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Exploring European Eel *Anguilla anguilla* (L.) Habitat Differences Using Otolith Analysis in Central-Western Mediterranean Rivers and Coastal Lagoons from Sardinia

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Abstract: An otolith shape and morphometric analysis was performed on European eel (*Anguilla anguilla*) subpopulations from five rivers and three coastal lagoons of Sardinia (central-western Mediterranean) to assess the role of different habitats on otolith development. Sagittal otolith shape was described by 11 harmonics from elliptic Fourier descriptors. Comparisons among the harmonics were run through canonical discriminant analyses (CDAs). The CDA reclassification rate (75.7%) demonstrated a spatial environmental discrimination among local eel subpopulations of Sardinia. The Euclidean distance values demonstrated a dissimilarity between the river and lagoon groups. The form factor and roundness shape indices were significantly higher in the river group than in the lagoon group. The distances of the first three rings to the otolith core revealed site-specific otolith development. Moreover, the annual otolith growth rate was faster in the lagoon group than in the river group. The differences among the studied sites in terms of sagittal otolith shape could relate to changes in different local stocks potentially related to environmental peculiarities. Establishing a direct correlation between otolith morphology and environmental factors is challenging, and further studies are needed to investigate the relationship between habitat type/environmental variation and growth/body characteristics of eels. Nevertheless, the achieved results suggest that this method can be considered to be a valuable tool for studying the ontogeny of the European eel.

Keywords: European eel; sagittal otoliths; shape analysis; morphometry; growth; continental waters; Mediterranean

Key Contribution: An otolith shape analysis was performed on European eel subpopulations from five rivers and three lagoons of Sardinia. The obtained differences could lead to changes in different local stocks related to environmental peculiarities.

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1. Introduction

Otoliths are biomineralized-crystalline-organic complexes composed mainly of calcium carbonate [1]. With a metabolically inert structure, otoliths are less vulnerable to chemical and structural modifications and grow throughout the life of fish in response to several environmental influences and seasonality [2–5].

Although otoliths typically exhibit a species-specific morphological structure, they may also exhibit intraspecific changes in shape and size in relation to physiological and environmental factors [6]. Variations in otolith morphology have been observed among several populations or stocks of the same species [7–10], as well as within a species depending on factors such as sex [11,12], diet [13,14], and ontogeny [15,16].

For these reasons, otoliths can be defined as among the most useful anatomical structures for studying fish growth [17–19] in the field of ichthyology, ecology [20,21], fisheries biology [22–27], population age structure [28,29], fisheries management [30,31], and the study of fish adaptations to different environmental conditions [32,33].

Otolith shape has widely been used as a phenotypic marker to study variations in the development of populations of the European eel (*Anguilla anguilla* L. 1758) (hereafter the eel), and otoliths are also commonly used for age and growth estimations [34–38]. The pertinence of using otoliths to study development and environmental adaptations in this species may be related to the conservation status of the eel, which is actually in decline and listed as critically endangered, and may support a better understanding of the roles of different habitats to sustain and conserve the species [39,40]. Due to its catadromous life cycle, its wide geographical distribution range, its genomic panmixia, and its ability to live in different aquatic environments, the eel is particularly well suited for understanding how growth is influenced by environmental conditions [28,29,41]. Nevertheless, the body growth of eels can vary significantly within the same subpopulation because of interindividual variation and geographically different habitats [24,29,38,42,43]. Furthermore, the species shows a marked sexual size dimorphism, with the female larger than the male of similar age [24,29,44]. Furthermore, it is also known that eel growth rates can vary across latitudinal gradients of different environmental factors, including, for instance, temperature, photoperiod, hydrology, and productivity [23,24,28,45]. Added to this is the fact that certain aspects, such as site-specific variability that could affect eel growth, have not yet been fully documented and do not provide an overview of the characteristics of the eel's life cycle [24].

The eel also has one of the most complex life cycles in the animal kingdom, which includes two migrations spanning ca. 6000 km from the spawning grounds in the Sargasso Sea to the European and northern African coasts [21,46–48]. Furthermore, the species undergoes a series of metamorphosis to adapt to several aquatic environments throughout its life [28]. Leptocephali (larvae) drift across the Atlantic Ocean and metamorphose into glass eels before entering the continental shelf. During the glass eel stage, the species colonizes continental waters (e.g., rivers, lakes, and lagoons), where it grows and lives from 2 to more than 4–20 years as the yellow eel stage. After this period, eels start to metamorphose into silver eels (adults) and return to their spawning ground [25,28,49–51].

Despite the need to better understand the roles of different habitats in the phenotypic development and growth success of the eel, and considering that an otolith analysis is particularly adapted to investigate this question, only a few studies have examined eels' growth in terms of otolith shape [37,52]. This knowledge gap highlights the need to investigate phenotypic variability in the development of this species in different habitats and across several spatial scales. In this context, the general aim of this study was to analyze otolith shape and growth variations among eel subpopulations inhabiting several rivers and lagoons of Sardinian (central-western Mediterranean).

2. Materials and Methods

2.1. Study Locations

Sampling was conducted in five rivers (the Pramaera, Tirso, Coghinas, Barca, and Mannu di Fluminimaggiore (hereafter UMannu) Rivers) and three lagoons (Calich, Porto Pino, and Sa Praia) in Sardinia. These locations were selected to cover several geographic areas of the island (Figure 1 and Table 1).

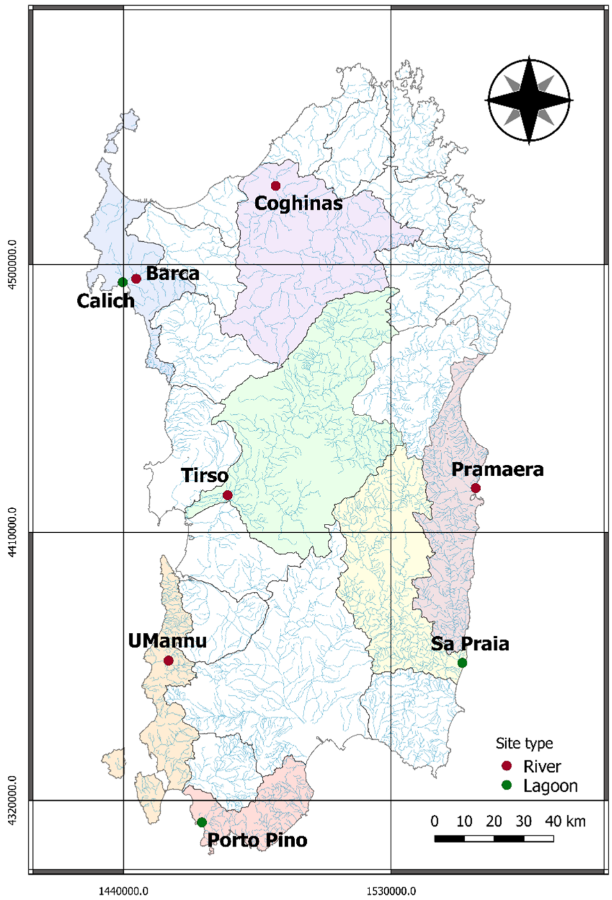

Figure 1. Locations of the eight studied sites within Sardinian continental waters. Rivers are indicated with red dots and lagoons are indicated with green dots.

