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SUMMARY

The impacts of non-native species might be noticeable years after their initial introduction. Determining the age of a recently established non-native fish population can be important to identify the year of its introduction. Although well-established, the accuracy and reproducibility of age determination techniques are not often discussed. In this manuscript, we propose a standardised method for the determination of age in scale-less fishes. We use the channel catfish *Ictalurus punctatus* as a model species and we highlight the importance of the pectoral spine section location to produce accurate age determinations. Our results show that decalcification is not necessarily needed and that cuts to the basal section of the spine shaft produce the clearest and most reliable results.

Keywords: Pectoral spine section location, annuli, population assessment, age determination, scale-less fish

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INTRODUCTION

Invasions of freshwater ecosystems by non-native species may go unnoticed due to the hidden nature of aquatic habitats (García-Berthou et al. 2005; Brundu et al. 2013). Invasions can often be stopped shortly after the introduction of a non-native species (Simberloff et al. 2013), however, due to delayed detection within aquatic ecosystems, eradication frequently becomes unfeasible.

One such invasion is by the North American channel catfish *Ictalurus punctatus* (Rafinesque, 1818), a species that has inconspicuously established dense populations in various European waters (Ligas 2008), being present in several countries, including Serbia (Olenin et al. 2008), Slovakia (Kosco et al. 2004), Greece (Zenetos et al. 2009), and Germany (Kottelat & Freyhof 2007). Moreover, non-native populations have also been known to occur in Mediterranean regions such as the Californian watersheds (Moyle 2002), Portugal (Banha et al. 2017) and Italy (Ligas 2008; Haubrock et al. 2018a, b). This species is thought to have been introduced in Europe for angling purposes (Appelget & Smith 1951; Rezk et al. 2003; Kottelat & Freyhof 2007) and aquaculture (Welcomme 1988; Elvira & Almodóvar 2001; Savini et al. 2010). However, due to its tolerance to various environmental conditions and high fertility rates, it has often become invasive (Moyle 2002). Although the optimal water temperature for this species is between 28 and 30 °C (Cheetham et al. 1976), it shows a wide tolerance for very low (2 - 4 °C; Moss & Scott 1961) and very high (36.6 – 37.8 °C; Allen & Strawn 1968) temperatures. This tolerance has allowed this species to endure the highly variable temperatures of Mediterranean riverine systems, thereby allowing it to become established.

Since the 1980s, *I. punctatus* has been reported in Spain, however, it has recently spread into other European freshwater systems including those in Portugal and Italy (Elvira & Almodóvar 2001; López et al. 2012; Banha et al. 2017). Following its introduction to Northern Italy in 1986 for aquaculture purposes (Nocita & Lenuzza 2016), *I. punctatus* now occurs in dense populations within the Po, Piave, Brante and Arno rivers. *Ictalurus punctatus* was first observed within the Arno River (Pisa, Italy) in 1998 (Nocita, personal communication). By 2004, its distribution had spread into the inner-Florence section of the Arno River (Nocita, personal communication), and is now one of several alien species that dominate this river's fish communities (Table 1). Furthermore, *I.* punctatus is the dominant species within the inner-city section of the Arno River and is likely causing adverse negative impacts on the native ecosystem through predation (Boersma et al. 2006; Endo et al. 2015), competition (Hackney 1975; Durham et al. 2005; Shephard & Jackson 2006) and disease transmission (Tyus & Nikirk 1990; Townsend & Winterbourne 1992; Nocita 2007; Nocita & Zerunian 2007; Pool 2007).

Identifying the age of non-native fishes can be a valuable tool when confronted with a new occurrence, as it can provide an indication of when a species was introduced (e.g. Gkenas et al. 2015). Moreover, age determinations can be used to determine if a species is reproducing and how its population is structured (in relation to age-classes or maturity). Thus, it can be shown if a studied population behaves differently, i.e. in different growth patterns, population structure or age at maturity compared to populations in the native environment (De Roth 1965; Ribeiro & Collares-Pereira 2010). Lastly, knowing the age structure of a population can improve the evaluation of a species' invasiveness through specific characteristics (e.g. age at maturity, length-weight relationships) compared with native populations parameters (Bøhn et al. 2004). Hence, information on the age structure of a non-native population is often of considerable importance for management and stakeholders but must be comparable among the study and research field.

