RESEARCH ARTICLE

Hearing capacities and otolith size in two ophidiiform species (*Ophidion rochei* and *Carapus acus*)

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ABSTRACT

Numerous studies have highlighted the diversity of fish inner ear morphology. However, the function of the shape, size and orientation of the different structures remains poorly understood. The saccule (otolithic endorgan) is considered to be the principal hearing organ in fishes and it has been hypothesized that sagitta (saccular otolith) shape and size affect hearing capacities: large sagittae are thought to increase sensitivity. The sagittae of many ophidiids and carapids occupy a large volume inside the neurocranium. Hence they are a good structure with which to test the size hypothesis. The main aim of this study was to investigate hearing capacities and inner ear morphology in two ophidiiform species: *Ophidion rochei* and *Carapus acus*. We used a multidisciplinary approach that combines dissections, μCT-scan examinations and auditory evoked potential techniques. *Carapus acus* and *O. rochei* sagittae have similar maximal diameters; both species have larger otoliths than many non-ophidiiform species, especially compared with the intra-neurocranium volume. Both species are sensitive to sounds up to 2100 Hz. Relative to the skull, *O. rochei* has smaller sagittae than the carapid, but better hearing capacities from 300 to 900 Hz and similar sensitivities at 150 Hz and from 1200 to 2100 Hz. Results show that hearing capacities of a fish species cannot be predicted only based on sagitta size. Larger otoliths (in size relative to the skull) may have evolved mainly for performing vestibular functions in fishes, especially those species that need to execute precise and complex movements.

KEY WORDS: Audition, Morphology, Otolith, Pearlfish

INTRODUCTION

Natural abiotic and biotic sounds as well as anthropogenic sounds are very abundant in underwater environments (Slabbekoorn et al., 2010; Ladich and Schulz-Mirbach, 2013). Animal sound sources mainly result from invertebrates (Fish, 1964; Cato, 1993), marine mammals (Fish, 1964; Cato, 1993) and fishes (Fish, 1964; Steinberg et al., 1965; Myrberg, 1981; Ladich et al., 2006). In this context, the ability to detect, discriminate and identify surrounding sounds is crucial for fish survival and reproduction (Fay and Popper, 2000; Slabbekoorn et al., 2010). During the last few decades, biologists have given increased attention to the hearing capacities of teleost fishes (Popper et al., 2005; Ladich and Fay, 2013). Although these studies have informed scientists on the hearing capabilities and inner ear morphology in fishes, they also raised new questions concerning

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hearing mechanisms and the evolution of the fish inner ear (Popper and Fay, 1999; Popper et al., 2005; Higgs and Radford, 2013).

The fish inner ear is composed of three semicircular canals and three otolith organs (Popper and Fay, 1999; Ladich and Popper, 2001; Popper et al., 2005). The semicircular canals and their associated sensory epithelium (cristae) are thought to help in the perception of angular accelerations, whereas the three otolith organs (saccule, utricule and lagena) are thought to be mainly involved in hearing and in the perception of linear accelerations (Popper et al., 2005; Webb et al., 2008). Among the three otolith organs, the saccule is often considered to be the most involved in hearing (Popper et al., 2005). Sensory hair cells of an otolith organ are grouped in an epithelium called the macula (Popper and Lu, 2000) and are sensitive to particle motion, being excited by the differences in the displacement between otoliths and soft tissues (Popper and Fay, 1999). In many fish species, the pressure component of sound intervenes only indirectly with hearing because it is converted into particle motion by means of displacement of a gas bubble (e.g. the swimbladder) (Popper and Fay, 1999). This indirect stimulation pathway mainly affects hearing capacities if such a gas bubble comes close to or is directly connected to the inner ears (Popper and Fay, 1999). The ability to efficiently process sound pressure generally improves fish hearing capacities (see Popper and Fay, 1999; Ladich and Wysocki, 2003).

Morphological specializations that improve hearing have been highlighted in many teleost families (Webb et al., 2008). Interestingly, many fishes evolved a simple way for enhancing their auditory sensitivity: they displaced or extended the anterior part of the swimbladder toward the otic capsule (Coombs and Popper, 1979; Ramcharitar et al., 2006a; Braun and Grande, 2008; Parmentier et al., 2011a; Parmentier et al., 2011b; Ladich and Schulz-Mirbach, 2013), improving sound transmission from gas bubbles to the inner ear (see Popper and Fay, 1999; Ladich and Wysocki, 2003). Otophysans (catfish, carp, piranhas and relatives) have developed a specialized hearing structure called the Weberian apparatus (Evans, 1925; Ladich and Wysocki, 2003; Braun and Grande, 2008; Lechner and Ladich, 2008). This structure consists of a modified chain of bones (Weberian ossicles) that connects the swimbladder to the otic capsule (Bridge and Haddon, 1889; Ladich and Wysocki, 2003). Historically, fish species displaying anatomical structures that connect or bring a gas bubble in close proximity to the inner ears were called 'hearing specialists', and species without such features were termed 'hearing generalists' (Popper and Fay, 1999; Lechner and Ladich, 2008; Popper and Fay, 2011). This dichotomy was also based on the belief that the absence of specific otophysic connections ('hearing generalists') made fishes insensitive to the pressure component of sound (Popper and Fay, 2011). However, some species without accessory hearing specializations were shown to respond to the pressure component of sound (Popper and Fay, 2011). Their sensitivity to this sound component was low and varied between species (Ladich and Fay, 2013). Popper and Fay (Popper and Fay, 2011) proposed to illustrate the relative importance

of pressure detection mechanisms in fish hearing by a continuum of variations between two extreme situations: fishes with no gas bubbles (only particle motion detection) and otophysan fishes (extensive use of the pressure component).