Table 1. Characterization of the investigated rivers and lagoons, and the number of sampled eels at each site.

Site	River (R) Lagoon (L)	Regime (Only R)	Area (km^2)	Dams (Yes/No) (Only R) [1]	Number of Eels
Pramaera	R	Perennial	184 *	no	26
Tirso	R	Perennial	2043 *	yes	13
Coghinas	R	Perennial	1836 *	yes	10
Barca	R	Intermittent	355 *	yes	10
UMannu (Mannu di Fluminumaggiore)	R	Intermittent	126 *	no	11
Calich	L		0.9		10
Porto Pino	L		0.5		10
Sa Praia	L		0.86		10

[1] [53] * Area of catchment basin.

The Sardinian hydrographic network is characterized by a limited presence of perennial streams and a prevalence of intermittent streams. Most watercourses are located in close proximity to the coast and exhibit irregular flow patterns and significant seasonal hydrological fluctuations [53–58]. These characteristics are further amplified by the presence of steep slopes and short downstream sections. The Pramaera River is a typical Mediterranean small watercourse located in central-eastern Sardinia. This river is characterized by not having fluvial interruptions of anthropogenic origin (i.e., dams or other anthropogenic barriers) [57]. The Tirso River is the main watercourse of the island; it rises in the center of Sardinia and develops from northeast to southwest. Its course differs considerably as it proceeds from its source to the mouth of the river, differentiating in the upstream part with a winding path and considerable slopes, taking on a regular appearance in the central part, and then presenting minimal slopes and large meanders in the downstream section. An important element is the presence of numerous artificial reservoirs that are relevant from the point of view of the quantity of invasable water. Meanwhile, the coastal area has a series of coastal lagoons, some of which dry up completely in the summer. The Coghinas River is the third mainstream in Sardinia; it is located in the northern part of the island. Along its course, the Coghinas River is regulated by two dams, and then it flows into the sea in the Asinara Gulf. The Barca River is found in northwestern Sardinia and is a first-order watercourse. Its downstream trait flows into the Calich lagoon. In the Barca river basin, there are several reservoirs and the natural lake of Baratz. The UMannu River is located in southwestern Sardinia and is a first-order watercourse belonging to the Riu Mannu basin.

Sardinian coastal lagoons extend for a total area of about 120 km^2 and are particularly interesting for their naturalistic value and productivity [59]. The Calich lagoon is located on the northwestern coast of Sardinia. It communicates with the sea through a channel located in the northwestern area of the lagoon. The main tributaries are the Barca and the Calvia Rivers, and the Oruni canal. The continuous tidal flow and the freshwater inputs result in a very variable brackish condition which results in fishing yields that do not exceed 50 kg ha^{-1}. The salinity can vary from 5 in the winter season to 38 in the summer [59]. The Porto Pino lagoon is located on the southern coast of Sardinia; it consists of a series of small basins (Porto Pino, Maestrale, Is Brebeis, Foxi, and Corvo) in communication with each other and used as tanks pre-evaporating from the saline. The salinity can vary from the marine values (ca. 37) and can increase up to 50 [60]. In this lagoon, good integration has been achieved between salt production and fishing activities through the management of bulkheads that regulate the water flow. Fishing activity is carried out using artisanal gill nets, pots, and fyke nets. The Sa Praia lagoon is located on the southeastern coast of Sardinia. It is provisioned by the Gironi River and is connected to the sea by a canal on which a traditional downstream trap called a "lavoriero" is positioned. The salinity ranges from 22.3 to 39.3 (Fish Products Service of Agricultural Research Agency of Sardinia, Agris).

2.2. Eel Samples

Eels were collected from June 2015 to February 2020 during the dry seasons (summer, autumn). In the Pramaera, Barca, and Coghinas Rivers, eels were caught by using experimental fyke nets (2 mm mesh size), while in the Tirso and UMannu Rivers, eels were captured using low-frequency, pulsed DC electrofishing. Lastly, in the Calich, Porto Pino, and Sa Praia lagoons, eels were caught with professional fyke nets (10 mm mesh size).

Individual eel samples were immediately stored in cool and aerated water and anesthetized by immersion in a bath of MS 222 (230 mg L^{-1}) until the termination of opercular movements [58], and then measured for total length (TL, cm) and total weight (TW, g). Subsequently, the eels were sacrificed in situ by decapitation, according to the European Community regulation and Italian legislation for the protection of animals used for scientific purposes (Directive 2010/63/UE L 276 20/10/2010, implemented by Italian Legislative Decree 26/2014). Individuals were kept frozen until head dissection for otolith extraction and gonad dissection for sexual characterization (female, male, undifferentiated).

Sex was determined macroscopically whenever possible, or through histological examination of gonads [61,62]. A preliminary exploratory analysis was carried out on the shape indices of the Pramaera River, which provided the largest number of samples (26 eels) and on which the sex of eels was determined both on a macro- and microscopic basis to test the influence of sex on the shape of sagittal otoliths. In the remaining sites, only macroscopic sex evaluation was possible.

2.3. Otolith Extraction and Shape Analysis

The right and left sagittal otoliths of each eel were extracted for the analysis, cleaned with distilled water to remove remaining adhering tissues, and then placed dry in tubes. Each dried sagittal otolith was observed in the dorsal position under a stereomicroscope equipped with a digital camera (Leica S9i Stereozoom LSR w/TL3000 ergo, Wetzlar, Germany). The digital images were acquired using the Leica LAS 4.12 software to obtain the most highly contrasted images. The extraction and preparation of the sagittal otoliths were developed according to the methodology defined in the *Manual for the Ageing of Atlantic Eel* [36]. For the age reading [36], the sagittal otoliths were prepared by grinding and polishing along the sagittal plane, followed by staining (EDTA and Toluidene Blue, 0.1 M). The sagittal otoliths of eels under 5 years were analyzed without any preparation (in toto), except for their immersion in thyme essential oil to improve the visualization of growth marks.