Little is known about the use of different age determination methods in non-native scale-less fishes, particularly those that have recently been introduced into southern European watersheds, including fish in the Ictaluridae Gill, 1861 [*Ameriurus melas* (Rafinesque, 1820), *Ameiurus nebulosus*(Lesueur, 1819), *Ictalurus furcatus* (Valenciennes, 1840), *I. punctatus*], Siluridae Rafinesque, 1815 [*Silurus glanis* Linnaeus, 1758] and Cobitidae Swainson, 1838 [*Misgurnus anguillicaudatus* (Cantor, 1842)]. Compared to scaled fish (see e.g. Lux 1971), determining the age of scale-less fish, such as catfish, is usually accomplished by an analysis of vertebrae (Marzolf 1955), otoliths (Sakaris & Irwin 2008) or the less conventional pectoral spines (see e.g. Penha et al. 2004; Carol et al. 2009). Since Siluriformes have relatively small otoliths (Sakaris & Irwin 2008), vertebrae and pectoral spines have been the method of choice in determining their age (Michaletz et al. 2009; Colombo et al. 2010). However, pectoral spines are generally considered as being easier to analyse due to their external positioning (Alexander 1966; Olivev et al. 2011). Surprisingly, various studies use the pectoral spine age determination method but fail at explicitly explaining how samples were prepared (e.g. if samples were decalcified or not; Ashley & Garling 1980; Buckmeier et al. 2002; Penha et al. 2004). The use of pectoral spines has its advantages (e.g. spines are easily accessible, the use of annuli is well practiced) and disadvantages (e.g. discrepancies from estimations using otoliths, variability in young and old specimens), but can be considered the easiest method to apply (Ashley & Garling 1980; Sakaris & Irwin 2008; Olivev et al. 2011). However, there is a lack of detailed information regarding the location of the section used for analysis in order to increase accuracy in age determination (Buckmeier et al. 2002).

Table 1. Fish-species present in the sampled inner-Florence section of the Mediterranean Arno river (Nocita 2001; Nocita 2007; Nocita & Zerunian 2007; pers. observation Phillip Haubrock).

Family	Species	Common name	Abundance	Status
Anguillidae	*Anguilla anguilla* (Linnaeus, 1758)	European eel	Low	Native
Cyprinidae	*Abramis brama* (Linnaeus, 1758)	Common bream	Low	Non-native
	Alburnus alburnus (Linnaeus, 1758)	Common bleak	High	Non-native
	Barbus barbus (Linnaeus, 1758)	Common barbel	Low	Non-native
	Carassius auratus (Linnaeus, 1758)	Goldfish	Low	Non-native
	Cyprinus carpio Linnaeus, 1758	Common carp	High	Non-native
	Pseudorasbora parva (Temminck & Schlegel, 1846)	Topmouth gudgeon	High	Non-native
	Squalius cephalus (Linnaeus, 1758)	Common chub	Low	Non-native
	Tinca tinca (Linnaeus, 1758)	Tench	Low	Non-native
Ictaluridae	*Ictalurus punctatus* (Rafinesque, 1818)	Channel catfish	High	Non-native
Siluridae	*Silurus glanis* Linnaeus, 1758	European catfish	High	Non-native
Poeciliidae	*Gambusia holbrooki* Girard, 1859	Eastern mosquitofish	Low	Non-native
Gobiidae	*Padogobius bonelli* (Bonaparte, 1846)	“Ghiozzi Padano”	Locally high	Non-native
	Padogobius nigricans (Canestrini, 1867)	“Ghiozzo dell' Arno”	Low	Native
Centrarchidae	*Lepomis gibbosus* (Linnaeus, 1758)	Pumpkinseed	Locally high	Non-native
Percidae	*Sander lucioperca* (Linnaeus, 1758)	Pikeperch	Low	Native

With the invasion by *I. punctatus* gaining attention in Europe (Ligas 2007, 2008; Banha et al. 2017; Haubrock et al. 2017, 2018a, b), a clear description of how pectoral spines can be analysed for an exact age determination is needed for future studies. For this purpose, we compared age determination based on different spine characteristics, such as section location and thickness. We also present the first age information about this non-native fish in a Mediterranean freshwater ecosystem in Southern Europe. With this information, we aim to propose a replicable basis for future age and growth studies on scale-less fish.

