RESEARCH ARTICLE

Sea chordophones make the mysterious /Kwa/ sound: identification of the emitter of the dominant fish sound in Mediterranean seagrass meadows

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ABSTRACT

The /Kwa/ vocalization dominates the soundscape of *Posidonia oceanica* meadows but the identity of the species emitting this peculiar fish sound remains a mystery. Information from sounds recorded in the wild indicates that the emitting candidates should be abundant, nocturnal and benthic. *Scorpaena* spp. combine all these characteristics. This study used an interdisciplinary approach to investigate the vocal abilities of *Scorpaena* spp.; morphological, histological and electrophysiological examinations were interpreted together with visual and acoustic recordings conducted in semi-natural conditions. All observed *Scorpaena* spp. (*S. porcus*, *S. scrofa* and *S. notata*) share the same sonic apparatus at the level of the abdominal region. This apparatus, present in both males and females, consists of 3 bilaterally symmetrical muscular bundles, having 3–5 long tendons, which insert on ventral bony apophyses of the vertebral bodies. In all chordophones (stringed instruments), the frequency of the vibration is dependent on the string properties and not on the rate at which the strings are plucked. Similarly, we suggest that each of the 3–5 tendons found in the sonic mechanism of *Scorpaena* spp. acts as a frequency multiplier of the muscular bundle contractions, where the resonant properties of the tendons determine the peak frequency of the /Kwa/, its frequency spectra and pseudo-harmonic profile. The variability in the length and number of tendons found between and within species could explain the high variability of /Kwa/ acoustic features recorded in the wild. Finally, acoustic and behavioural experiments confirmed that *Scorpaena* spp. can emit the /Kwa/ sound.

KEY WORDS: Bioacoustics, Passive acoustic monitoring, Sound production, Acoustic communication, Scorpaenidae

INTRODUCTION

Passive acoustic monitoring (PAM) of fish populations (i.e. the monitoring of vocal fish by means of hydrophones) has been used for analysing fish presence, distribution and relative abundance, examining their diel, lunar and seasonal cycles of activity, delimitating spawning areas and studying wild fish spawning behaviour (Mann and Lobel, 1995; Fine and Thorson, 2008; Locascio and Mann, 2008; Luczkovich et al., 2008; Picciulin et al., 2013; Wall et al., 2013; Ruppé et al., 2015; Wang et al., 2017; Lindseth and Lobel, 2018). In the Mediterranean Sea, several studies have applied passive acoustics to the monitoring of different vocal fish species, mainly the brown meagre, *Sciaena umbra* (Bonacito et al., 2002; Picciulin et al., 2012, 2013; Parmentier et al., 2018), the cusk-eel *Ophidion rochei* (Parmentier et al., 2010a; Kéver et al., 2016; Picciulin et al., 2018) and the dusky grouper, *Epinephelus marginatus* (Bertucci et al., 2015). Mediterranean shallow waters are characterized by an alternation of sandy, rocky bottoms and seagrass meadows, where the Neptune seagrass *Posidonia oceanica* (L.) Delile (an endemic and protected species; Habitat Directive 92/43/EU) is the dominant seagrass species. Neptune seagrass meadows are crucial habitats that provide refuge, nursery and food to several fish and invertebrate species (Gobert et al., 2006; Abadie et al., 2018). It is somehow surprising that the first descriptions of the Neptune seagrass soundscape and of its fish biophonical component have been published only very recently (Ceraulo et al., 2018; Di Iorio et al., 2018). Di Iorio et al. (2018) recorded the soundscape of nine Western Mediterranean meadows (over a 200 km range) over 7 months (March–October). All nine sites were dominated by one particular fish sound type, the /Kwa/, which was 20 times more abundant than any other fish sound. The /Kwa/ possesses unique features which set this sound type apart from other Mediterranean fish calls, especially in terms of pulse shape, spectral content (bandwidth, peak frequency, pseudo-harmonic contour) and intrinsic variability (Di Iorio et al., 2018). The /Kwa/ is a fast, amplitude-modulated pulse train of 13±6 pulses repeated every 13±4 ms with a peak frequency of 747±136 Hz characterized by pseudo-harmonics around the 800 Hz contour in the spectrographic view (Di Iorio et al., 2018; Fig. S1). All known Mediterranean fish sounds are generally characterized by a lower frequency content (i.e. <500 Hz; Desiderà et al., 2019), and only a few show harmonic and pseudo-harmonic contours. The /Kwa/ has been registered over a wide geographical range, from French and Corsican coastlines (Di Iorio et al., 2018) to Sicily (Ceraulo et al., 2018; the /Kwa/ was here called 'medium frequency fish sound') and Ustica Island (Hermand, 2006) and from Crete (M.B., personal observation) to the Azores (Rita Carriço, personal communication), passing through the Balearic islands (M.B., personal observation; Correa et al., 2018, 'croak sound'). None of the 38 currently identified Mediterranean vocal fish species produces the /Kwa/ (Di Iorio et al., 2018). The specific identity of the fish species emitting this dominant and peculiar fish sound remains a mystery.