Some adaptive changes of the inner ear, especially at the level of the otolith organs, should also improve hearing capacities. To date, at least eight out of the 33 species of Carapidae (Nielsen et al., 1999; Parmentier, 2004; Anderson, 2005; Parmentier, 2012) have been studied because of their wide otic cavity (Parmentier et al., 2001). The sagittae (saccular otoliths) and, consequently, the saccules of these species are particularly large and occupy nearly the entire otic cavity, pushing the brain forward with only a thin myelencephalon at the level of the otic cavity (Parmentier et al., 2001). Large saccules or sagittae were also observed in other Ophidiiformes including some Ophidiidae, e.g. *Acanthonus armatus* (Fine et al., 1987) and *Ophidion rochei* (Lychakov and Rebane, 2000). Some authors (Wilson, 1985; Lombarte and Lleonart, 1993; Nolf, 1993; Paxton, 2000; Parmentier et al., 2001; Cruz and Lombarte, 2004; Lombarte and Cruz, 2007; Lombarte et al., 2010) have suggested that sagitta shape and size are adaptations to different habitats or ecological niches. In carapids, the thickest and heaviest sagittae are found in benthic or parasitic species whereas thin otoliths are found in pelagic species (Parmentier et al., 2001; Parmentier et al., 2002). It was also hypothesized that otolith size and shape may affect hearing capacities (Wilson, 1985; Nolf, 1993; Paxton, 2000; Popper et al., 2005). For example, the greater the otolith mass, the better the hearing sensitivity at lower frequencies (Lychakov and Rebane, 2000). However, the factors that drive the evolution of fish inner ear morphology remain poorly understood.

This study focuses on two vocal ophidiiform species (Parmentier et al., 2003; Parmentier et al., 2006b; Parmentier et al., 2010; Kéver et al., 2012): *Carapus acus* (Brünnich 1768) (Carapidae) and *O. rochei* Müller 1845 (Ophidiidae). *Carapus acus* is a holothurian commensal from the Eastern Atlantic Ocean and Mediterranean Sea (Nielsen et al., 1999). *Ophidion rochei* is a sand-dwelling species found from 0 to 150 m in the Mediterranean and Black Seas (Jardas, 1996; Matallanas and Casadevall, 1999). Both species are nocturnal and can leave their 'shelter' in order to seek food or sexual partners (Meyer-Rochow, 1979; Matallanas and Riba, 1980; Jardas, 1996). Despite their particular anatomy, hearing abilities have never been described in these two families. The main aim of this study was to investigate hearing capacities and inner ear morphology in these two ophidiiform species. These data should provide a basis to consider the possible benefit offered by the presence of large sagittae in the fish inner ear.

RESULTS
Morphology

The combination of dissections and CT scans allowed a precise description and illustration of structures of interest. Position, shape and size of the brain, semicircular canals, otoliths and otolith organs are quite similar in both species. Sagittae occupy a substantial portion of the otic capsule while the brain is mainly restricted to the anterior part of the skull (Figs 1–3). The maximal diameter and thickness of the sagittae were similar between *C. acus* and *O. rochei*, while their volumes were higher in *O. rochei* (Table 1). However, the sizes relative to the skull showed differences in diameter [sagittae maximal diameter/head length (SD/HL)], thickness [sagittae maximal thickness/head length (ST/HL)] and volume [volume of the sagittae/intraneurocranium volume: (VolS/VolINC)]: *C. acus* has larger sagittae than *O. rochei*. The ophidiiform sagittae (including *Onuxodon fowleri*) were more voluminous proportionally to the head than in other measured species (Tables 1, 2). The sagitta volume was between 12.7% and 25.2% of the neurocranium volume in Ophidiiformes and less than 4% in other species tested (Tables 1, 2). *Carapus acus* showed the highest VolS/VolINC ratio, with a mean value of 23.4%.

According to Volpedo and Echeverría (Volpedo and Echeverría, 2003), the E index corresponds to overall otolith shape: sagittae with high E index values tend to be more circular (versus elongate). Sagittae of *O. rochei* were rounder than those of *C. acus* (E index of ca. 74% and 55%, respectively).

In *C. acus* and *O. rochei*, asterisci (lagenar otoliths) and lapilli (utricular otoliths) are extremely small compared with the sagittae (Figs 2, 3). Their position, shape and location in the neurocranium are similar in both species (Figs 2, 3). Otolith organs appeared to be well individualized in *C. acus* and *O. rochei* (Fig. 3). Lagenae, for example, were connected to the rest of the inner ear by a long canaliculus (Fig. 3).

The semicircular canals of *C. acus* and *O. rochei* have a similar orientation, but they appeared relatively different. In the former species, they were longer along the antero-posterior axis but lower in the dorsoventral axis.

There are several bony structures or extensions inside the neurocranium of *C. acus* and *O. rochei* (Fig. 2G,H, Fig. 3). The basioccipital stalk, for example, maintains the lagenae close to a
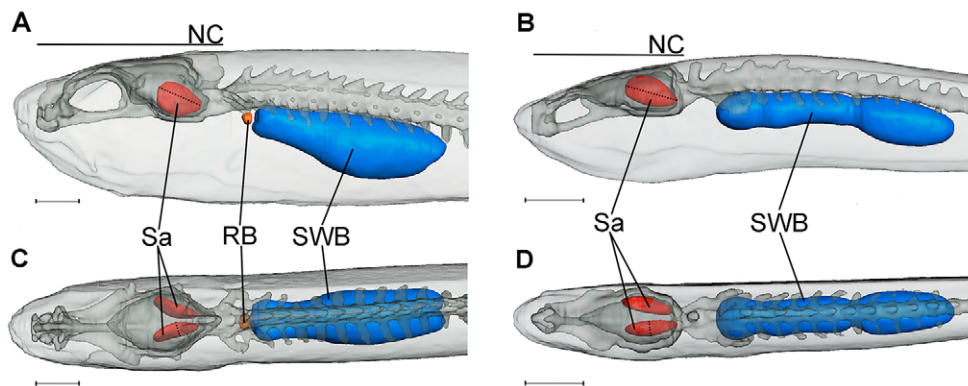