MATERIALS AND METHODS

Study Area

The Arno River is 241 km long and its watershed covers approximately 8200 km². It is the most important freshwater river in Central Italy after the Tiber River, with a mean annual discharge of about 110 m³/s (see Nocita & Zerunian 2007). It presents a discharge regime that is typical of Mediterranean systems due to the severe flow reduction during summer. The Arno River is sectioned by various weirs in Florence and presents a high density of aquatic vegetation during spring, although the riparian vegetation is almost always absent from riverbanks. Furthermore, the Bilancino reservoir in the north of Tuscany opens into the Sieve River, a tributary of the Arno river. With the use of floodgates, the water regime is controlled during winter periods to prevent floods and in summer, to regulate water levels in dry periods. In summer, the water of the Arno River in the inner-city section of Florence has a mean temperature of 29.6 °C (min: 27.2, max: 32.4; data from 2016-2018 http://www.arpat.toscana.it), while information on water temperatures from the winter period are not available. However, sheets of ice and temperatures below the thermocline are not uncommon in January (personal communication Gianna Innocenti).

Sampling

Samples of *I. punctatus* from the inner-city section of the Arno River (Figure 1; 43°45'49.9"N, 11°18'04.2"E) were collected in April 2018 using standard fishing rods (2.40 m), 0.42 mm braided line and size 2 fishing hooks baited with various items (cuttlefish, worms, liver, etc.). Bait was cast into the faster flowing current in the middle-water to drift downstream with the current. A total of 28 fish were caught, placed on ice, and transported to the laboratory of the Department of Biology at the Natural History Museum "La Specola" in Florence.

