Received: 1 March 2020 | Revised: 10 January 2021 | Accepted: 12 January 2021
DOI: 10.1111/jai.14174

SHORT COMMUNICATION

Journal of Applied Ichthyology DWK WILEY

Regional variation in the otolith chemistry of age-0 atlantic bluefin tuna from nurseries in the mediterranean sea

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Funding information
European Union; Ministry of Economy and Competitiveness (MINECO), Grant/Award Number: CTM2011–27505

Keywords: Atlantic bluefin tuna, Mediterranean Sea, otolith, stock structure

1 | INTRODUCTION

Atlantic bluefin tuna (*Thunnus thynnus*) is an oceanic predator characterized by highly complex migratory behavior and trans-oceanic movement (Block et al., 2001, 2005; Rooker et al., 2007). Several lines of evidence support the existence of two separate regions of spawning or production: Gulf of Mexico (western stock) and Mediterranean Sea (eastern stock) (Block et al., 2005; Stokesbury et al., 2004; Teo et al., 2007). This species represents an important fishing resource both in the Atlantic Ocean and Mediterranean Sea, and its exploitation during several centuries has led, in the last decades, to severe declines in both western and eastern populations (Longo & Brett, 2012). Even though stocks show signs of increasing in recent years, as reported by the International Commission for the Conservation of Atlantic Tunas (ICCAT Report 2018–2019), more information on the structure and dynamics of both populations is required in order to develop an efficient management system of this resource. The ICCAT currently manages *T. thynnus* as two separate stocks even though some degree of mixing is known to occur (Block et al., 2001, 2005; Quílez-Badia et al., 2016; Rooker et al., 2014; Rooker, Secor, De Metrio, Kaufman, et al., 2008; Rooker, Secor, De Metrio, Schloesser, et al., 2008), which has caused some to question the two-stock management approach (Goldstein et al., 2007).

In the Mediterranean Sea, principal spawning areas of *T. thynnus* occur in western (Balearic Islands), central (Tyrrhenian Sea), and eastern (Levantine Sea) regions of the basin (Karakulak et al., 2004; Oray & Karakulak, 2005), albeit other spawning sites may also exist (Arrizabalaga et al., 2018; Rooker et al., 2007). A significant fraction of the *T. thynnus* produced in the Mediterranean Sea is known to migrate through the Strait of Gibraltar as juveniles, supporting an important fishery in the Bay of Biscay and other locations in the eastern Atlantic Ocean (Aranda et al., 2013; Fraile et al., 2015). To date, the origin of recruits in this region is largely unknown. Our current understanding of the relative importance of the three spawning areas to fisheries, both within and outside the Mediterranean Sea, is critical to the management of the eastern stock. Moreover, some recent findings, based on thousands of genome-wide single-nucleotide polymorphism (SNP) markers, have shown that Mediterranean samples are genetically indistinguishable (Rodríguez-Ezpeleta et al., 2019) in contrast to previous results based on few molecular markers (Boustany et al., 2008; Riccioni et al., 2010). Therefore, this found underline the importance of additional tools to account for the contribution of each spawning area to the overall population.

The chemical composition of calcified structures such as otoliths (ear stones) represents a promising alternative to tagging or genetics studies, particularly for determining the natal origin and movement of *T. thynnus* (Fraile et al., 2015; Rooker et al., 2014, 2019;

Rooker & Secor, 2019). It is generally accepted that resorption or remobilization of elements in the otoliths during ontogeny is minimal (Thresher, 1999) and the accreted material reflects the physicochemical conditions of the seawater inhabited by the individual (Elsdon & Gillanders, 2003).

Therefore, material deposited during the *early life* period can provide information about physicochemical conditions of the nursery area or place of origin (Rooker & Secor, 2019; Rooker, Secor, De Metrio, Kaufman, et al., 2008). Here, we assess the utility of trace elements in the otoliths of age-0 T. *thynnus* from different nurseries in the Mediterranean Sea. This work builds on earlier assessments of trace element chemistry (Rooker et al., 2003) and further evaluates the promise of using trace elements to complement stable carbon and oxygen isotopes for research on the origin and mixing of *T. thynnus* (Rooker & Secor, 2019).

2 | MATERIALS AND METHODS

Sixty age-0 T. *thynnus* (20–46 cm fork length) were collected from August to October 2012 from the primary nursery areas (20 individuals per nursery) in three regions of the Mediterranean Sea: western (Balearic Sea), central (Tyrrhenian Sea) and eastern (Levantine Sea). All the three regions were sampled independently by commercial long-line fishermen. Age-0 T. *thynnus* were collected under the provision of the ICCAT Atlantic Wide Research Program for Bluefin Tuna (GBYP) (Figure 1).