Fig. 1. Three-dimensional reconstruction of the anterior part of *Ophidion rochei* and *Carapus acus*. (A,B) Left lateral and (C,D) dorsal views of the reconstructed anterior part of (A,C) *O. rochei* and (B,D) *C. acus*. The 3D reconstruction of saccular otoliths (red), swimbladder (blue), neurocranium (gray), vertebral column (gray) and body limits (light gray) is based on CT scans. For *O. rochei*, the growing rocker bone (orange) was also reconstructed. Dotted lines correspond to (A,B) the maximal diameter or (C,D) the maximal thickness of the sagittae. NC, neurocranium; RB, rocker bone; Sa, sagitta; SWB, swimbladder. Scale bars: 5 mm.
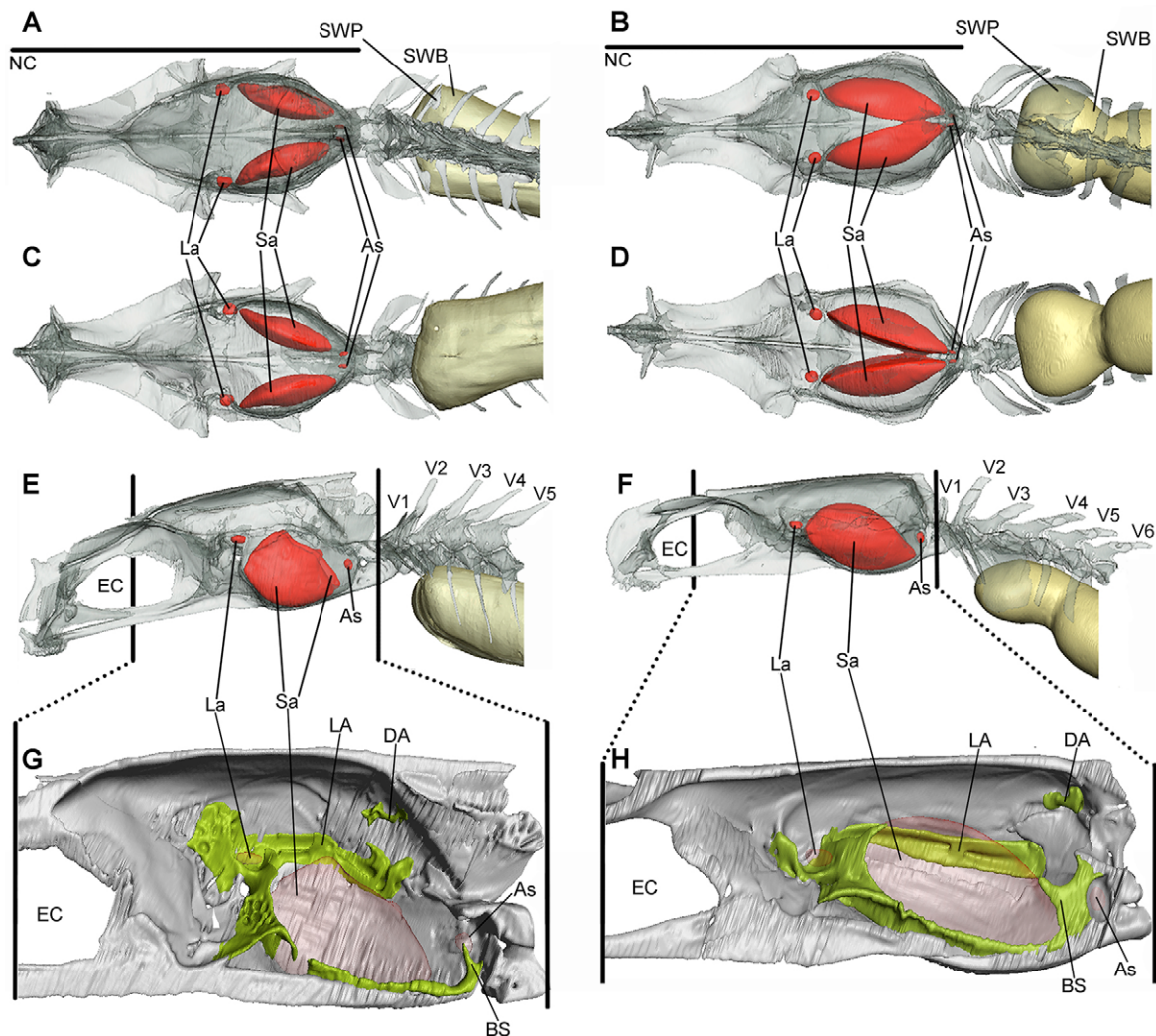
Fig. 2. Three-dimensional reconstruction of the neurocranium and otoliths of *Carapus acus* and *Ophidion rochei*. (A) Dorsal, (C) ventral and (E) left lateral views of the reconstructed anterior part of a female *O. rochei*. (B) Dorsal, (D) ventral and (F) left lateral views of the reconstructed anterior part of a *C. acus*. (G,H) Left lateral views of the right half of the intra-neurocranium of (G) *O. rochei* and (H) *C. acus*. The bones that help to maintain inner ear structures are highlighted in green. The 3D reconstruction is based on µCT scans. As, asteriscus; BS, basioccipital stalk; DA, dorsal arch; EC, eye cavity; La, lapillus; LA, lateral arch; NC, neurocranium; Sa, sagitta; SWB, swimbladder; SWP, swimbladder plate; V1–6, vertebrae 1–6. Scale data: maximal diameter of sagitta is 4.3 and 5.5 mm in *O. rochei* and *C. acus*, respectively.

small depression called the foramen of the asteriscus, located ventrally to the foramen magnum (Figs 2, 3). In addition, two bony arches were observed on each side of the neurocranium of both species (Figs 2, 3). The posterior arch is on the epiotic and is crossed by the posterior semicircular canal. The lateral arch is found on the pterotic and is crossed by the lateral semicircular canal (Figs 2, 3). Lastly, a small groove that corresponds to the position of the lateral semicircular canal in the neurocranium was observed in *C. acus* and *O. rochei* (Fig. 2). Thus, the main function of bony structures inside the neurocranium is to preserve the configuration of the inner ears.

The remaining INC spaces are devoted to the brain, which is small and mostly restricted to the anterodorsal region in both species (Fig. 3). At the level of the lagenae and the posterior half of the saccules, there is only the medulla oblongata (Fig. 3).

Hearing

Carapus acus and *O. rochei* exhibited clear responses to sound frequencies ranging from 150 to 1800 Hz (Fig. 4). In addition, 10 of the 12 *O. rochei* and nine of the 10 *C. acus* showed a response to

high intensity sounds at 2100 Hz (Fig. 4). Although *C. acus* and *O. rochei* are sensitive to the same frequency range (Fig. 4), they showed significant differences in their overall mean auditory thresholds (Table 3). *Post hoc* tests (Tukey's HSD) revealed that *O. rochei* was more sensitive than *C. acus* at 300 Hz ($P<0.04$), 600 Hz ($P<0.001$) and 900 Hz ($P<0.001$). At 600 Hz (*O. rochei* best hearing sensitivity), the difference between the mean hearing thresholds exceeded 20 dB SPL (Table 4).