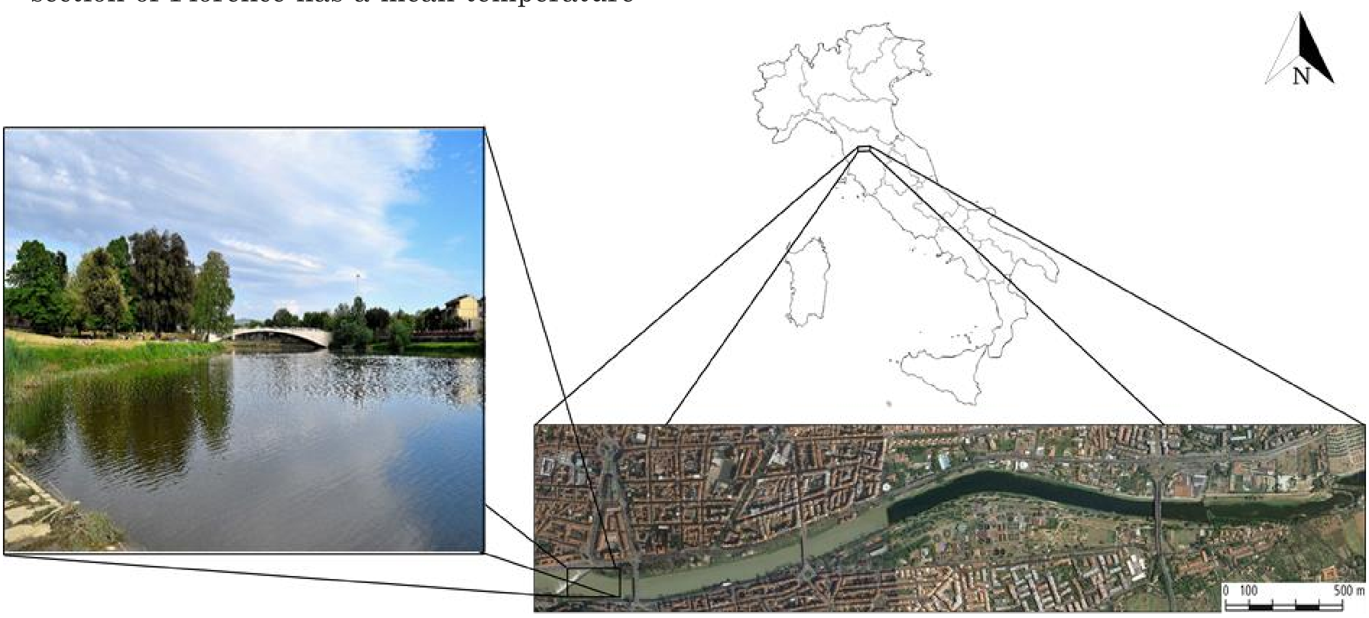

Figure 1. Sampled inner-Florence stretch of the Arno river.

Sample preparation

Total length (TL, nearest cm) as well as Total weight (TW, nearest g) were measured and are here reported as mean ± SD. Sex was determined using pelvic fin morphology (see Norton et al. 1976) before the pectoral spines were disarticulated from the pectoral girdle by clockwise twisting (Mayhew 1969). Spines were manually cleaned to remove all soft tissues and air-dried overnight without storage in ethanol before being placed into resin filled silicone cups.

Once the resin had cured, spines were sectioned several times (Figure 2). From each section, a thin transversal cut (ideally close to 2mm) was cut off with a standard jewellery saw and adhered to a microscope slide. These sections were sanded down to ~0.5 mm, photographed and subsequently further ground down to ~0.2 mm using fine sandpaper. All spine slices were analysed under a standard stereoscopic microscope with ×6.3 – ×20 magnification. The annuli were identified along the anterior radius (SA), previously indicated by Marzolf (1995) as the most reliable section. Each annulus was assumed to be the end of one growth period. All size measurements were taken with an electronic calliper.

The observed results were then used to determine if differences in the positioning of the cuts can lead to varying age determinations and how the thickness of the spine (~0.2-0.5 mm) slides can affect the readability. Lastly, with this information on the readability of different sections, the estimated ages of most reliable identified section were plotted against two sections that provided highly differing results. The reliability of sections was evaluated based on i) the easiness of preparation and ii) the readability determined as the ability to differentiate / count annuli and an observed consistent distance between annuli.