Sagittal otoliths were extracted from each *T. thynnus*. A single otolith (i.e. right or left sagittae) was used for the analysis based on random assignment. Otoliths were then cleaned of adhering tissues, soaked in 3% hydrogen peroxide for few minutes, and rinsed with Milli-Q water. Each otolith was embedded in Struers epoxy resin (EpoFix™) and sectioned using a low speed diamond-blade ISOMET saw to obtain 1.5 mm transverse sections that included the core (Rooker et al., 2014). Sections were rinsed (MilliQ water) and mounted (5 per slide) on a glass slide with Crystalbond™. Otolith thin sections were polished to the core using wet-dry sandpaper and 3 μm aluminum oxide, triple rinsed and air dried under a laminar flow hood. Only one otolith from each individual (i.e., right or left) was used for trace analysis based on random assignment. Trace elements (Li, Mg, Mn, Zn, Sr, Ba) commonly used in chemistry application as tracers of natal habitats (Campana et al., 2000; Miller et al., 2005; Patterson et al., 2004; Rooker et al., 2001) were determined from polished otoliths using a laser ablation inductively coupled plasma mass spectrometer, LA-ICP-MS (XSeries 2, Thermo Scientific ICP-MS and New Wave Research NWR 213 laser system) at Texas A&M University at Galveston. The calibration of the instrument was achieved using certified reference material (NIST 614) distributed by the National Institute of Standards and Technology. The standard was analyzed before and after each otolith. ^{44}Ca was used as internal standard and its concentration normalized to be 380,000 μg/g. Concentration of all the other elements were calculated as ratio to Ca. Estimated detection limits (LOD_S) (μg/g) for examined elements were estimated as the quantity of analytes required to produce a signal equivalent to three times standard deviation of the NIST 614 ($n = 30$). These LOD_S were estimated as: ^{7}Li: 0.48, ^{24}Mg: 2.08, ^{55}Mn: 0.33, ^{66}Zn: 1.17, ^{88}Sr: 3.71, ^{137}Ba: 1.24. Trace element chemistry of each otolith was based on averaging element:Ca ratios from 7 laser ablation spots carried out across the otolith surface, extending out ~150 μm from the focus (i.e., central point) on both dorsal and ventral ridges. All analyses were conducted at a repetition rate of 10 Hz and at 70% power. Each spot had an ablation diameter of 50 μm with an ablation dwell time of 12 s. The same parameters were applied to NIST 614 standard except that the ablation dwell time was 20 s. Here, the same portion of the otolith attributable to age-0 class was considered, meaning that the time interval surveyed with LA-ICP-MS (portion of otolith) for each fish was the same. The age was evaluated on the age-length relationship and, based on data by La Mesa et al. (2005), mean size was within a month for all three regions.

Differences in trace element chemistry of age-0 T. *thynnus* among regional nurseries were evaluated with one-way tests for independent groups. Shapiro-Wilk test and Bartlett test (normal variables) or Leven test (non-normal variables) were performed to assess normality and variance homogeneity respectively. In addition, the potential influence of size of the individuals on elemental levels were investigated. An analysis of covariance (ANCOVA) using

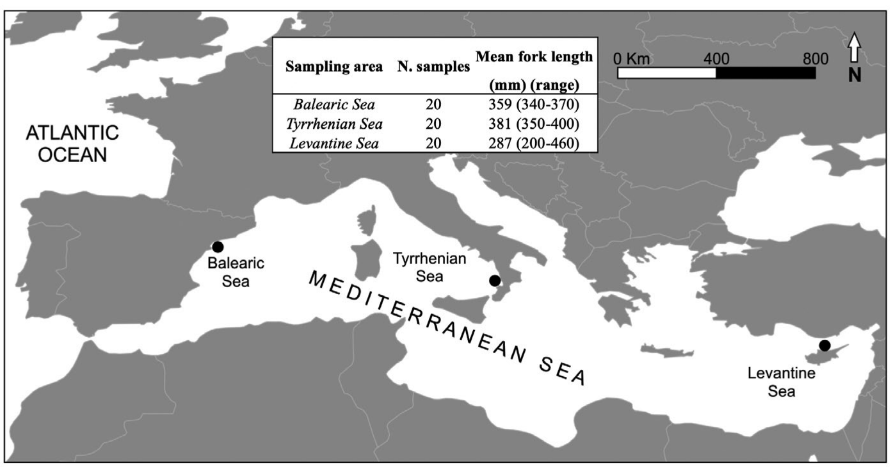

FIGURE 1 Sampling areas for *Thunnus thynnus* in the Mediterranean Sea and collection information