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Cluster analysis carried out by Di Iorio et al. (2018) on a consistent sample of /Kwa/ (i.e. $N_{/Kwa/}$=23,566) revealed a high degree of /Kwa/ variability. This in turn suggests a mechanism which allows for high variability and/or multiple species emitting the /Kwa/. The unique characteristics of the /Kwa/ suggest that the morphological structures allowing its emission differ from stridulatory mechanisms, swimbladder forced-response mechanisms and swimbladder rebound mechanisms (Parmentier et al., 2017). Finally, the impressive abundance of /Kwa/ and its wide geographical range of distribution combined with the increasing number of studies which reported its presence suggest that the emitting species should be abundant and widespread. Information from recorded sounds indicates that the emitting candidates should be found in Mediterranean Neptune seagrass meadows, and be abundant, nocturnal and benthic. According to these criteria, *Scorpaena* spp. deserve attention, as these species are the only ones that combine all the above-mentioned characteristics. Five *Scorpaena* species (*S. scrofa*, *S. porcus*, *S. notata*, *S. maderensis* and *S. elongata*) are present in the Mediterranean Sea, where especially *S. porcus* and *S. scrofa* are ubiquitous in the meadows (Ferri et al., 2012; Matić-Skoko et al., 2015). These fish are a monophyletic group of sedentary, solitary and benthic predators, often characterized by overlapping areas of distribution, which reproduce from June to September (Bradai and Bouain, 1990; La Mesa et al., 2005; Turan et al., 2009; Saju et al., 2014; Šantić et al., 2011).

The baseline hypothesis of this study is that *Scorpaena* spp. emit the /Kwa/ (Fig. 1). Evidence for the identity of the species that emits a sound type recorded in the wild is often obtained by comparing this sound type with those recorded by auditioning fish species in captivity. However, fish sound production is dependent on the right environmental and social conditions, which are sometimes difficult to recreate in artificial settings. The result is that some species do not vocalize in captivity or show a more restricted acoustic repertoire than in the field. For example, *Dascyllus aruanus* produced agonistic sounds in tanks (Parmentier et al., 2006a) but did not perform the typical associated behaviour recorded in the field, i.e. the signal jump (Parmentier et al., 2009); *Carapus mourlani* produced two sound types in a first study (Parmentier et al., 2006b) but four sound types in a second study (Parmentier et al., 2016). Preliminary captivity recordings of *Scorpaena* spp. were carried out in 2017 at STARESO (Calvi, Corsica, France) and at the Aquarium of Cala Gonone (Sardinia, Italy); no /Kwa/ were recorded in these

settings. This absence of the /Kwa/ sound could simply reflect our lack of success in recreating the optimal environmental and social conditions for inducing *Scorpaena* spp. voluntary sound production. We therefore adopted a sharper hypothesis formulation and a refinement of the experimental settings. Two specific hypotheses were tested in this study: (1) *Scorpaena* spp. possess a sonic mechanism with morphological characteristics that differ from those of other Mediterranean vocal fish species; (2) this sonic mechanism allows the emission of /Kwa/. In order to answer our experimental hypotheses, we used an inter-disciplinary approach; morphological, histological and electrophysiological examinations (hypothesis 1) were interpreted together with the results of visual and acoustic recordings conducted in semi-natural conditions (hypothesis 2).

