:Ca Area	6	7.44	0.000003**	GSA4>GSA15*, GSA4>AtlN*, GSA4>GSA20*, GSA4>GSA1*, GSA12>AtlN*, GSA15>GSA20*, GSA12>GSA20*,AtlS* >GSA20 GSA12>GSA1*, GSA12>GSA15*, GSA12>AtlS*

The canonical variate plots of the otolith edge chemistry indicated weak clusters of the samples from GSAs 1 and 4 and AtlN and AtlS compared to those from GSAs 12, 15 and 20 along the canonical variate 1 (Figure 3).

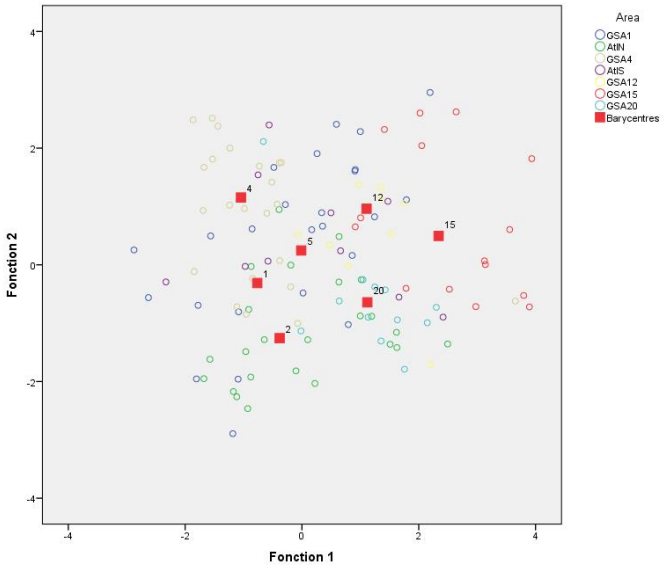

Figure 3. Scatter plot of scores obtained by DA of multi-element chemistry of otolith edges of blackspot seabream collected from GSAs 1, 4, 12, 15 and 20 and AtlN and AtlS.

The jacknifed classification procedure with all areas included was moderately accurate in assigning individuals to their collection area based on Mg:Ca, Sr:Ca, Ba:Ca and Mn:Ca ratios of their otolith edge. Classification accuracy was highest for the GSA 12 samples followed by GSAs 15 and 20. The lowest classification was observed in AtlS and GSA 1. Classification error in GSA 12 was due to misclassification especially in AtlN, and classification error in GSA 20 was due to misclassification of individuals to GSAs 15, 4 and 1.

Table 4. Results of jackknife classification of individuals based on multi-element chemistry of otolith edges.

	Area classified to (% sample)						
Jacknife classification	GSA1	AtlN	GSA4	AtlS	GSA12	GSA15	GSA20
GSA1	**20**	8	20	12	12	12	16
AtlN	12.5	**37.5**	0	0	41.7	0	8.3
GSA 4	8.7	17.4	**65.2**	4.3	0	4.3	8.3
AtlS	20	0	30	**10**	10	30	0
GSA12	0	11.1	0	0	**88.9**	0	0
GSA15	0	0	0	13.3	6.7	**73.3**	6.7
GSA20	8.3	0	8.3	0	0	8.3	**75**

Discussion and conclusions

Pagellus bogaraveo is an important commercial demersal species in the Mediterranean Sea and in the Atlantic. As juveniles, they live mainly in shallow and coastal zones, pre-adults in intermediate zones, and adults in deeper and offshore zones to 400 meters in the Mediterranean but down to 700-800 meters in the Atlantic (Fischer *et al*. 1987; Mytilineou *et al*., 2013; Froese and Pauly, 2019). Little is known on the stock structure of blackspot seabream in the Mediterranean and Atlantic areas. Some genetic studies on population structure of this species had shown low level of genetic differentiation (Bargelloni *et al*., 2003; Stockley *et al*., 2003), except for the Azores areas (Pinera *et al*., 2007). However, morphological and parasitological studies indicated some degree of dissimilarity in Mediterranean and Atlantic stocks (Palma and Andrade, 2004; Hermida *et al*., 2013). Multivariate comparison of chemical element concentrations between core and edge zones of the otolith was applied to discriminate stocks between the different fishing areas.