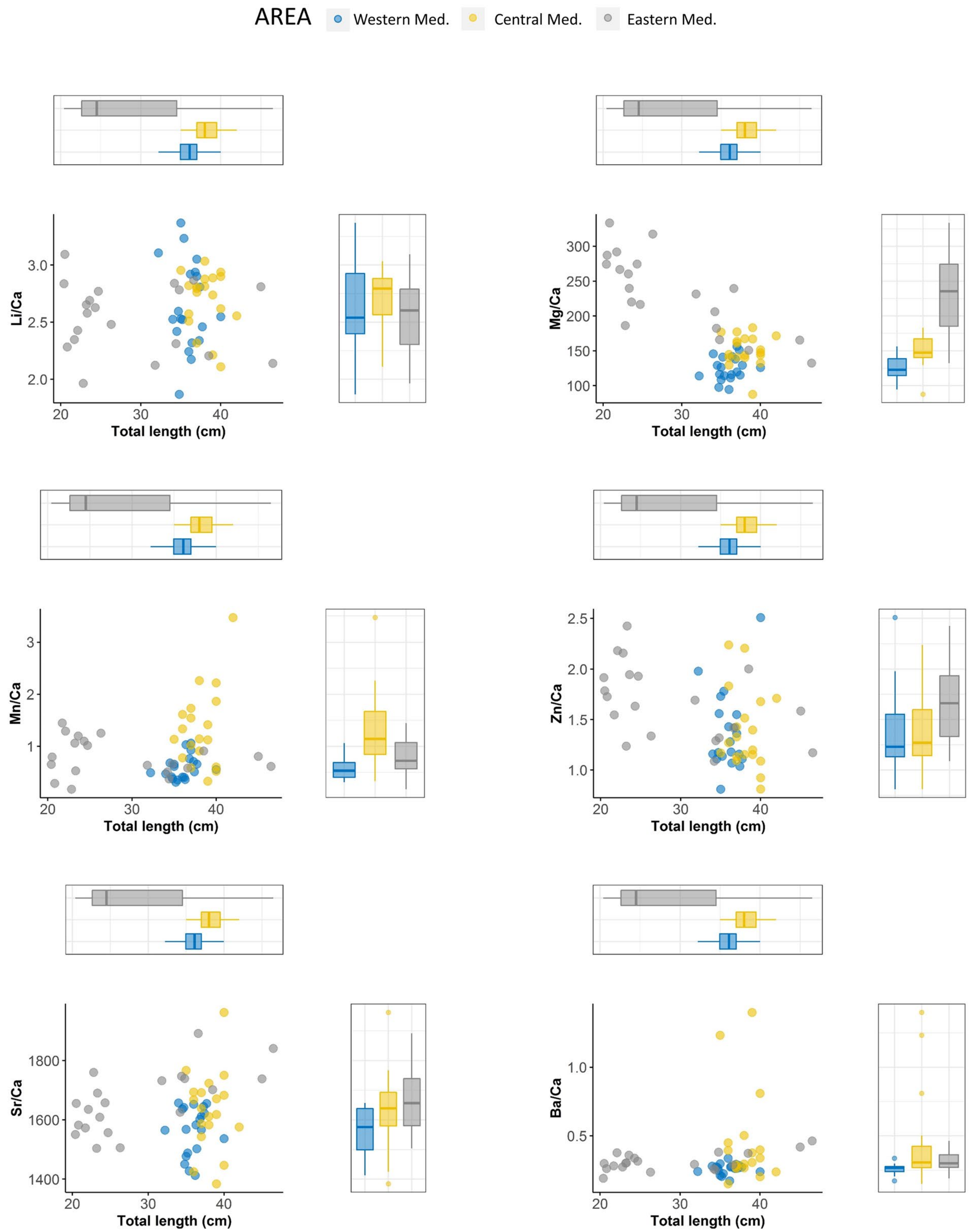

FIGURE 2 Scatterplot of fork length (cm) versus element:Ca ratios (μmol/mol) for otoliths of *Thunnus thynnus*. Colors of points correspond to the regional nurseries (western, central and eastern Mediterranean Sea). Boxplots (showing median, 10th, 25th, 75th, 90th percentiles and outliers) of fork length (*x*-marginal plot) and otolith element:Ca ratios (*y*-marginal plot) by areas were also reported at the top and the right respectively

fish length as covariate was performed for Mg, significantly influenced by the size, in order to adjust the elemental levels in sampling sites. Normality and homoscedasticity were tested by residuals analysis. An analysis of variance (ANOVA) model, with Tukey's post hoc tests, was applied for elements that satisfy both assumptions (Li and Sr), while non-parametric Kruskal-Wallis test and pairwise