MATERIALS AND METHODS
Fish collection
In August 2017, two *Scorpaena porcus* Linnaeus 1758 and four *Scorpaena scrofa* Linnaeus 1758 were collected by night-time free-diving at STARESO. Fish were collected on a rocky bottom adjoining *P. oceanica* meadows at depths ranging from 1 to 7 m using hand-nets and lights. Immediately after collection, fish were placed in a plastic bucket filled with seawater in order to be identified to species level. Subsequently, their acoustic behaviour was monitored in semi-natural conditions (SNC1, see below). Afterwards, fish were killed by immersion in a saturated MS-222 seawater solution. Fish were measured (standard length, SL) and weighed. Fish were fixed in a 7% formaldehyde solution and then transferred, after 2 weeks, to a 70% ethanol solution. Fish were taken to the Laboratory of Functional and Evolutionary Morphology (University of Liège). Additional specimens of *S. scrofa*, *S. porcus* and *Scorpaena notata* Rafinesque 1810 (Table 1) were collected at La Ciotat (Bouches-du-Rhône, France) and prepared for analysis as described above. During July 2018, three additional *S. porcus* specimens were collected during night-time free-diving at STARESO. Acoustic and visual behaviour was observed for two individuals during SNC2 (see below) before they were released, while the third specimen was electro-stimulated (see below) and then killed as described above (Table 1). All experimental procedures followed a protocol that was approved by the local ethics committee of the University of Liège (no.1759). *Scorpaena* spp. are not endangered or protected species, and specimens were not caught in protected areas.

Gross morphology of the sonic apparatus
Gross morphological examinations were carried out on 11 individuals belonging to three species (*S. scrofa* N=5, *S. porcus* N=4, *S. notata* N=2; Table 1). Fish were carefully dissected and observed under a binocular microscope (Leica MS5), with particular focus on potential sound-producing structures. Sex was determined through examination of gonad cross-sections; sex was indeterminate (NA in Table 1) when fish were released after the SNC (i.e. without immersion in MS-222 and subsequent dissection) or when fish were too small (<11 cm).

Histological and functional comparison of sonic and epaxial musculature
Cross-sections of sonic and epaxial muscles were obtained from one individual of *S. porcus* collected at La Ciotat (Table 1) that was later identified as a female. Samples were fixed with cacodylic acid (1%). After fixation, these samples were dehydrated in a series of ethanol–propylene oxide and embedded in epoxy resin (SPI-Pon 812).
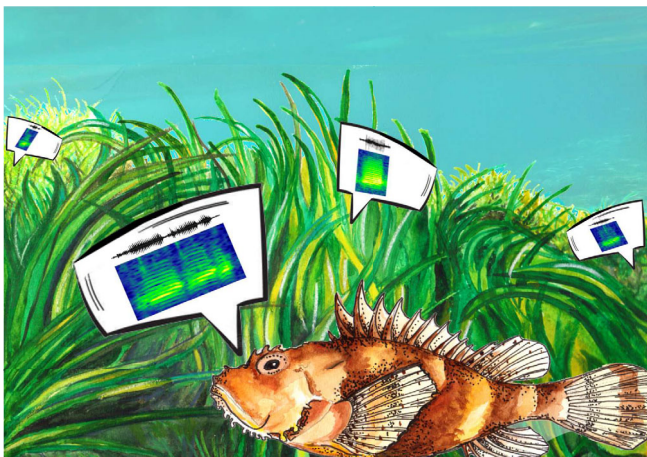

Fig. 1. The experimental hypothesis. *Scorpaena* spp. emit the /Kwa/ sound.