Standard deviations were relatively high in both species (Table 4): from 8.1 dB SPL (at 150 Hz) to 16.5 dB SPL (at 900 Hz) in *O. rochei* and from 9.1 dB SPL (at 2100 Hz) to 14.1 dB SPL (at 900 Hz) in *C. acus*. The general linear model with repeated measures indicated no effect of total length (TL) on overall mean thresholds, but a significant ($P=0.045$) effect of TL by frequency suggested an effect of size at some frequencies (Table 3). In both species, linear regressions were performed between fish TL and hearing thresholds at each frequency tested. The slopes of the linear regressions did not differ significantly from 0, and there was no correlation between fish size and hearing threshold ($r^2<0.2$).
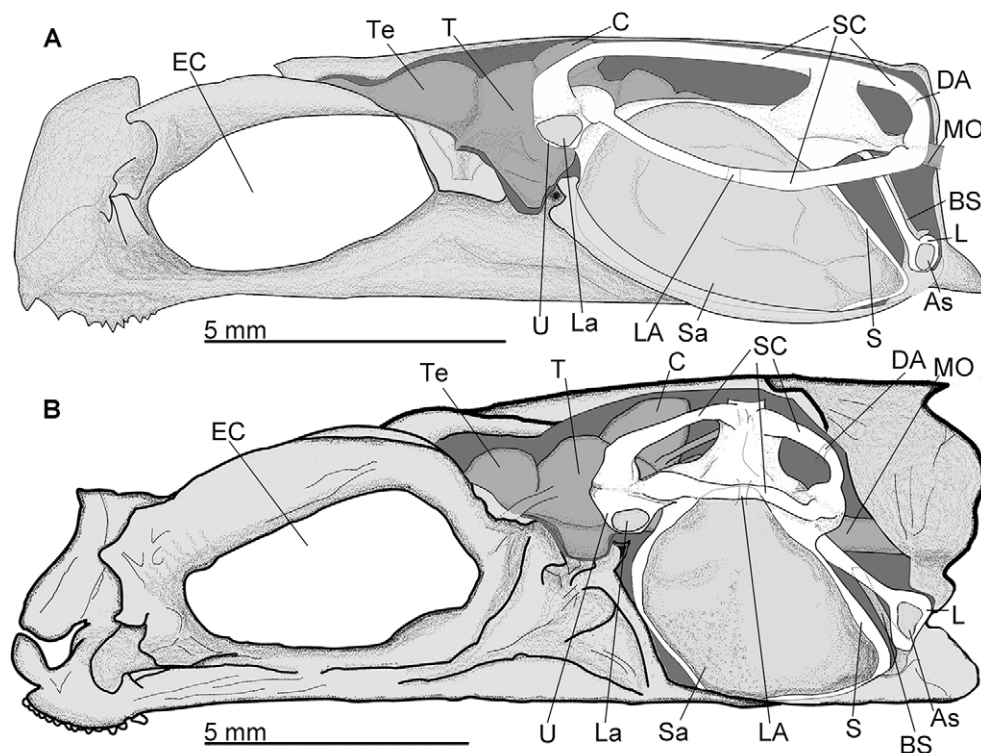
Fig. 3. Morphology of the inner ear, brain and skull of *Carapus acus* and *Ophidion rochei*. Left lateral views show the morphology and the position of the brain and the left inner ear in the neurocranium of (A) *C. acus* and (B) *O. rochei*. As, asteriscus; BS, basioccipital stalk; C, cerebellum; DA, dorsal arch; EC, eye cavity; L, lagena; La, lapillus; LA, lateral arch; MO, medulla oblongata; S, saccule; Sa, sagitta; SC, semicircular canals; T, tectum; Te, telencephalon; U, utriculus.

DISCUSSION

Sagitta morphology: general considerations

According to Paxton (Paxton, 2000) and Lombarte et al. (Lombarte et al., 2010), interspecific differences in otolith size are more likely related to environmental and physiological constraints than phylogeny. Nolf (Nolf, 1985; Nolf, 1993) has also reported examples of convergence in otolith shape among unrelated teleost families sharing similar ecological niches during their life span. In Carapidae, pelagic Pyramodontinae have thinner sagittae and smaller labyrinths than the other carapids, and the shape of these organs resembles that of fast swimmers such as Gadidae, Merlucciidae and Macrouridae (Parmentier et al., 2001; Parmentier et al., 2002). In contrast, commensal and parasitic species have the thickest sagittae, lodged in the proportionally largest otic cavity (Parmentier et al., 2001; Parmentier et al., 2002). Volpedo and Echeverría (Volpedo and Echeverría, 2003) also studied sagitta shape and noticed that sagittae with a relatively high E index and with an absent or dull rostrum generally belong to benthic species associated with soft substrates. *Carapus acus* and *O. rochei* are benthic species associated with soft substrates and the shape of their sagittae is in agreement with these observations (Fig. 2). The sagittae of both species are unusually large compared with head length and neurocranium volume, limiting the space devoted to the remaining inner ear structures and brain (Table 1, Figs 1–3). In the present study, the volume of sagittae (*O. rochei*, *C. acus* and *O. fowleri*) occupied at least 12.7% (up to 25.2% in *C. acus*) of the INC volume in ophidiiform species, whereas sagittae volume of the other species is less than 4% of the INC volume (Tables 1, 2). Other authors also highlighted that saccules are large relative to the skull in all ophidiid (Marshall, 1971; Fine et al., 1987) and carapid (Parmentier et al., 2001) species investigated to date. Unfortunately, the VolS/VolINC ratio has never been reported in previous studies. However, comparisons with other teleost inner ears photographed or drawn together with the neurocranium seem to reinforce our observations (e.g. Wilson, 1985; Popper and Lu, 2000; Yan and Curtsinger, 2000; Yan et al., 2000; Lovell et al., 2005; Webb et al., 2006; Maruska et al., 2007; Popper and Fay, 2011; Schulz-Mirbach et al., 2011; Schulz-Mirbach et al., 2012).