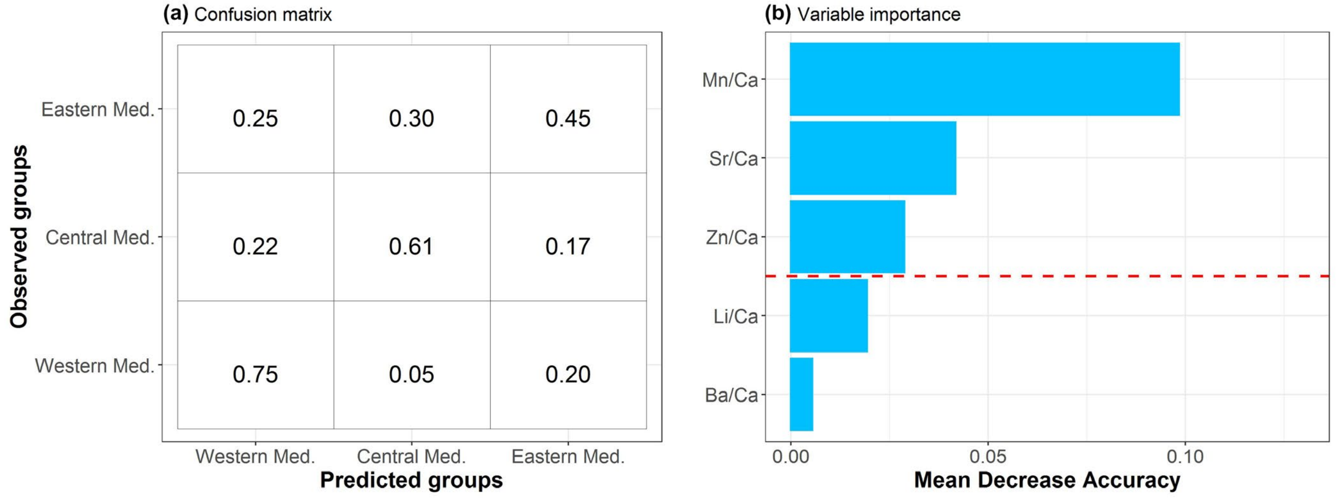

FIGURE 3 Random Forest results: (a) Confusion matrix showing the classification results, where numbers in the square are indicative of the percentage agreement between observed groups (*x*-axis) and predicted groups (*y*-axis) (b) Barplot of mean decrease accuracy of analyzed parameters. The variables above the dotted line are those identified by the backwards selection procedure

Wilcoxon Rank Sum Tests were applied for non-normal variables (Zn). Differences in Mn and Ba ratios were assessed with Welch's heteroscedastic with post-hoc Duncan test due to the violation of variance homogeneity assumption. Afterwards, a Random Forest analysis (RF) was performed in order to verify if the trace elements were able to discriminate the regional nurseries and assess their importance for the discrimination. The RF is a powerful ensemble machine-learning method used in classification, which constructs from the input variables a multitude of decision trees to output predicted classes. The number of trees for the classification is selected using a stepwise forward procedure to pick models with smaller estimation of error rate. The RF provides the confusion matrix, that expresses the percentage agreement between observed groups and groups predicted by the model. It also produces the variable importance according to the higher values of Mean Decrease in Accuracy (MDA). This index is the normalized difference between the classification accuracy when the variable is included as observed and the classification accuracy when the variable is randomly permuted. The more the accuracy of the random forest decreases due to the permutation of a single variable, the more important that variable is deemed in the classification (Liaw & Wiener, 2002). The choice of discriminant variables was carried out using backwards elimination with the minimization of error rate as selection criterion.

All statistical testing was performed by R using statistical software R 3.6.3 (R Development Core Team, 2020).