Table 1. *Scorpaena* spp. observed as part of this study

Species	Collection site	SL (cm)	Sex	Analysis	Tendon length (cm)				Tendon no.
					α	β	$\delta1$	$\delta2$	
S. porcus	STARESO	13.5	NA	SNC1-GM	1.49	2.03	1.83	2	4|4
	STARESO	16	NA	SNC1-GM	1.94	1.97	1.95	1.59	5|4
	La Ciotat	9	NA	GM	0.9	0.89	1.1	1.13	4|4
	La Ciotat	8	NA	GM	0.78	0.88	0.67	0.84	5|4
	La Ciotat	13	♀	HIS					
	STARESO	13	NA	SNC2					
	STARESO	9	NA	SNC2					
	STARESO	10	NA	E					
S. scrofa	STARESO	16	♂	GM	1.65	1.3	1.46	2.06	4|4
	STARESO	18	♀	GM	1.59	1.11	1.22	2.06	4|4
	STARESO	11.5	♀	SNC1-GM	1.05	1.03	1.33	1.62	4|4
	STARESO	18.5	♂	SNC1-GM	1.95	1.79	2.03	2.83	4|4
	La Ciotat	16	♀	GM	1.62	1.44	2.05	2.44	5|5
S. notata	La Ciotat	12	♂	GM	1.08	1.14	1.17	1.35	4|5
	La Ciotat	10	NA	GM	1.17	0.92	1.10	1.57	4|4

Collection site (STARESO, Calvi, Corsica, France; La Ciotat, Bouches-du-Rhône, France), standard length (SL), sex (♀, female; ♂, male; NA, indeterminate), type of procedure carried out (SNC1/2, acoustic recording in semi-natural condition without/with video recording; GM, gross morphological examination; HIS, histological examination; E, electrostimulation), absolute length of tendons in the sound-producing apparatus and number of tendons on each body side of the same individual are shown.

Semi-thin sections (0.1 µm) of muscles were coloured with Toluidine Blue (0.5% in a 1% borax solution) and photographed (Leica MC170HD; software LAS EZ) under an optical microscope (Leica DM1000). Fibre size (i.e. area of the perpendicular section) and the ratio of the surface of myofibrils and sarcoplasmic reticulum per fibre were measured in the muscle cells using ImageJ software and compared between muscle types.

In order to compare the response of epaxial and sonic muscles to electrical stimulation, one *S. porcus* specimen collected at STARESO (Table 1) was anaesthetized in a saturated MS-222 seawater solution. The fish was then placed in a plastic tray; respiration and anaesthetization were ensured by a pipette inserted into the mouth and connected to a gravity-fed circulation system containing an MS-222–seawater solution. The sonic and epaxial muscle were exposed through dissection and were individually stimulated via electrodes connected to a stimulator (HUGO Sachs Elektronik Type 215-T), which generated the electric stimuli. Sonic and epaxial muscles were stimulated in sequence using the same settings, i.e. 7 ms long stimuli at 1 V repeated every 25 ms. The muscle response was recorded in air using a hydrophone (HTI-96-Min, High Tech Inc., Long Beach, MS, USA; −164.4 dB re. 1 V µPa^{-1}, placed <5 cm from the fish) connected to a digital recorder (DR-05, Tascam, Montebello, CA, USA). At the end of the experiment, the fish was killed by immersion in a saturated MS-222 seawater solution. The recorded .wav files (sample rate 44.1 kHz, 16 bit) were down-sampled at 4 kHz and analysed using RAVEN Pro version 1.4 (Cornell Laboratory of Ornithology) to compare the waveform generated by the response of the epaxial and sonic muscles.

Statistical analysis

Statistical analysis was carried out in STATISTICA 7.0. Mean and s.d. of measured morphological and histological features was calculated. As normal distribution assumptions were not met by morphological data (Shapiro–Wilk, $P<0.05$), non-parametric statistics were used (Kruskal–Wallis, Mann–Whitney) to compare morphological measurements between four *Scorpaena* spp. As fibre area and the percentage of myofibrils were normally distributed (Shapiro–Wilk, $P>0.05$), a paired Student's t-test was used to compare these two fibre measurements between epaxial and sonic muscle.