Different measurements were performed on each sagittal otolith to calculate the shape indices [63] by using the software ImageJ (National Institutes of Health, USA). The following five indices were derived from the area (A), the perimeter (P), the Feret length (L), and the Feret width (w) of otoliths: form factor ($4\,\pi A/P^2$), circularity (P2/A), roundness ($4\,A/\pi L^2$), ellipticity $(L - w)/(L + w)$, and rectangularity (A/Lw). All indexes ranged from 0 to 1.

The sagittal otolith shape was first compared between males and females from the Pramaera River, which had the largest number of samples, and on which were conducted a microscopic analysis of the gonads to better assess the sex. As no significant differences were observed between sexes (K–W, $p > 0.05$), shape analyses were conducted on both the males and females together for all investigated sites. The five shape indices as well as the measured distances of the first three rings to the core and the annual otolith growth rate (cm year^{-1}) were compared between the river and lagoon groups. The assumption of linearity (normality and homoscedasticity) was rejected, and therefore a nonparametric Kruskal–Wallis (K–W) test followed by a pairwise comparison post hoc Dunn's (Z) test were performed to test for differences in the median values between the studied sites.

According to the methodology of a shape analysis previously described in Morat et al. [7] and in Mérigot et al. [64], an elliptic Fourier analysis [7,64–66] and comparisons of the shape indices [60] were conducted. The elliptic Fourier analysis describes the outline of sagittal otoliths as several components named harmonics. Each harmonic is characterized by 4 coefficients, derived from the projection of each point along the x- and y-axes. The Fourier coefficients were calculated using the software Shape 1.3 (Tokyo, Japan) [67]. The Fourier power spectrum was calculated for each sagittal otolith to determine the best number of harmonics for the optimal reconstruction of the otolith outline [68,69] for both the right and left sagittal otoliths of the same individual separately, as well as combined. In order to define the suitable number of harmonics to be considered in the analyses, the minimum number of harmonics was set up to obtain a threshold of 99.99% of the outline. For this reason, a total of 11 harmonics of the right sagittal otoliths were selected. Because the first harmonic was not considered (representing a simple ellipse), a total of 40 Fourier coefficients were used to describe each sagittal otolith.

The shape differences between each river and lagoon were determined using a canonical discriminant analysis (CDA) performed with the 40 Fourier coefficients. This classification method investigates the groups' integrity (each river and lagoon) by finding a linear combination of the descriptors that maximizes Wilk's lambda (λ) obtaining values ranging from 0 (low discrimination) to 1 (high discrimination) [70]. The Cohen kappa statistic was used to estimate the global reclassification rate of all groups [71]. The dissimilarity between groups was evaluated by using the Euclidean distance (d) between the barycenters of each group.

For the analysis of the shape indices, pairwise collinearity was investigated by examined scatter plots, excluding redundancy between paired variables using Spearman's $\rho > 0.7$. Shape indices were discarded from the pairwise combination based on the variance inflation factor (VIF) discarding observation with VIF > 3 [72]. Then, differences in shape indices between the river and lagoon groups were analyzed to describe and compare the sagittal otoliths in the different study sites (each river and lagoon). In the Pramaera river, comparisons were also carried out between sexes.

Significance was set at $p < 0.05$. All statistical and shape analyses were performed with the software R 4.3.1 (R Development Core Team) [73].

3. Results

A total of 100 eels were collected for the sagittal otolith analysis (Tables 1 and 2).

Table 2. Biometric ranges (minimum–maximum) of sampled eels per sex (female, male, and undifferentiated eels) for each studied river and lagoon. Total length (TL) and total weight (TW).

	Females		Males		Undifferentiated	
Site	**TL (cm)**	**TW (g)**	**TL (cm)**	**TW (g)**	**TL (cm)**	**TW (g)**
Pramaera	49.50–65.00	190.00–497.70	31.10–41.90	50.80–152.20	6.80–31.60	0.21–51.00
Tirso	/	/	/	/	16.30–32.30	6.70–71.90
UMannu	/	/	26.40–34.20	20.0–58.40	/	/
Barca	77.20	945.65	29.30–43.20	43.78–143.50	28.00	31.00
Coghinas	/	/	/	/	11.00–20.50	1.22–10.66
Calich	47.30–56.00	229.97–450.51	33.40–38.40	68.70–101.95	27.50–30.60	40.02–50.40
Porto Pino	26.50–75.50	24.50–636.80	/	/	/	/
Sa Praia	56.00–56.50	326.30–342.80	32.00–39.50	51.18–104.40	32.1	43.58

Comparisons of shape indices between male and female eels from the Pramaera River revealed no significant sex-dependent differences in sagittal otolith morphology from eels of the same study site (K–W test, $p > 0.05$).

Based on this result, the Fourier coefficients of the right sagittal otoliths were used in the CDA to assess the relative classification of the eight study sites in terms of otolith shape (Figure 2). The CDA revealed Wilks λ values equal to 0.06 and 0.17 for the x- and y-axis, respectively, indicating a relatively low discrimination between groups, while the percentage of reclassification assessed with Cohen's kappa test was 75.7%.

The Euclidean distance values between the barycenters of each group (rivers and lagoons), obtained from the CDAs, showed a distinct clustering pattern. The Pramaera, Tirso, and UMannu Rivers exhibited a close grouping with values of $d < 0.9$. Similarly, the three lagoons (Calich, Porto Pino, and Sa Praia) formed a distinct grouping with d values less than 0.4. The Barca River displayed intermediate characteristics, sharing similarities with both the river and the lagoon groups. In contrast, the Coghinas River did not group with any other site showing a high dissimilarity with d values greater than 2.4 compared to all other sites (Table 3).

Shape indices were analyzed through Spearman's rank correlation ($\rho < 0.7$) (Figure 3) and a VIF score threshold of 3. Based on these criteria, only form factor (VIF = 1.70), roundness (VIF = 1.57), and rectangularity (VIF = 1.18) were considered for the subsequent analysis. Area, perimeter, and the remaining shape indices (Feret length, Feret width, and circularity) showed a correlation higher than 0.7 and VIF greater than 3, and thus were discarded from subsequent analyses.