3 | RESULTS

In Figure 2 the correlation of element ratios with fish length was analyzed. The median length of the individuals from eastern Mediterranean was significantly lower compared to other areas (*x*-marginal plot in Figure 2). Only the Mg ratios were negatively correlated with fish length ($r = -.76$; p-value $<.05$). All tested elements, except Li:Ca, were different among the three regional nurseries in the Mediterranean Sea (*y*-marginal plot in Figure 2; $p < .05$). The ANCOVA results for Mg:Ca indicated that the individuals from eastern Mediterranean has significantly higher concentrations, compared to the other two areas . A similar trend was observed for Zn:Ca ratios. In contrast, otolith Mn:Ca ratios for age-0 T. *thynnus* from the central Mediterranean were significantly higher than the eastern and western regions ($p < .05$; Figure 2). Otolith Sr:Ca ratio differed between samples of age-0 T. *thynnus* from eastern Mediterranean and western Mediterranean with higher values in samples from eastern region, while otolith Ba:Ca ratios was lower in western Mediterranean compared with the other two regions ($p < .05$; Figure 2).

Mg:Ca would seem a good discriminator but the negative correlation with fish size suggests that the variability could have reflected the inherent instability of Mg in otoliths as an effect of growth (Javor & Dorval, 2016), therefore, it was chosen to exclude the Mg:Ca ratio in the RF. The RF model results demonstrated that the element:Ca ratios were useful for discriminating age-0 T. *thynnus* to the three nurseries with a total classification success of 60.3%. The confusion matrix showed that RF model and data from western, central, and eastern Mediterranean agree on 75%, 61%, and 45% cases, respectively (Figure 3a). The backwards selection identified Mn:Ca, Sr:Ca and Zn:Ca as the relevant element ratios for discriminating age-0 T. *thynnus* from the three regions (Figure 3b).

4 | DISCUSSION

Trace elements ratios in the otoliths of age-0 T. *thynnus* varied among the three regional nurseries, and the observed classification success to the three regions was moderate (60%), suggesting that several of the elements assayed could be informative markers for retrospectively establishing the nursery origin of this species. Several of the element:Ca ratios examined were distinct among the regions, and particularly, Mn:Ca, Sr:Ca and Zn:Ca ratios contributed most to variation between groups.

Several element:Ca ratios (Mg:Ca, Mn:Ca, Sr:Ca and Ba:Ca) in the otoliths of age-0 T. *thynnus* from the western Mediterranean were lower than the two other regions. The western area of the basin is considered an oceanographic transition zone where waters from the Mediterranean Sea mix with oceanic waters from the northeast Atlantic Ocean (García Lafuente et al., 1995; Pinot et al., 2002). Oceanic waters are typically lower in trace element concentration than marginal seas because of their proximity to continental sources of metals brought to the sea as fluvial or atmospheric inputs (Desboeufs et al., 2005). Therefore, the lower concentration of most elements could be related to the inflowing North Atlantic waters that plays an important role in the seawater chemistry of the western Mediterranean Sea (Elbaz-Poulichet et al., 2001; Riso et al., 2004).

We also observed conspicuous differences in otolith chemistry of age-0 T. *thynnus* in the central and western regions of the Mediterranean Sea. Otoliths Mn:Ca ratios were significantly higher for *T. thynnus* from the central Mediterranean region. This difference may be related in part to variation in water chemistry due to aeolian particulates from industrialized northwest European countries, as well as from the Sahara Desert because both are characterized by relatively high Mn concentration in aeolian particulates, and relevant dissolution kinetics of these elements in seawater from the dust (Elbaz-Poulichet et al., 2001). In past investigations, Mn has been used as a potential indicator of natal origin of *T. thynnus* (Rooker et al., 2001, 2003). Nevertheless, mechanisms regulating Mn are not fully understood (Miller, 2009) and further investigations are needed.

Elevated Mg:Ca in the otoliths of age-0 T. *thynnus* distinguishes the eastern Mediterranean region. This element is involved in a number of biological processes and in stabilizing amorphous mineral phases during otolith biomineralization, thus is tightly regulated in the body (Weiner, 2008). Furthermore, the observed negative correlation with length suggests the role of combined effects of extrinsic and intrinsic factors on otolith signature. The concentration of this metal in otoliths and its correlation with environmental variables (e.g., seawater chemistry, temperature) or intrinsic factors (e.g. physiology) has been investigated by several authors with different results (Di Maria et al., 2010; Javor & Dorval, 2016; Mazloumi et al., 2017; Miller, 2011; Sarimin & Mohamed, 2014; Sturrock et al., 2015).