Ideally, the study of the relationships between the characteristics of a species and the particularities of one of its morphological systems requires firstly a complete knowledge of all the biological roles the morphological system fulfills in the natural environment of

Table 1. Absolute and relative sizes of sagittae in *Ophidion rochei* and *Carapus acus*

	TL (mm)	HL (mm)	SD (mm)	ST (mm)	VolS (mm³)	SD/TL (%)	SD/HL (%)	ST/HL (%)	VolS/VolINC (%)
C. acus A	149	15.3	4.8	1.4	16.9	3.2	31.4	9.2	25.2
C. acus B	143	17.3	4.7	1.3	14.4	3.3	27.1	7.5	22.1
C. acus C	161	21.2	5.5	1.6	21.6	3.4	25.8	7.5	22.8
Mean values	151	17.9	5.0	1.4	17.6	3.3	28.1	7.8	23.4
O. rochei A	161.5	25.9	5.3	1.5	27	3.3	20.5	5.8	16.1
O. rochei B	185	32.8	5.7	1.8	35.7	3.1	17.3	5.5	14.1
O. rochei C	135	22.7	4.3	1.3	17.4	3.2	19	5.7	14.5
Mean values	160.5	27.2	5.1	1.6	26.7	3.2	18.9	5.9	14.9

HL, head length; SD, maximal diameter of the sagitta; ST, maximal thickness of the sagitta; TL, total length; VolS, volume of two sagittae; VolS/VolINC, volume of the two sagittae divided by intra-neurocranium volume.

Table 2. Absolute and relative sizes of sagittae in five teleost species: *Onuxodon fowleri* **(Carapidae),** *Epinephelus marginatus* **(Serranidae),** *Chromis chromis* **(Pomacentridae),** *Plectroglyphidodon lacrymatus* **(Pomacentridae) and** *Amphiprion clarkii* **(Pomacentridae).**

	TL (mm)	HL (mm)	SD (mm)	ST (mm)	VolS (mm³)	SD/TL (%)	SD/HL (%)	ST/HL (%)	VolS/VolINC (%)
O. fowleri A	79	10.4	2.1	0.7	1.3	2.7	20.1	6.7	17.4
O. fowleri B	79	9.3	1.8	0.5	2.3	2.3	19.4	5.4	12.7
E. marginatus A	420	143.9	8.9	2.1	195	2.1	6.2	1.5	2.6
E. marginatus B	574	184.2	9.2	3.7	489	1.6	5	2	3.5
C. chromis	ND	23.6	3.6	0.9	7.9	ND	15.3	3.8	3.1
P. lacrymatus	ND	16.9	3.1	0.8	5.3	ND	18.2	4.7	3.6
A. clarkii	84	24.7	3.2	0.7	4.6	3.8	13.1	2.8	1.6

HL, head length; SD, maximal diameter of the sagitta; ST, maximal thickness of the sagitta; TL, total length; VolS, volume of two sagittae; VolS/VolINC, volume of the two sagittae divided by intra-neurocranium volume.
ND, not determined.

the studied species. Moreover, an organism does not consist of one single morphological complex, but of many such complexes, interacting to improve the fitness of the individual in given ecological conditions. The morphology of a species consequently appears as an assembly of functional characters interacting with environmental factors (Parmentier et al., 2002). It results from these considerations that large saccules are expected to have a functional significance for the fishes. This suggests that the sagittae of *O. rochei*, *C. acus* and *O. fowleri* evolved under strong and potentially similar selective pressures. Because otolith organs have two major functions (sound detection and vestibular cues), both should be considered when functional morphology of otoliths is debated.

Sagitta size and hearing capacities

Ophidion rochei and *C. acus* were able to detect sounds up to 2100 Hz (Fig. 4), which is quite high for fishes lacking accessory hearing structures (Ramcharitar et al., 2006a; Vasconcelos and Ladich, 2008; Wysocki et al., 2009; Popper and Fay, 2011). The best sensitivity for *O. rochei* was at 600 Hz. At higher frequencies, the hearing thresholds almost match those of *Eucinostomus argenteus* (Fig. 4), which has swimbladder horn-like extensions corresponding to accessory hearing
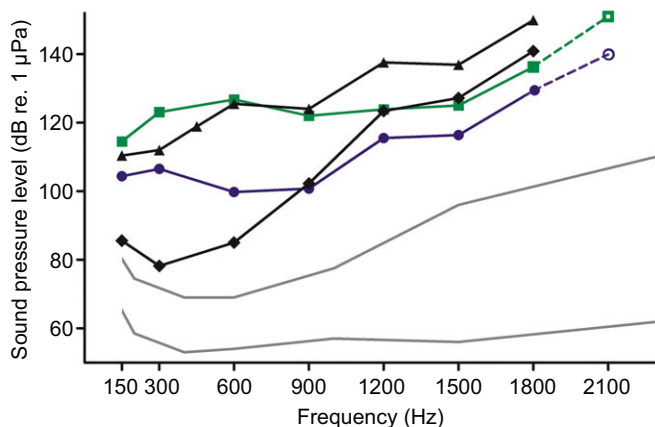

Fig. 4. Audiograms of *Carapus acus*, *Ophidion rochei*, *Eucinostomus argenteus* **(Parmentier et al., 2011a),** *Amphiprion clarkii* **(Parmentier et al., 2009) and** *Carassius auratus* **(adapted from Ladich and Wysocki, 2009).** Mean hearing thresholds of *C. acus* (green squares), *O. rochei* (blue circles), *E. argenteus* (black diamonds) and *A. clarkii* (black triangles). Dashed lines and open symbols were used for frequencies over 1800 Hz because not every fish responded. The area between the grey lines contains the hearing thresholds (between 150 and 2100 Hz) obtained for *C. auratus* by several authors using different techniques (adapted from Ladich and Wysocki, 2009). For greater clarity, standard deviations were not represented.

structures (Parmentier et al., 2011a). A relatively short distance between the swimbladder and otic capsule can increase indirect sound detection, as shown for holocentrids (Coombs and Popper, 1979), sciaenids (Ramcharitar et al., 2006b) and cichlids (Schulz-Mirbach et al., 2012). Two out of the five sciaenid species tested by Ramcharitar et al. (Ramcharitar et al., 2006b; Ramcharitar et al., 2006a) showed a response to sound over 1500 Hz. In these two species, the swimbladder was less than 3 mm away from the otic cavity (Ramcharitar et al., 2006b; Ramcharitar et al., 2006a). In *C. acus* and *O. rochei*, this distance was 2.9±0.2 and 5.3±2.5 mm, respectively (extracted from µCT scans of six fishes).