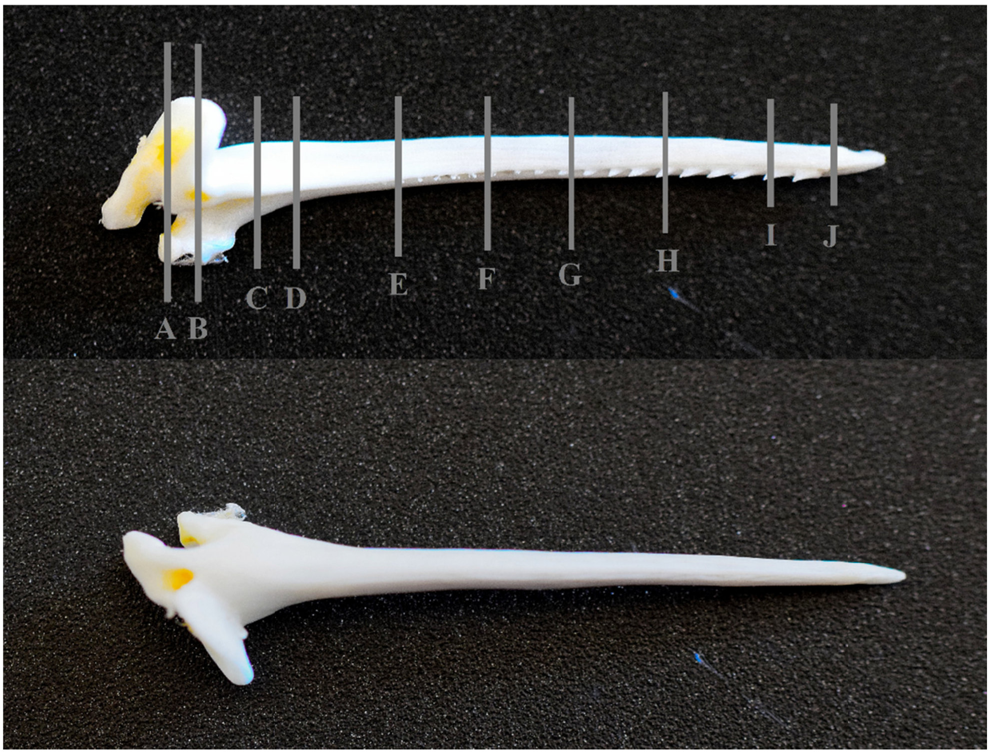

Figure 2. Spine of *Ictalurus punctatus* and indications of the transversal cuts (A-J) made to analyse the annuli, i.e. age

RESULTS

The sampled specimens (n = 28; 17 females, 11 males) had total lengths ranging from 13.5 to 64 cm (33.5 ± 14.4 cm) and total weights between 20 and 3500 g (511.9 ± 106.3 g). From one individual (TL = 64 cm; TW = 3503 g; sex = female), ten successful cuts were produced for each spine and analysed at ~5 and ~0.2 mm (Table 2), revealing equal age determinations between cuts of both pectoral spines and a better readability at ~0.5 mm.

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In 28 specimens, we compared the estimated ages among the cut sections / thickness between 1+ (cut J) and 5+ (cut A, B) years. Considering the spine sections from all specimens, cut section E at the beginning of the closed lumen with a thickness of ~0.5 mm produced the most reliable results. This was based on the clear display of readable annuli at the anterior radius, as well as closed and clearly distinguishable annuli.

Table 2. Results from visual determination of the age from specimen #1. Ten transversal cut sections were chosen (A-J) and analysed at a thickness of ~0.5mm and ~0.2mm.

Section	Thickness		Estimated age	
	~0.5mm	~0.2mm	~0.5mm	~0.2mm
A			4+	4+
B			4+	4+
C			4+	3+
D			3+	3+
E			3+	3+
F			4+	2+
G			2+	2+
H			2+	2+
I			1+	2+

In contrast, annuli in cut sections A-D were not closed, negatively affecting the cutting process and readability. Moreover, annuli at different radiuses were variable in their clarity and definition. Plotting the estimated ages (~0.5 mm) at cut position E from all specimens against the respective ages at cut sections I (distal) and A (basal) revealed considerably varying results for the determination of age (Figure 3, Table 3). The varying trends highlighted higher age determinations when using cut section A, compared to that of the relied upon distal cut section I.