Although data on metabolic rate of specimens and water chemistry are not available in this work, it is quite reasonable to speculate that the Mg content in otoliths from the eastern Mediterranean could be a result of different metabolic rates, which is in turn controlled by external factors (e.g., temperature and salinity). The eastern Mediterranean basin is known to show an average higher temperature than other areas (Tanhua et al., 2013), which may affect the chemical incorporation indirectly through its influence on fish metabolism or directly through kinetic effects.

Regional variation in otolith Sr:Ca was also observed for age-0 T. *thynnus* with values increasing from west to east across the three nurseries in the Mediterranean Sea (Figure 2). Because Sr is often incorporated into otoliths in direct proportion to ambient conditions (Farrell & Campana, 1996; Secor & Rooker, 2000), it probably reflects the correlation with salinity as suggested from several authors (Panfili et al., 2015; Walther & Limburg, 2012). The Levantine Sea (i.e., eastern nursery) is indeed the most saline portion of the Mediterranean Sea and it is documented that the concentration of Sr is often higher in marginal seas characterized by high evaporation or low freshwater input (Talley et al., 2011). Our finding of increased otolith Sr:Ca for age-0 T. *thynnus* from the eastern region is in accord with elevated salinity and possibly higher seawater Sr:Ca in the Levantine Sea.

It is widely recognized that composition of calcified structures in fish is influenced not only by exogenous factors (e.g., salinity) but also by mechanisms mediated by endogenous aspects (e.g., growth) (Kalish, 1991; Walther et al., 2010). To date, the extent to which environmental and physiological factors affect elemental uptake in otoliths remains partially unresolved (Tanner et al., 2016) and can vary among species, habitats, populations and life stages (Clarke et al., 2011; Walther et al., 2010). Since the relationships to otolith chemistry are not well established for many elements (i.e., Mg, Mn and Zn) further investigations are needed to provide a direct interpretation of results. An understanding of factors contributing to chemical differences and variability in chemical signatures can be helpful; however, for the purpose of stock identification, recognition of between group differences in chemical signatures is sufficient (Campana et al., 2000; Elsdon et al., 2008). Thus, beyond the potential interpretation of mechanisms related to elemental uptake in otoliths, the results of this work allowed for a discrimination between groups with a moderate degree of success (RF 45%–75%)

As widely reported, this species performs large-scale feeding migrations during the summer months to the Bay of Biscay and surrounding waters in the northeastern Atlantic Ocean (Fromentin & Powers, 2005), and this fishery appears to be supported almost exclusively by recruits of Mediterranean Sea origin (Fraile et al., 2015). The relative contribution rates of the three different nurseries within the basin, however, are currently unknown, and our findings support the application of using trace element signature to better understand the importance of different putative nurseries or production zones on the population dynamics of *T. thynnus* (Karakulak et al., 2004; Oray & Karakulak, 2005; Rooker et al., 2003).

Hence, the analysis of age-0 fish can be useful to define a baseline characterization of the chemical fingerprint for a stock, and is quite certain that a further examination of multiple year-classes is important to establish the temporal stability of elemental signatures (Gillanders, 2002; Rooker, Secor, De Metrio, Kaufman, et al., 2008). Although this study is limited to otoliths of age-0 T. *thynnus*, the application developed here could shed light on the relative contribution of the different Mediterranean spawning and/or nursery areas to different Atlantic Ocean and Mediterranean Sea fisheries, allowing for better management and conservation of this species.

ACKNOWLEDGEMENTS

The authors would like to thank Mario Sprovieri from IAS-CNR for valuable comments and suggestions, as well as N. Goñi, I. Onandia and T. Ortega for their help in sampling the otoliths. We also acknowledge staff at Fisheries Ecology Laboratory at Texas A&M University at Galveston for valuable assistance.

This work was carried out under the provision of the ICCAT Atlantic Wide Research Programme for Bluefin Tuna (GBYP), funded by the European Union, by several ICCAT CPCs, the ICCAT Secretariat and by other entities (see: https://www.iccat.int/gbyp/en/overview.asp). The contents of this paper do not necessarily reflect the point of view of ICCAT or of the other funders, which have not responsibility about them, and in no ways, anticipate the Commission's future policy in this area. This work was also supported by grant CTM2011–27505 from the Spanish Ministry of Economy and Competitiveness (MINECO).

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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How to cite this article: Traina A, Quinci E, Fraile I, Oray IK, Arrizabalaga H, Rooker JR. Regional variation in the otolith chemistry of age-0 atlantic bluefin tuna from nurseries in the mediterranean sea. *J Appl Ichthyol*. 2021;37:318–325. https://doi.org/10.1111/jai.14174