Among inner ear structures, the saccule is thought to be the main structure implicated in fish hearing (Lu and Xu, 2002; Popper et al., 2005; Sisneros and Bass, 2005). Among fish species, the sagitta shows an important diversity in shape and size (Volpedo and Echeverría, 2003; Popper et al., 2005) that could influence the ability to respond to acoustic stimuli (Paxton, 2000; Cruz and Lombarte, 2004; Ramcharitar et al., 2004). Absolute (e.g. Lychakov and Rebane, 2000; Schulz-Mirbach et al., 2010) and relative (e.g. Paxton, 2000; Lombarte and Cruz, 2007) sizes were both considered in the literature in order to determine the function of otolith size in fish hearing. Larger otoliths (absolute or relative size) are commonly considered to improve hearing capacities (Lychakov and Rebane, 2000; Paxton, 2000; Cruz and Lombarte, 2004; Lombarte and Cruz, 2007).

Heavier sagittae should improve hearing capacities, especially at low frequencies (Lychakov and Rebane, 2000): the sensitivity increases and the frequency at which the otolith amplitude displacement is maximal shifts toward low frequencies with otolith mass. This assumption has, however, never been experimentally demonstrated. Although the mass was not considered in the present study, Lychakov and Rebane (Lychakov and Rebane, 2000) showed

Table 3. General linear model using 'frequency' (150–2100 Hz) as repeated measurements (Rm)

Source	d.f.	MS	F	P
Between-subjects effects				
TL	1	475.201	1.129	0.304
Species	1	9924.425	23.573	**<0.001**
Error	16	421.006		
Within-subjects effects				
Rm (Frequency)	7	87.65	1.085	0.378
Rm (Frequency)×TL	7	172.31	2.134	**0.046**
Rm (Frequency)×Species	7	371.54	4.601	**<0.001**
Error	112	80.76		

The model was calculated with auditory threshold (sound pressure level) as a dependent variable and species and total length (TL) as fixed factors. Significant P-values are in bold.

Table 4. Hearing thresholds of *Ophidion rochei* and *Carapus acus*

	O. rochei			*C. acus*		
	Mean (SPL)	s.d. (SPL)	N	Mean (SPL)	s.d. (SPL)	N
150 Hz	104.4	8.1	12	114.5	12.7	10
300 Hz	106.6	13.0	12	123.1	10.6	10
600 Hz	99.8	14.2	12	126.8	13.6	10
900 Hz	100.8	16.5	12	122.0	14.1	10
1200 Hz	115.5	15.7	12	123.9	10.1	10
1500 Hz	116.4	12.7	11	125.0	11.1	10
1800 Hz	129.3	13.4	12	136.3	13.3	10
2100 Hz	139.9	15.9	10	151.0	9.1	9

SPL, sound pressure level in decibels relative to 1 µPa.

that otolith mass is related to its area and depends on otolith type (sagittae, asteriscus or lapillus) but not on fish species. Larger sagittae are also heavier. Tables 1 and 2 show that *O. rochei* and *C. acus* have larger sagittae (14.4 to 35.7 mm^3) than the *Chromis chromis* (7.9 mm^3) and the *Amphiprion clarkii* (4.6 mm^3) investigated in the study. *Poecilia mexicana* has very small sagittae: mean area values varied from 1.11 to 1.24 mm^2 in three distinct populations (Schulz-Mirbach et al., 2010). This area was 9.2±1.9 mm^2 in *C. acus* and 13.2±3 mm^2 in *O. rochei* (extracted from µCT scans). Despite their larger sagittae, *O. rochei* had a higher frequency of best sensitivity (600 Hz) than *C. acus* (150 Hz), *A. clarkii* (150 Hz), *C. chromis* [200 Hz (Wysocki et al., 2009)] and *P. mexicana* [200 Hz (Schulz-Mirbach et al., 2010)]. It is reasonable to state that large sagittae in absolute size are not synonymous with lower best frequency of sensitivity.

Fine et al. (Fine et al., 1987) speculated that *Acanthonus armatus* (Ophidiiformes) would have a good sensitivity to low-frequency sounds because of its large saccular otoliths relative to the neurocranium. Several other authors (Paxton, 2000; Cruz and Lombarte, 2004; Lombarte and Cruz, 2007) also suggested that relatively (generally compared with body length) larger sagittae improve hearing capacities. Unfortunately, to our knowledge, the way relative sagitta size affects hearing has never been addressed from a theoretical view. Relative to the neurocranium, *C. acus* had larger sagittae than *O. rochei* (Table 1), but it shows a significantly higher hearing threshold from 300 to 900 Hz (Table 3, Fig. 4) while the differences observed for the other frequencies were not significant. Schulz-Mirbach et al. (Schulz-Mirbach et al., 2010) investigated the morphology of otoliths and hearing abilities in cave- and surface-dwelling ecotypes of the Atlantic molly, *P. mexicana*. They found numerous differences in otolith morphology between both ecotypes but not in hearing capacities, concluding that these otolith differences do not affect hearing sensitivities, at least in terms of auditory thresholds. Results from the present study also found no direct link between otolith size and hearing capacities of fishes.

Comparisons performed in this study were only based on sound pressure audiograms. Future studies on hearing capacities in Ophidiiformes would ideally determine hearing threshold using pressure and particle motion of sound because it is not possible to separate both components of sound when a speaker is used to test hearing capacities (Radford et al., 2012). However, the experimental setup used in this study was the same as in Anderson and Mann (Anderson and Mann, 2011), which observed similar audiogram shapes for each acoustic modality (pressure and particle motion) and concluded that similar observations can be made for numerous other studies. In addition, the estimated value of the particle motion threshold is highly affected by the choice of the calibration

technique (Radford et al., 2012). In a previous study, goldfish hearing capacities based on auditory evoked potentials (AEP) were tested by a single team using different techniques for particle motion calibration; depending on the technique employed (Euler equation, shaker table or accelerometer), differences in thresholds reached 60 dB at some frequencies (Radford et al., 2012).

Sagitta morphology and vestibular function

Fishes generally use visual and vestibular senses for postural equilibrium maintenance and orientation (Anken and Rahmann, 2002). Because *O. rochei* and *C. acus* are active in dark environments, these functions will mainly rely on the vestibular sense. They need very acute vestibular cues, mainly for their posture and the precise movements they have to execute. *Carapus acus* swim head down when they are searching for host but switch to a tail down (almost vertical) position to enter into it (Parmentier and Vandewalle, 2003; Parmentier and Vandewalle, 2005; Schwarz et al., 2012). *Ophidion rochei* generally follow the sea floor with their barbel-like pelvic fins and keep their tail slightly higher than the head (Codina et al., 2012). However, they also enter the sand tail first (Codina et al., 2012) in an almost vertical posture (L.K., personal observation). A similar behavior was also described in *Ophidion scrippsae* (Greenfield, 1968). Once in the sand, *O. rochei* are also able to travel more than 1 m horizontally in a 10 cm deep layer of sand (L.K., personal observation). This set of observations strongly suggests that *C. acus* and *O. rochei* need an accurate perception of their body positioning.