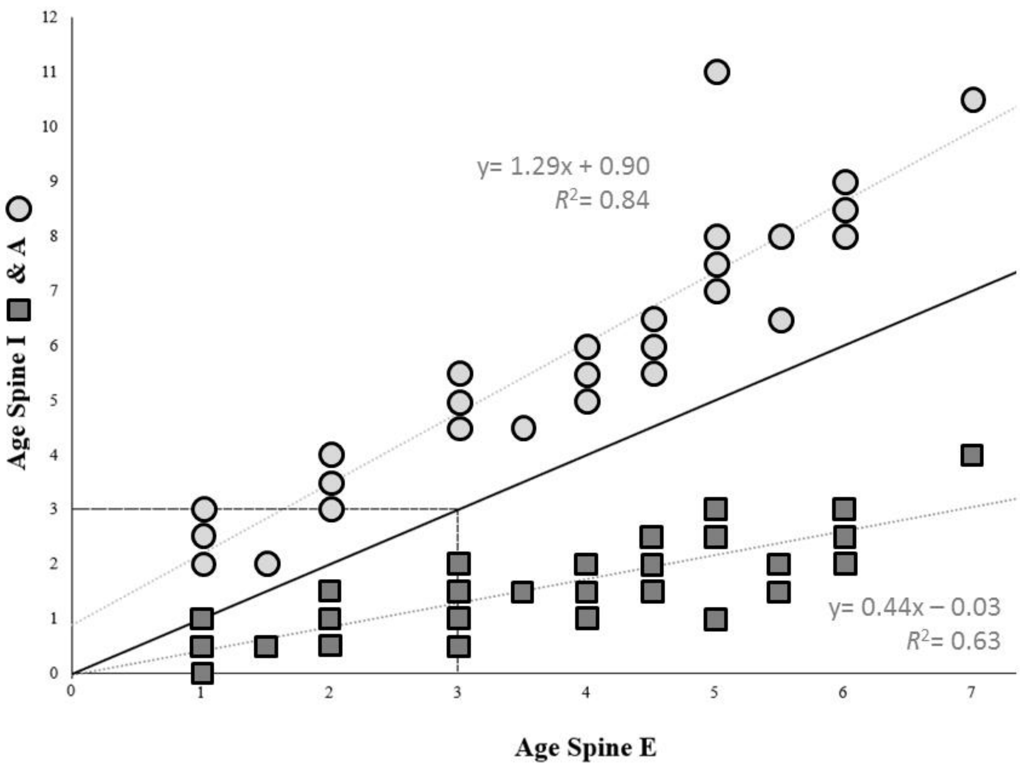

Figure 3. Age determined for section E (interpreted as the most reliable) plotted against the two variable sections I (dark grey squares) and A (light grey dots), also showing the 1:1 line (solid line).

DISCUSSION

Our results highlight the importance of a standardized method in determining the age of scale-less fishes, by selecting the same spine section and a repeatable protocol in spine section thickness. Moreover, this work presents, for the first-time, data on age and length (and thus, growth) of *I. punctatus* in Europe, which must be addressed in more detail in future research.

Considering the high abundance of *I. punctatus* in the study area, suitable growing conditions can be assumed. The investigated individual's spines that were cut at ten different sections was identified to be 3+ years of age. This individual was considerably larger than the other sampled individuals of the same age (Figure 4) as well as individuals from native populations (Finnel & Jenkins 1954; Bouska et al. 2011). The growth rates of *I. punctatus* tend to be affected by water temperature, length of vegetative season, availability of prey and competition, accounting for the high variability in growth rates of this species in its native range. Therefore, warmer water released from pipelines at multiple locations in the Arno River offers abundant prey throughout the year (algae, small fish, etc.), allowing for faster growth. This links to observations by Reebs (2002), who states that these warmer water areas are monopolized by large *I. punctatus* individuals, potentially leading to exaggerated growth rates in individual specimens.

Table 3. Age determination of 28 specimens of *I. punctatus* at a spine thickness of ~0.5 mm for three different pectoral spine cut sections (E, I & A); the "+" indicates that the specimen has entered a new growth / vegetative season.

Specimen	Age I	Age E	Age A	Total length (cm)
1	1+	3+	4+	64.0
2	1	3	5	27.1
3	2	3	4+	18.4
4	0+	2	3+	17.8
5	1	2	3	22.3
6	1+	2	4	19.4
7	1	1	2	13.5
8	0+	1	2+	15.2
9	0	1	3	14.2
10	0+	1+	2	16.3
11	1	4	6	29.5
12	1+	4+	5+	45.5
13	2	4	5	28.3
14	2+	4+	6+	29.7
15	3	5	11	42.1
16	2+	5	7	40.9
17	2	5+	6+	47.4
18	1+	5+	8	48.5
19	1	5	7+	37.3
20	0+	3	5+	24.3
21	2+	6	8	43.3
22	2	6	8+	36.2
23	3	6	9	55.6
24	1+	4	5+	37.8
25	1+	3	5+	25.6
26	2	4+	6	30.1
27	3	5	8	59.0
28	4	7	10+	48.3