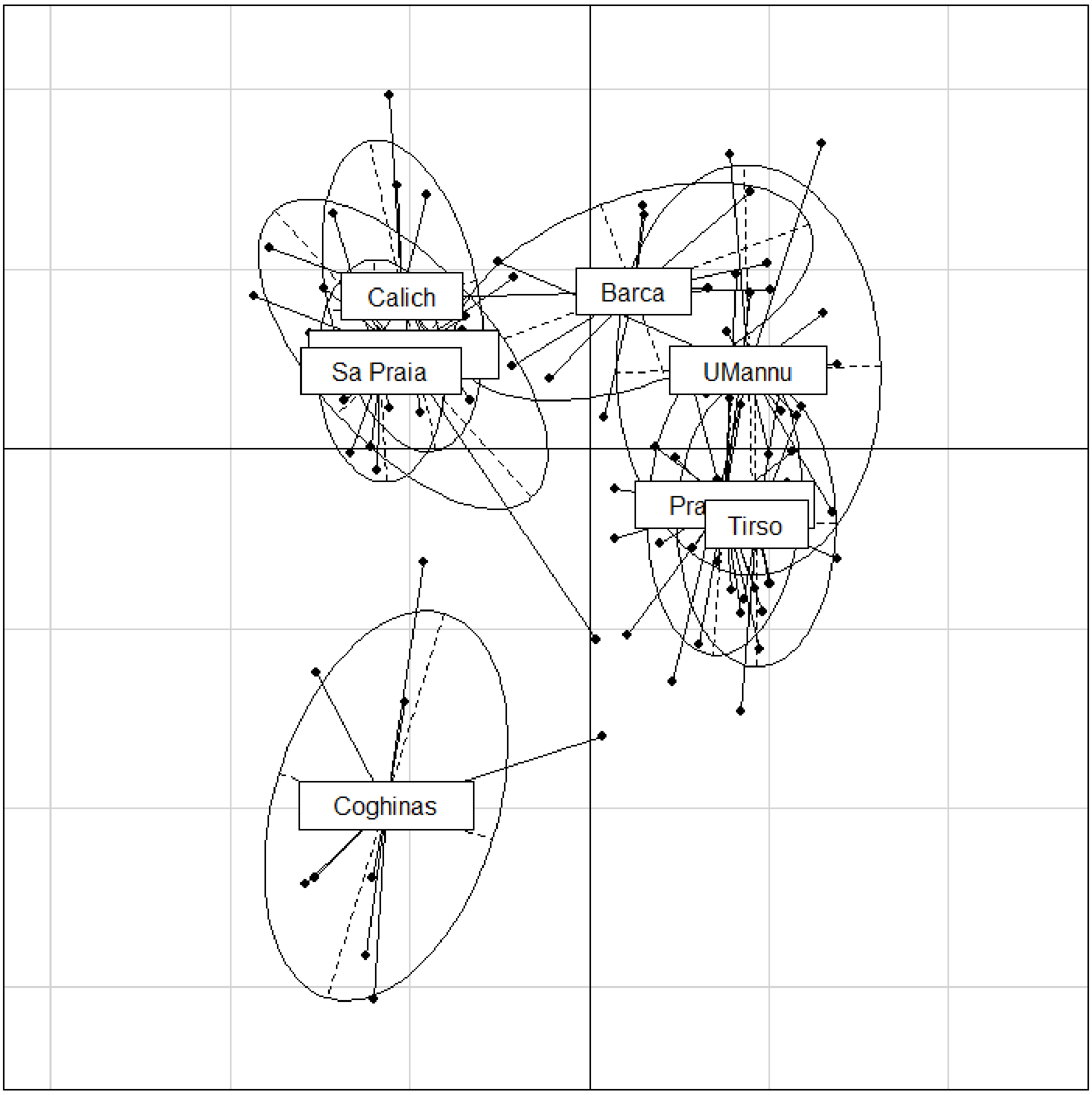

Figure 2. Canonical discriminant analysis (CDA) output achieved with Fourier coefficients for the five rivers (Pramaera, Tirso, UMannu, Barca, and Coghinas) and three lagoons (Calich, Porto Pino, and Sa Praia) investigated in the present study.

Table 3. Euclidean distance values between the barycenters of the study sites resulting from the CDAs performed with right sagittal otoliths (Euclidean distance values < 1, shown in bold, represent strong clustering between sites).

Site	Pramaera	Tirso	UMannu	Barca	Coghinas	Calich	Porto Pino	Sa Praia
Tirso	**0.21**							
UMannu	**0.76**	**0.86**						
Barca	1.29	1.47	**0.78**					
Coghinas	2.52	2.59	3.16	3.18				
Calich	2.14	2.34	1.97	1.28	2.84			
Porto Pino	1.98	2.18	1.93	1.33	2.52	**0.32**		
Sa Praia	2.06	2.26	2.05	1.47	2.42	**0.43**	**0.16**	

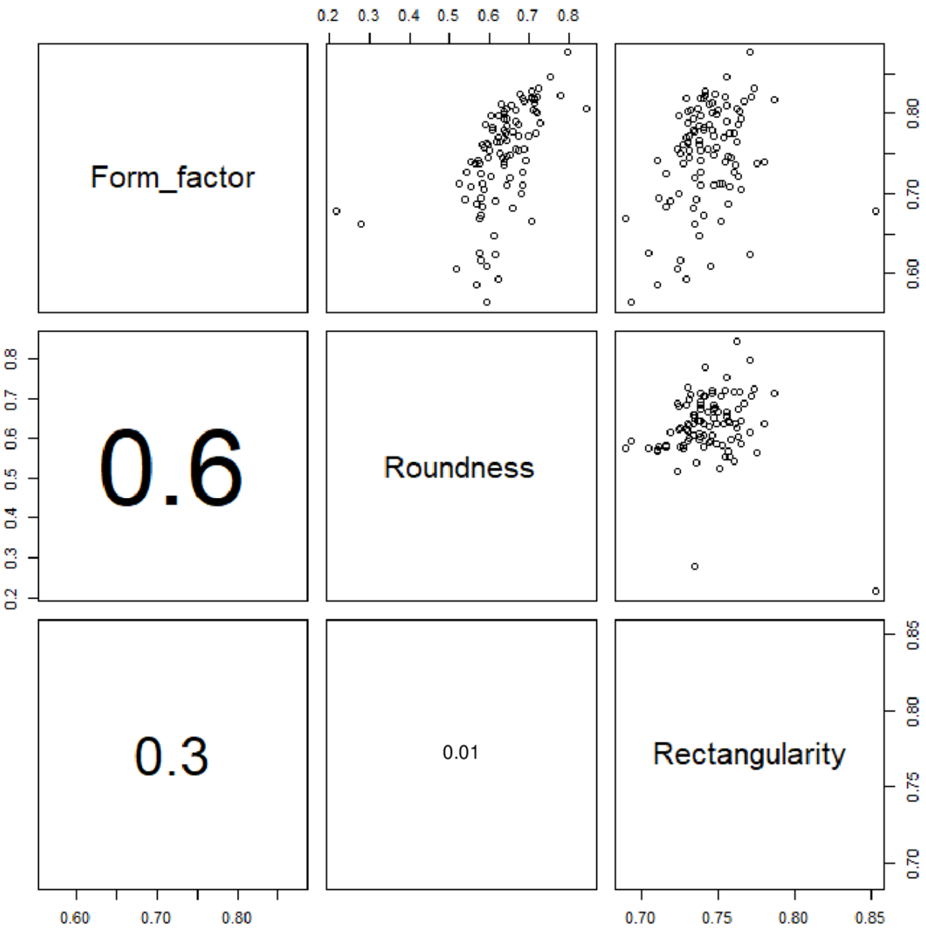

Figure 3. Spearman rank correlation and ρ values for shape otoliths' indexes.