Anken et al. (Anken et al., 1998; Anken et al., 2000) conducted experiments on otolith growth under altered gravity (hyper- and micro-gravity) and proposed a feedback loop between the brain and the ear that controls otolith size in order to maintain an equilibrium in the force exerted on underlying hair cells. These data lead us to consider that otolith mass, and consequently volume, affects hair cell excitation by gravity. Thus, it is conceivable that fishes exhibiting slow swimming patterns and fishes that need precise information about the gravity vector would have bigger otoliths (Parmentier et al., 2001).

According to Lychakov and Rebane (Lychakov and Rebane, 2000), the smaller the mass of the otolith, the wider the range of accelerations to which the otolith organ is sensitive. However, the wide perceptible acceleration range in the otolithic organs with small otoliths leads to a loss of resolving power (Lychakov and Rebane, 2000). Schulz-Mirbach et al. (Schulz-Mirbach et al., 2011) observed no difference in hearing capacities of cave- and surface-dwelling *P. mexicana*, despite that the former population had thicker otoliths. Consequently, they suggested that thicker otoliths may have evolved in cave populations, where an acute sense of balance based on the inner ear would be advantageous. In carapids, the thickest and heaviest sagittae are found in benthic or parasitic species, whereas thin otoliths are found in pelagic species (Parmentier et al., 2001; Parmentier et al., 2002). Large relative saccule thickness and length in *C. acus* and *O. rochei* might have evolved to respond to non-acoustic constraints.

Hearing capacities of *O. rochei* and *C. acus*: ecological implications

One of the main purposes of studies on animal hearing ability is to determine what kind of sounds the different species are able to detect in their natural habitat. In *O. rochei*, the best hearing capacities were obtained between 300 and 1200 Hz. This is higher than the first peak frequency (ca. 200 Hz) of many male sounds and higher than the fundamental frequency (ca. 250 Hz) of female

sounds (Kéver et al., 2012). However, it matches pretty well the second peak frequency (ca. 380 Hz) of male sound and the first and second harmonics (ca. 500 and ca. 750 Hz, respectively) of female sounds (Parmentier et al., 2010; Kéver et al., 2012). Consequently, *O. rochei* are able to process sounds of their conspecifics, but seem more sensitive to the higher-frequency components of these sounds. Rogers and Cox (Rogers and Cox, 1988) stated that it would be advantageous for shallow-water fishes to have adaptations for high-frequency sounds because their habitat prevents the propagation of low-frequency sounds. For example, in 5 m water depth with a sandy bottom, sounds of ca. 215 Hz or below suffer very strong attenuation (Mann, 2006). In 100 m water depth, this 'cut-off frequency' is ca. 11 Hz (Mann, 2006). Fine and Lenhardt (Fine and Lenhardt, 1983) investigated the propagation of *Opsanus tau* sounds in very shallow water and observed that the fundamental frequency attenuated faster than the second harmonics. Given that *O. rochei* specimens were caught in a maximum of 10 m water depth, relatively good hearing capacities around 600 Hz could allow conspecific sounds to be detected from further away. Additionally, *O. rochei* is also known to live at 150 m of water depth (Jardas, 1996), where all the sound frequencies of their call should be detected by their conspecifics.

In *C. acus*, the best hearing capacities are between 150 and 300 Hz, while sound energy ranges from 250 to 1600 Hz (Parmentier et al., 2006b). This means that sound characteristics and auditory sensitivity do not match perfectly. However, the threshold was relatively flat between 300 and 1500 Hz, which should enable *C. acus* to detect sound from closely located conspecifics. Similar observations were performed for other species (Myrberg, 1981; Ladich, 1999). In such cases, it may be more important for fish survival to detect sounds from other sources rather than from conspecifics. Although sounds of *C. acus* were not recorded while fish were in their host holothurians (Parmentier et al., 2006b), acoustic communication between conspecifics through the host seems possible because holothurian tissues are almost transparent to sounds (Parmentier et al., 2006b).

Conclusions

Ophidion rochei and *C. acus* have larger otic cavities and sagittae compared with many non-ophidiiform species. Sagitta size (relative, absolute or both) does not seem to be a sufficient criterion to predict fish hearing abilities. Relative sagitta size could be related to an acute sense of equilibrium, which is needed in both species because of their particular way of life.

MATERIALS AND METHODS
Fish collection

Specimens of *O. rochei* were collected during five field campaigns that took place in two different locations: Dùlce-Glàva (43°26N, 16°40E; Croatia) and Banyuls-sur-mer (42°28N, 3°08E; France). In Dùlce-Glàva (May 2010, July 2010 and September 2011), fish were trapped between 21:30 and 02:00 h with a beach seine (22 m long, mesh size of 4 mm at the outer wing and 2 mm at the central part) in shallow waters (<2 m depth). In Banyuls-sur-mer (August 2010 and 2012), fish were collected by SCUBA diving (5 to 10 m depth) after sunset (between 21:30 and 00:00 h) with hand nets.

Ten *C. acus* were extracted from ca. 250 *Holothuria tubulosa*, which were collected by SCUBA diving (40 m depth) in October 2011 and March 2012 in front of the marine research center STA.RE.SO. in Calvi Bay (42°34N, 8°43W; Corsica, France).

At the laboratory (University of Liège, Belgium), fish were kept in a 1000 l tank (14 h:10 h light:dark cycle, internal filter) with a 0.1 m high sandy bottom and filled with seawater.

Inner ear morphology

All fish investigated were euthanized with an overdose of MS-222 (Sigma-Aldrich, St Louis, MO, USA) and fixed in formalin (7%) for 15 days before being transferred to 70% ethanol.

Dissections

The skulls of 15 *O. rochei* were opened dorsally and dissected in order to investigate inner ear and brain morphologies. According to the nomenclature of Kéver et al. (Kéver et al., 2012), this sample included two juveniles [87 mm and 114 mm total length (TL)], three males (169, 184 and 201 mm TL) and 10 females (from 151 to 217 mm TL).

The inner ears and brains of three *C. acus* were also dissected. Fish measured 149, 169 and 177 mm TL. Sex was not determined for this species, but *Carapus* species generally do not have intersexual differences in their sonic apparatus (Emery, 1880; Courtenay and McKittrick, 1970). Dissections were performed under a stereoscopic microscope (Leica, Wild M10) coupled to a camera lucida.