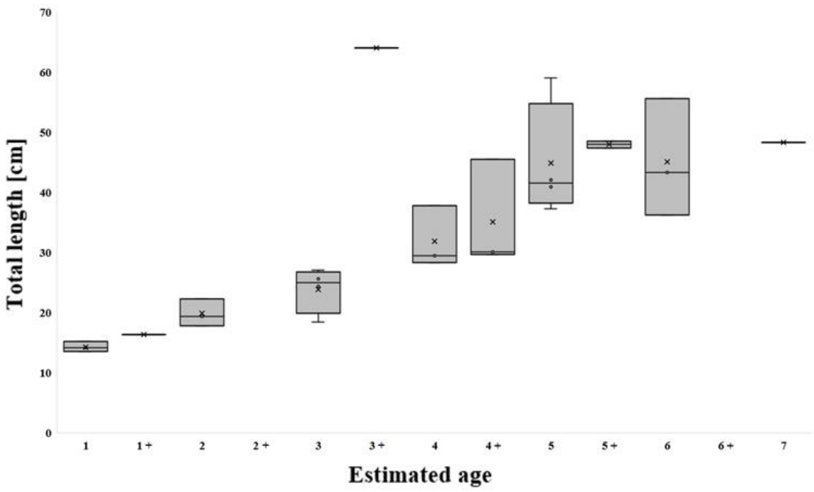

Figure 4. Channel catfish ages determined at section E in relation to total length of the respective age groups. Whiskers indicate minimum and maximum lengths and boxes indicate 25% and 75% quartiles. The "+" indicates that the specimen has entered a new growth / vegetative season

Our results highlight the high variability between the number of observed annuli at the basal sections A-C in respect to the more distal sections G-J. However, the readability of annuli is considerably affected by the open shaft at basal cut sections, limiting the surface at which annuli can be counted. Additionally, the thickness of produced cuts affected the readability, because cuts that were too thin resulted in indistinguishable annuli. This high variability between estimated ages among sections means that the introduction of *I. punctatus* goes back between 11 (section A), 7 (section E) or 3 years (section I). As no individuals older than 14 years were identified in our study, our results imply that this species either reproduced or it was continually reintroduced after 2004, the year it was first observed in the inner-city of Florence. Furthermore, the observed 8-year variability in specific sections of spines underpins the importance of exact age determination by following a reproducible protocol. Moreover, the observed differences have implications for other studies investigating the age of Ictaluridae by means of pectoral spines. While Buckmeier et al. (2002) followed a similar procedure for the preparation and reading of the pectoral spines for the age determination of *I. punctatus*, the authors used the distal end of the spine and cut through the dorsal and anterior processes. As a result, it was noted that spines and otoliths led to varying but not significantly different results. The differences between the otoliths and pectoral spines highlighted by these authors likely originated because section A was the spine used for age determination. Our results verify this due to the variability of annuli among different spine sections, specifically section A.

In conclusion, we highlight the variability of results obtained from different sections of *I. punctatus* spines and difficulties interpreting the annuli, especially those in cuts from distal spine sections. We propose a standardized procedure to accurately determine the age of scale-less fish by cutting the pectoral spine along the upper most distal shaft with a closed lumen (position E), without the need for decalcification. Lastly, due to the small sampling size, future stud-

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ies should enlarge the dataset and broaden the number fish species specifically, catfishes. Future research in this field could aid in the evaluation of the invasiveness of this species, particularly in Mediterranean rivers, allowing for the improvement of management and risk assessments in areas with highly endangered endemic fishes (Smith & Darwall 2006).

AUTHORS CONTRIBUTIONS

E.T., A.I. and P.J.H. designed the study and E.T. and A.I. supervised the concept and laboratory work. A.N. supervised the sampling. P.J.H., P.B. and I.F. conducted the laboratory work with valuable input from I.J. and A.N. Spines were analysed and interpreted by P.J.H., P.B., I.F., I.J. N.J.B. and A.N. P.J.H. and N.J.B. were responsible for the writing and editing of the manuscript.

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