The form factor showed significant differences between the river and lagoon groups (K–W = 29.34, p = 0.00012) (Figure 4). Specifically, the form factor values were found to be higher in the river group than in the lagoon group (Table 4).

This result was also confirmed by the post hoc Dunn's test revealing significant differences, especially between the Calich and Porto Pino lagoons compared to the Coghinas, Pramaera, and Tirso Rivers (Z tests, $p < 0.05$).

A similar outline was obtained for the roundness index (Figure 4), highlighting statistical differences detected between sites (K–W = 42.11, $p < 0.0001$) (Figure 4), with greater values of roundness in rivers than in lagoons, especially the Coghinas River (Table 5).

Table 4. In the grey boxes, median values ± standard deviation (SD) of the form factor shape index are described. Below the grey boxes, the p values are reported with significant values ($p < 0.05$) shown in bold, and above the grey boxes, asterisks indicate the significance level, i.e., $p < 0.05$ = *, and $p > 0.05$ = ns (not significant).

	Pramaera	Tirso	UMannu	Barca	Coghinas	Calich	Porto Pino	Sa Praia
Pramaera	0.767 ± 0.063	ns	ns	ns	ns	ns	*	ns
Tirso	1	0.777 ± 0.044	ns	ns	ns	ns	*	ns
UMannu	1	1	0.794 ± 0.041	ns	ns	ns	ns	ns
Barca	1	1	1	0.763 ± 0.067	ns	ns	ns	ns
Coghinas	1	1	1	1	0.794 ± 0.038	*	*	ns
Calich	0.14	0.08	0.19	1	**0.04**	0.704 ± 0.055	ns	ns
Porto Pino	**0.03**	**0.02**	0.06	0.90	**0.01**	1	0.695 ± 0.055	ns
Sa Praia	0.44	0.24	0.49	1	0.14	1	1	0.720 ± 0.071

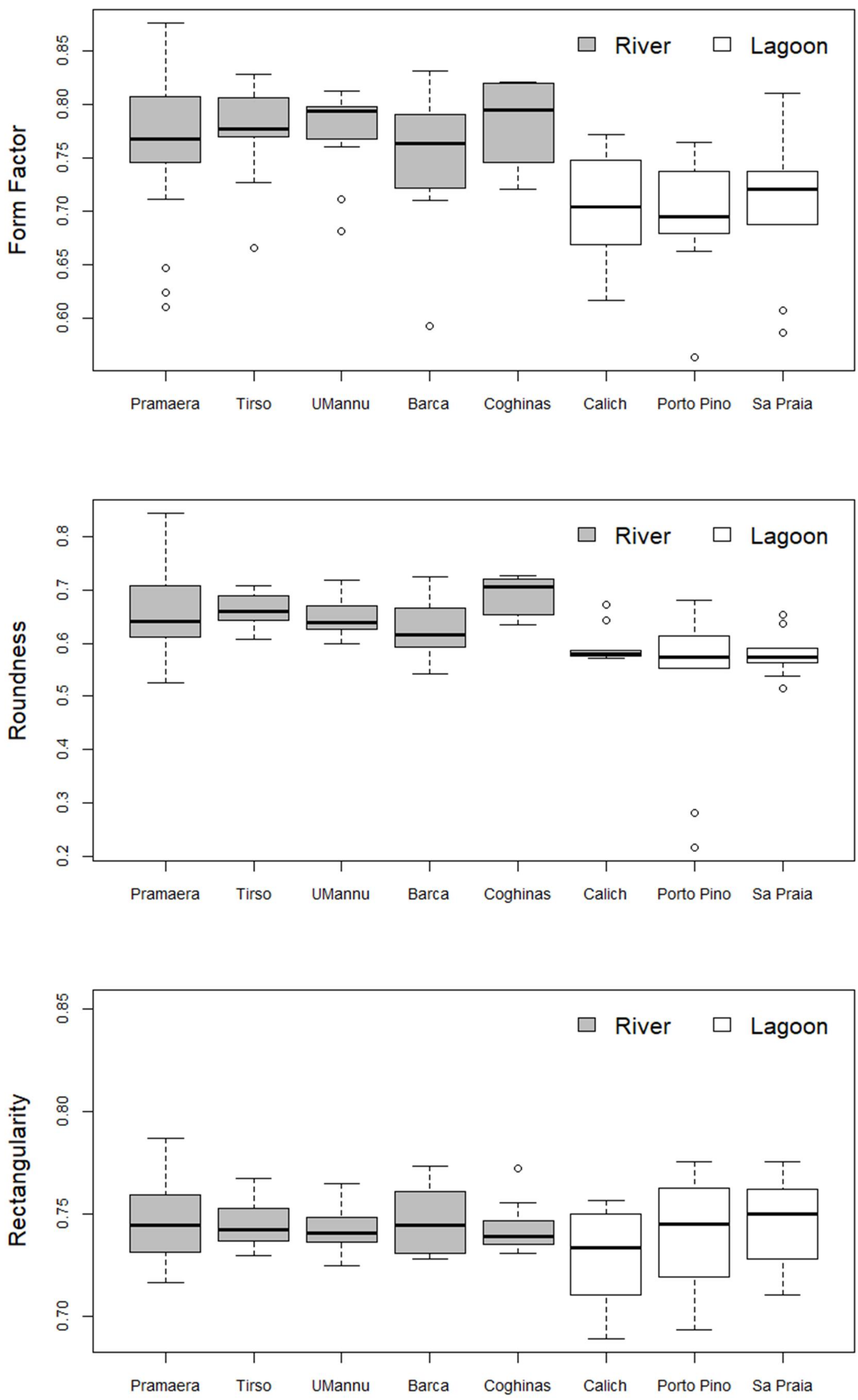

Figure 4. Boxplots for form factor, roundness, and rectangularity shape indices for the rivers (grey) and for the lagoons (white).