Laser ablation ICPMS is used to analyse the elemental composition of different parts of the otolith. The core represents the first weeks of age when the eggs and larvae of the blackspot seabream were pelagic, and the edge represents the signal in recently deposited material. The multi-element signals may reflect populations that live and grow in a discrete area or follow set migration routes also can characterize the nursery areas of the species, clarify the connectivity between nursery and recruitment and therefore could be useful for discriminating between stocks.

The four elements Ba:Ca, Mg:Ca, Mn:Ca and Sr:Ca demonstrated variation among the different fishing areas.

For Ba, there is a great evidence across multiple species that incorporation into otolith is driven principally by ambient concentration (Bath *et al*., 2000; Hamer *et al*., 2003, 2006). Differences in otolith Ba can therefore be assumed to be indicative of variation in the ambient Ba level that the fish was exposed to. This element is often associated with high primary productivity and exhibits a nutrient-type profile, with surface depletion and enrichment at depth and potential to be enriched in inshore coastal waters and marine dominated bays and estuaries

So the different level of otolith Ba between the core (low value) and the edge (high value) among the different areas could be related to shifts in the spatial and or depth distribution between larvae (shallow waters) and adult stage (deeper waters). Among the areas, the levels of Ba in the otolith core were the highest in GSAs 15 and 20.

Otolith Mg is not affected by either temperature and/or salinity but is correlated with otolith precipitation and somatic growth rate (Artetxe-Arrate *et al*., 2019; Bath, Martin and Thorrold, 2005). The patterns of otolith Mg variation among areas were relatively different between core and edge however the highest level of Mg was observed in GSA 1 and AtlN both in the core and edge.

Mn incorporation into the otolith is physiologically regulated and sensitive to growth effects. According to Ruttenberg (2006), high level of Mn near the otolith primordium were related to maternal transfer. The level of otolith Mn in the core was relatively equivalent among GSAs 15 and 20 and AtlN. the different level of otolith Mn between the core (high value) and the edge (low value) among the different areas could be related to shifts in the spatial and or depth distribution between larvae (shallow waters) and adult stage (deeper waters).

Sr incorporation into otoliths has been shown to be correlated with ambient concentration, and thus appear to be reliable 'geographical marker' (Zimmerman, 2005). The concentrations of Sr reflect salinity changes.The different level of otolith Sr between the core and the edge among the different GSAs characterized by high level of Sr in the edge compared to the otolith core could be related to a shift in a spatial and or depth distribution between larvae and adult life stage that inhabiting different salinity and temperature regimes indeed this species migrates to deeper waters as adult. The level of Sr in the otolith edge differed significantly among the areas. The highest concentration of Sr was observed in GSA 12 and 15. In the core there is no significant differences in the Sr;Ca ratio between the different areas however the concentration of Sr in GSA 15 and GSA 20 are the most comparable in Sr concentration.

Although, analysis of MANOVA and ANOVA among areas showed in a statistical sense that blackspot seabream from the different areas would be from different stocks, discriminant analysis showed that there is a considerable overlapping between individual from the different areas.

The discriminant analysis plot showed a significant overlapping among the different areas. Especially between samples from GSAs 1 and 12 and AtlN and to a lesser degree with GSA 4 and AtlS. However, samples from GSAs 15 and 20 tend to be separated from the other areas. This overlapping was behind the high level of misclassification of blackspot seabream to their area of origin. Two possible causes of misclassifications which are: (1) similarity of the environmental condition influencing otolith chemistry and or (2) mixing of the individual between areas. The otolith edge chemistry indicated tighter clustering of the samples from

GSAs 12, 20 and 15 with the highest classification accuracy of 88.9%, 75% and 73.3%, resepectively, more than GSA 1, AtlN, GSA 4 and AtlS, which overlapped between each other and seemed to be relatively closer. The relatively separation between samples from GSAs 12, 20 and 15 and the other areas suggests that there is limited or no mixing of the adult blackspot seabream from these areas with the other population.

Overall, the results of the core otolith chemistry were not sufficiently different for consistent discrimination between blackspot seabream stocks from the different sampling sites as well as from Atlantic or Mediterranean. This could be related to a common origin of the blackspot seabream or due to limited variability of the chemical composition of their ambient environment owing to the interchange of water between Atlantic and Mediterranean. On the other hand, the composition of the otolith margin showed some separation in adult blackspot seabream mainly for GSAs 12, 15, and 20 from the other areas. This separation assumes that these blackspot seabream remained separated for the recent period of time over which the otolith edge material was precipitated.

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