CT scans

The internal morphology of three *O. rochei* and three *C. acus* was investigated with computed tomography (CT) imaging systems. (1) An *O. rochei* (161.5 mm TL) and a *C. acus* (149 mm TL) were scanned at the Veterinary Institute of the University of Liège. Structure details were limited by the maximal resolution (isotropic voxel size: 600 μm) of the scanner (Scanner Siemens Somaton Sensation 16-slice, Siemens AG, München). (2) Two *O. rochei* (135 and 185 mm in TL) and two *C. acus* (143 and 161 mm in TL) were scanned at the National Museum of Natural History in Paris with a μCT scan (v|tome|x 240 L, GE Sensing & Inspection Technologies phoenix|x-ray), which allowed greater precision. Depending on fish size, the imaging system was set at 42 or 70 kV and specimens were scanned at an isotropic voxel size between 22.4 and 60.4 μm.

Specimens were submerged in a 5% phosphomolybdic acid solution for 10 days before scanning to improve contrast between the different tissues. Volume and surface rendering was performed with AMIRA 5.4.0 (VSG, FEI Company). Structure measurements were also performed in AMIRA. Several authors (e.g. Paxton, 2000) have expressed the relative size of the sagitta by comparing its largest diameter (usually length, rarely height) to fish standard length. However, this may lead to a bias and would suggest anguilliform fishes have small otoliths compared with other fish species that have bigger skulls but are of a similar length. In the present study, we compared sagitta length with TL as well as head length. In addition, the ratio of the volume of the two sagittae to the overall intra-neurocranium (INC) volume was calculated. In order to make comparisons and because this kind of data are completely unknown in the literature, the same measurements were performed on fish CT scans from different vocal species (Courtenay and McKittrick, 1970; Picciulin et al., 2002; Parmentier et al., 2006a; Parmentier et al., 2007; Bertucci et al., 2013): two *O. fowleri*, two *Epinephelus marginatus*, one *Plectroglyphidodon lacrymatus*, one *Chromis chromis* and one *Amphiprion clarkii*. Hearing capacities of the last two species are known from the literature (Parmentier et al., 2009; Wysocki et al., 2009).

Hearing capacities

Twelve *O. rochei* (106 to 210 mm TL) and 10 *C. acus* (127 to 177 mm TL) were used to measure their hearing capacity. Following the AEP technique (Corwin et al., 1982; Kenyon et al., 1998), bulk neural responses elicited by sounds were recorded near the brainstem. The presence or absence of a response for sounds at different intensities and frequencies thus allows the determination of hearing thresholds. Our experimental setup was similar to that of Parmentier et al. (Parmentier et al., 2009; Parmentier et al., 2011a). However, Ophidiiformes were harder to immobilize because of their anguilliform shape, the absence of hard spines in fins, and the substantial mucus production. Thus, fishes were anesthetized with 150 mg l^{-1} of MS-222, which allowed us to wrap the posterior two-thirds of the fish body in cellophane. They were then placed in a custom-made harness allowing respiration. The harness was closed dorsally with small pliers and used to suspend the fish 10 cm below the water surface in a steel tube (115 cm high, 22 cm diameter, 0.7 cm thick). This tube was placed in a soundproof booth, oriented vertically, closed at the bottom with a square steel plate (40×40 cm),

and filled to a constant height (105 cm) with saltwater kept between 20 and 22°C. A UW-30 underwater speaker (Lubell Labs, Colombus, OH, USA) was placed on the bottom of the steel tube.

Three subdermal stainless steel needle electrodes (Rochester Electro-Medical, Lutz, FL, USA) were used for recording electrical signals. The first or recording electrode was inserted approximately 1 mm into the head, over the otic region. The reference electrode was placed within the fish epaxial musculature while the ground electrode was in the water. These electrodes were placed while fishes were still anesthetized but recordings were performed with the animal being awake.

Eleven different frequencies were presented to each fish: 150, 300, 600, 900, 1200, 1500, 1800, 2100, 2400, 2700 and 3000 Hz. For a detailed description of sound stimuli generation, see Parmentier et al. (Parmentier et al., 2011a). At each frequency, sound levels were presented at up to 179 dB re. 1 V μPa^{-1} and were decreased in 6 dB steps (dB values are dB re. 1 μPa). Evoked potentials recorded by the electrode were amplified (TDT HS4-DB4 amplifier; 10,000 gain), connected to an RP2.1 enhanced real-time processor, routed into the computer, and averaged using BioSig software. For each sound level and at each frequency, the signal was presented 500 times. For each stimulus presentation, signal phase was alternated (−90 and 90 deg) to reduce stimulus artifacts. Sound pressure levels of the acoustic stimuli were calibrated with a Brüel and Kjær 8101 hydrophone (Nærum, Denmark; sensitivity −184 dB re. 1 V μPa^{-1}; bandwidth 0.1 Hz to 200 kHz) connected to a calibrated Brüel and Kjær 2610 amplifier that gave the absolute pressure level of sound. Evoked responses were averaged and power spectra were calculated using a 4096-point fast Fourier transform. These spectra were analyzed for the presence of peaks at twice the frequency of the stimulus that were at least 3 dB above background levels. The lowest sound level at which such a peak was apparent was defined as the audibility threshold. Dead specimens of both species were also tested to confirm that activity recorded by electrodes was caused by the fish brain being stimulated by the sound.

Statistical tests
The normality of the variables was investigated using Kolmogorov–Smirnov tests. A general linear model with repeated measures was performed to compare sound pressure thresholds (dependent variable) obtained at the different frequencies tested (repeated measures). Species and TL were selected as fixed factors. Tukey's HSD *post hoc* tests allowed for comparisons between species at each frequency. The relationship between fish size and auditory sensitivity at the different frequencies was examined using linear regressions. All statistics were performed in STATISTICA 10 (StatSoft Inc., Tulsa, OK, USA).

Ethics statement
All experimental procedures were approved by the University of Liège Institutional Animal Care and Use Committee (protocol no. LA1610430). *Ophidion rochei* and *C. acus* are not endangered or protected species and they were not caught in protected areas. No specific authorization was required to catch specimens of these species with hand nets. The Croatian Ministry of Science and Education delivered an authorization for using a beach seine during field campaigns conducted in Split.

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