Table 5. In the grey boxes, median values ± standard deviation (SD) of the roundness shape index are described. Below the grey boxes, the *p* values are reported with significant values ($p < 0.05$) shown in bold, and above the grey boxes, asterisks indicate the significance level, i.e., $p < 0.001$ = ***, $p < 0.01$ = **, and $p < 0.05$ = *, and $p > 0.05$ = ns (not significant).

	Pramaera	Tirso	UMannu	Barca	Coghinas	Calich	Porto Pino	Sa Praia
Pramaera	0.642 ± 0.073	ns	ns	ns	ns	ns	*	*
Tirso	1	0.660 ± 0.032	ns	ns	ns	*	*	**
UMannu	1	1	0.639 ± 0.040	ns	ns	ns	ns	ns
Barca	1	1	1	0.616 ± 0.051	ns	ns	ns	ns
Coghinas	1	1	1	0.30	0.705 ± 0.035	**	***	***
Calich	0.07	**0.03**	0.39	1	**0.002**	0.580 ± 0.034	ns	ns
Porto Pino	**0.03**	**0.01**	0.19	1	**<0.001**	1	0.573 ± 0.015	ns
Sa Praia	**0.01**	**0.007**	0.12	1	**<0.001**	1	1	0.574 ± 0.041

On the one hand, significant differences were evident in all three lagoons compared to the Pramaera, Tirso, and Coghinas Rivers (Z tests, $p < 0.05$). On the other hand, the rectangularity index (Figure 4) did not show significant differences in medians between the sites (Table 6) (K–W = 3.88, $p = 0.79$).

Table 6. In the grey boxes, median values ± standard deviation (SD) of the rectangularity shape index are described. Below the grey boxes, the *p* values are reported with significant values ($p < 0.05$) shown in bold, and above the grey boxes, asterisks indicate the significance level, and $p > 0.05$ = ns (not significant).

	Pramaera	Tirso	UMannu	Barca	Coghinas	Calich	Porto Pino	Sa Praia
Pramaera	0.744 ± 0.018	ns	ns	ns	ns	ns	ns	ns
Tirso	1	0.742 ± 0.012	ns	ns	ns	ns	ns	ns
UMannu	1	1	0.741 ± 0.012	ns	ns	ns	ns	ns
Barca	1	1	1	0.745 ± 0.016	ns	ns	ns	ns
Coghinas	1	1	1	1	0.739 ± 0.013	ns	ns	ns
Calich	1	1	1	1	1	0.733 ± 0.024	ns	ns
Porto Pino	1	1	1	1	1	1	0.745 ± 0.044	ns
Sa Praia	1	1	1	1	1	1	1	0.750 ± 0.021

Analysis of ring distances from the core of the first two years revealed significant differences in otolith growth. The median values were higher in lagoons compared to in rivers (Tables 7 and 8) (K–W = 22.57, $p = 0.0019$ and K–W = 19.30, $p = 0.0073$, respectively) (Figure 5). Pairwise significant differences were observed only between the Calich and Sa Praia lagoons compared to the UMannu River (Z tests, $p < 0.05$).

Table 7. In the grey boxes, the median values ± standard deviation (SD) of the first ring distance from the core of sagittal otoliths are described. Below the grey boxes, the *p* values are reported with significant values ($p < 0.05$) shown in bold, and above the grey boxes, asterisks indicate the significance level, i.e., $p < 0.05$ = *, and $p > 0.05$ = ns (not significant).

	Pramaera	Tirso	UMannu	Barca	Coghinas	Calich	Porto Pino	Sa Praia
Pramaera	0.326 ± 0.088	ns	ns	ns	ns	ns	ns	ns
Tirso	1	0.347 ± 0.069	ns	ns	ns	ns	ns	ns
UMannu	1	1	0.298 ± 0.061	ns	ns	ns	ns	*
Barca	0.45	1	0.15	0.424 ± 0.060	ns	ns	ns	ns
Coghinas	1	1	1	1	0.347 ± 0.68	ns	ns	ns
Calich	1	1	0.73	1	1	0.414 ± 0.166	ns	ns
Porto Pino	0.39	1	0.07	1	1	1	0.439 ± 0.116	ns
Sa Praia	0.07	0.61	**0.03**	1	1	1	1	0.460 ± 0.086

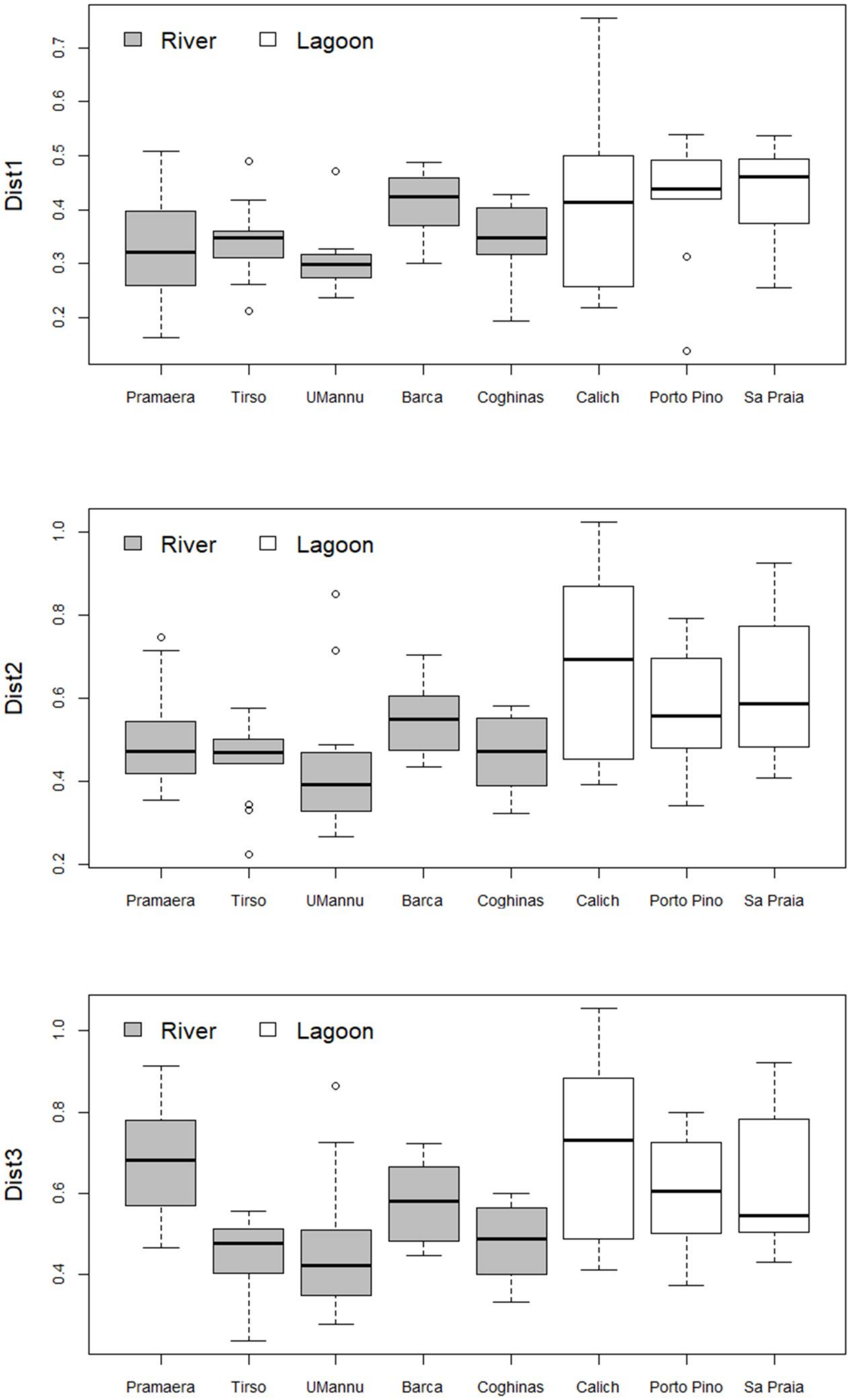

Figure 5. Boxplots for the first (Dist1), second (Dist2), and third (Dist3) ring distances from the sagittal otolith's core for rivers (grey) and for lagoons (white).

Table 8. In the grey boxes, median values ± standard deviation (SD) of the second ring distance from the core of sagittal otoliths are described. Below the grey boxes, the *p* values are reported with significant values ($p < 0.05$) shown in bold, and above the grey boxes, asterisks indicate the significance level, i.e., $p < 0.05$ = *, and $p > 0.05$ = ns (not significant).

	Pramaera	Tirso	UMannu	Barca	Coghinas	Calich	Porto Pino	Sa Praia
Pramaera	0.496 ± 0.126	ns	ns	ns	ns	ns	ns	ns
Tirso	1	0.469 ± 0.099	ns	ns	ns	ns	ns	ns
UMannu	1	1	0.390 ± 0.184	ns	ns	*	ns	ns
Barca	1	1	0.49	0.549 ± 0.088	ns	ns	ns	ns
Coghinas	1	1	1	1	0.471 ± 0.088	ns	ns	ns
Calich	1	0.36	**0.04**	1	0.89	0.694 ± 0.223	ns	ns
Porto Pino	1	1	0.31	1	1	1	0.556 ± 0.145	ns
Sa Praia	1	0.51	0.07	1	1	1	1	0.587 ± 0.188

For the third ring distances (K–W = 26.33, $p < 0.001$), differences in the distances were found between the Pramaera and the Tirso Rivers, and the Pramaera and the UMannu Rivers (Z tests, $p < 0.05$) (Figure 5 and Table 9).

Table 9. In the grey boxes, median values ± standard deviation (SD) of the third ring distance from the core of sagittal otoliths are described. Below the grey boxes, the *p* values are reported with significant values ($p < 0.05$) shown in bold, and above the grey boxes, asterisks indicate the significance level, i.e., $p < 0.01$ = ** and $p < 0.05$ = *, and $p > 0.05$ = ns (not significant).

	Pramaera	Tirso	UMannu	Barca	Coghinas	Calich	Porto Pino	Sa Praia
Pramaera	0.693 ± 0.154	**	*	ns	ns	ns	ns	ns
Tirso	**0.007**	0.476 ± 0.095	ns	ns	ns	ns	ns	ns
UMannu	**0.01**	1	0.421 ± 0.180	ns	ns	ns	ns	ns
Barca	1	1	1	0.581 ± 0.100	ns	ns	ns	ns
Coghinas	0.10	1	1	1	0.488 ± 0.094	ns	ns	ns
Calich	1	0.08	0.13	1	0.46	0.729 ± 0.234	ns	ns
Porto Pino	1	0.45	0.66	1	1	1	0.605 ± 0.140	ns
Sa Praia	1	0.52	0.75	1	1	1	1	0.546 ± 0.168

Significant differences in annual otolith growth values were found between the river and lagoon groups (Figure 6) (K–W = 58.27, $p < 0.0001$), with lagoons generally showing higher median values, except for the Pramaera River, which showed variation in annual growth with values close to those for the lagoons. The Coghinas River displayed the lowest median annual otolith growth values (Table 10) (Z tests, $p < 0.05$).

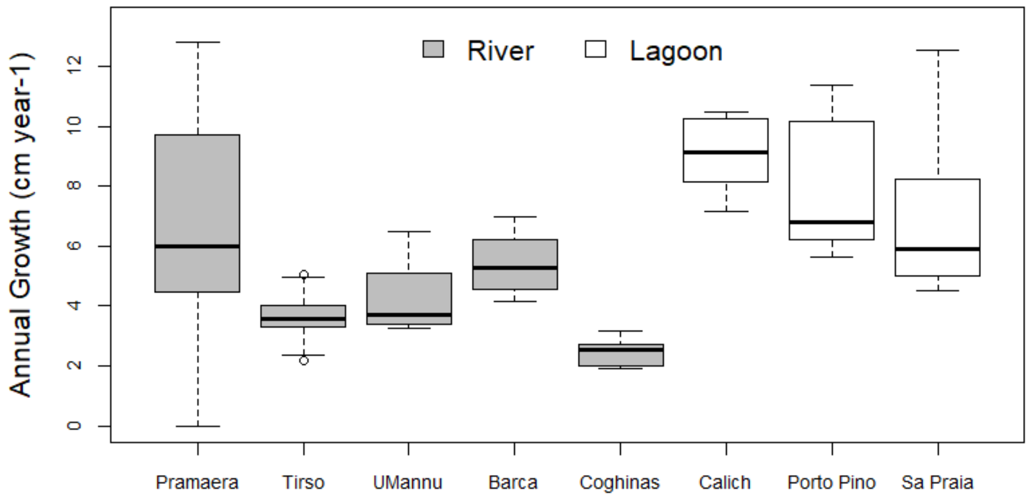

Figure 6. Boxplot for the annual otolith growth (cm year^{-1}) for rivers (grey) and for lagoons (white).

Table 10. In the grey boxes, median values ± standard deviation (SD) of the annual otolith growth (cm year^{-1}) are described. Below the grey boxes, the *p* values are reported with significant values ($p < 0.05$) shown in bold, and above the grey boxes, asterisks indicate the significance level, i.e., $p < 0.0001$ = ****, $p < 0.001$ = ***, $p < 0.01$ = **, and $p < 0.05$ = *, and $p > 0.05$ = ns (not significant).

	Pramaera	Tirso	UMannu	Barca	Coghinas	Calich	Porto Pino	Sa Praia
Pramaera	6.00 ± 3.50	*	ns	ns	****	ns	ns	ns
Tirso	0.01	3.58 ± 0.84	ns	ns	ns	****	**	ns
UMannu	0.33	1	3.70 ± 1.33	ns	ns	**	ns	ns
Barca	1	0.87	1	5.29 ± 1.03	*	ns	ns	ns
Coghinas	**<0.0001**	1	1	**0.03**	7.60 ± 2.86	****	****	***
Calich	0.72	**<0.0001**	**0.002**	0.31	**<0.0001**	9.15 ± 1.77	ns	ns
Porto Pino	1	**0.002**	0.57	1	**<0.0001**	1	6.81 ± 2.16	ns
Sa Praia	1	0.57	0.65	1	**<0.001**	1	1	5.90 ± 2.64

4. Discussion

In this study, for the first time, to the best of our knowledge, we aimed to investigate the subpopulations of *A. anguilla* from Sardinian continental waters (central-western Mediterranean) using an otolith shape analysis. Our goal was to understand the role of different habitat types (rivers and lagoons) on otolith shape and growth as a marker of eel subpopulation success. The results of the canonical discriminant analysis demonstrated a high value of reclassification of 75.7%, which suggests that the classification analysis and shape index comparisons can represent useful tools for discriminating eel subpopulations that inhabit different habitat typology. Similar findings have already been observed in other regional studies that have focused on Mediterranean eel stocks [37,74]. In particular, Capoccioni et al. [37] found differences in otolith morphology among three Mediterranean eel local stocks, including two brackish lagoons and one river. Additionally, Milošević et al. [75] investigated otolith shape variations between riverine and lacustrine habitats in the Adriatic Basin (Croatia and Montenegro), and their findings aligned with our study, highlighting the role of environmental variability in shaping otolith morphology and size during the growth of the species.

In the present study, we found that sagittal otoliths from rivers were generally rounder and less irregular compared with those from lagoons. This finding corroborated results obtained in previous shape otolith studies, as mentioned above. In addition, we obtained high variability in terms of annual otolith growth rates among several subpopulations as well as in single habitats, which has already been observed in previous studies all over Europe and in the Mediterranean basin, due to the multitude of used habitats by the species [38,76–79]. Our values were in accordance with those obtained in other particular Mediterranean lagoons (about 5 cm year^{-1}) (Valli di Comacchio [80], Vaccarés-Impérieux, Fumemorte [78], Aveiro [81], and Valle Nuova lagoons [80]). Eels from rivers showed lower annual growth rate values that appeared to be similar to values obtained in other studies (between 3 and 4.5 cm year^{-1} (Severn [82], Shannon [83], Frome [84], Koge Lellinge [85], Barrow [86], and Imsa [87] Rivers). We also confirmed that brackish habitats such as coastal lagoons could support faster growth rates than riverine habitats offering probably more suitable conditions to support eel growth and survival [78,88–90].

Furthermore, in our study, the sagittal otoliths from rivers maintained a more circular shape across their entire life, with the only exception of otoliths of the eels from the Coghinas River which showed a shape differentiation resulting in a more circular shape than all otoliths of the other rivers. This peculiar result can be attributed to the size of the sample that was possible to collect, consisting of only small specimens (TL ≤ 20.5 cm). Despite their reduced TL values, these eels showed ages ranging from two to five years. Even if coming from the same basin, sagittal otoliths of eels from the Barca River and Calich lagoon were an average distant, according to the Euclidean distance, sharing similarities both with the river and lagoon groups. However, differences found in the shape indices fit well between these two sites. This could be related to the resident behavior of the eels during the trophic stage that showed differences in development according to the habitat typology.

Indeed, several environmental abiotic characteristics (e.g., temperature, salinity, depth, food availability, and ecological niche) create habitats that are more suitable for eels, determining variability in their development that can also be reflected in otolith shape; therefore, more effort should be made to protect them [2,13,64,91–95]. Furthermore, different eels' development strategies in a variety of aquatic habitats located in different geographic areas can reflect the complexity of these environments, and therefore, can help to understand habitat suitability, the success of recruitment, and eel productivity [96,97]. However, these results, allowed the discrimination of local eel subpopulations, corroborating the hypothesis that ecological and morphological differences in otolith shape are influenced by the specific environments inhabited by the species [7]. Eels with rounder otoliths were found in freshwater riverine habitats. These environments generally tend to be less susceptible to variations in salinity or temperature and depth than brackish estuaries or lagoons [98]. However, it remains unknown how environmental abiotic variables may act together, influencing or limiting the growth of the species [29].

Although the European eel is protected according to regional, national, and international regulations, and despite its commercial importance, little has been published on the ecology of this species in Sardinia [53,57,60,98,99]. There are no studies that have analyzed eels' sagittal otolith shape in Sardinian continental waters, including rivers and lagoons. Furthermore, only one study has investigated the relationship between otolith and growth in the European eel in the Porto Pino lagoon [60].

All the differences found in sagittal otolith shape among the studied sites could lead to changes between different local stocks and they could be related to environmental peculiarities. However, establishing a direct correlation between environmental factors and variations in otolith morphology poses challenges. Further studies are necessary to investigate the relationship between habitat type/environmental variability and the growth/body characteristics of eels. Studies should also be conducted taking into consideration intraspecific variability in terms of sex and size, when possible, which is representative of an entire local eel subpopulation. Therefore, because otolith shape has been studied for the European eel in some European areas [37,52], successfully discriminating eels that grow in different habitat types, we also support that this method can be considered to be a valuable tool for studying the species' ontogeny.

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Institutional Review Board Statement: This study makes use of otoliths (or otolithic organs). For the study of otoliths, it is necessary to extract them from the head of dead animals. As reported in Directive 2010/63/EU and in the Italian Legislative Decree 26/2014 in the implementation of the Directive 2010/63/EU, the suppression of animals for the sole purpose of using their organs or tissues, as in our case, is excluded among the actions considered to be procedures. Therefore, we have not applied any procedures on animals to be killed. The ethical approval should be waived.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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