Acoustic and visual recordings in SNC

Data collection

During summer 2017, the acoustic behaviour of two *S. porcus* and two *S. scrofa* collected at STARESO was monitored (SNC1). Two Plexiglas aquaria (74×25×32 cm) were deployed underwater in the STARESO harbour (2 m depth; close to the pier's cement wall) (Fig. S2) and were filled with small rocks collected in the harbour in order to recreate the natural environment. Each aquarium was equally divided into two compartments by placing two removable Plexiglas partitions (one opaque and one transparent) together in the centre of the aquarium. Furthermore, each aquarium was equipped with a removable, transparent Plexiglas lid, which prevented the experimental animals from escaping. Immediately after species identification, two fish of the same species were placed in an aquarium (one in each compartment) and their acoustic behaviour was monitored during four consecutive nights. The first night of observation corresponded to the night on which fish were collected and identified. As fish collection was carried out after dusk, on the first night, acoustic behaviour was monitored from ca. 23:30 h to 01:30 h. On subsequent nights, fish acoustic behaviour was monitored for 6 h, from ca. 19:30 h to 01:30 h. During the first two nights of observation, both visual and tactile communication were prevented by the two partitions. During the third night, removal of the opaque partition allowed visual communication between the two individuals. Finally, the transparent partition was removed during the fourth night, allowing the fish to interact physically. All aquaria manipulations were carried out whilst free-diving. Acoustic behaviour was monitored by means of four cabled hydrophones (two per aquarium). One sensor was inserted in each aquarium compartment (from small holes present on both sides of the aquarium lid), and cables were run on the pier where recordings took place using portable digital recorders. Specifically, two HTI-96-Min hydrophones (−164.4 dB re. 1 V µPa^{-1} ±3 dB over 5 Hz to 30 kHz) were connected to two DR-05 digital recorders, and two H2a hydrophones (AquarianAudio, Anacortes, WA, USA; −180 dB re. 1 V µPa^{-1} over 10 Hz to 100 kHz) were connected to two ZOOM H1 digital recorders (Zoom, Hauppauge, NY, USA). An underwater recorder (BCB, Loggerhead Instruments, Sarasota, FL, USA; −164.4 dB re. 1 V µPa^{-1}, gain +4 dB) was placed on the sea bottom in front of the two aquaria, at a distance of 93 cm (SNC1)

to 135 cm (SNC2) from the internal hydrophones (see Fig. S2). While the datalogger collected data continuously for the entire duration of the experiment (5 min .wav file, 16 bit, 44.1 kHz), the digital recorders connected to the cable hydrophones placed inside the aquarium were stopped and re-started every 30 min (30 min .wav files, 16 bit, 44.1 kHz). After recorders were restarted, a loud acoustic signal was made close to the aquaria using a heavy metallic chain connected to a rope, repetitively lowered from the pier to the sea bottom. As the noise made by the chain was clearly recorded by each recording device, this acoustic signal was used to synchronize the recordings collected by the five recorders.

During July 2018, the same procedure with the same hydrophone configuration was repeated for one night (SNC2) but this time including a prototype of an underwater camera coupled with an HTI-96-Min hydrophone (High Tech Inc.; recording both outputs in the same .mp4 file), together with an underwater white light (Keldan Video 8X) at ca. 30 cm distance from the underwater aquarium. The camera filmed two *S. porcus* individuals interacting in the same compartment. The first fish was netted the night before and was left to acclimate in the experimental aquarium for 24 h, while the second fish was inserted during the visual and acoustic experiment. Video and audio observation lasted for ca. 3 h (19:30 h to ca. 22:30 h). Both fish were released the next day.

Data analysis

All collected .wav files (total of 836 min of recordings) were analysed using RAVEN Pro version 1.4 (Cornell Laboratory of Ornithology; Hanning window, FFT size 256) after being down-sampled at 4 kHz. Each 30 min .wav file was synchronized with the corresponding .wav files collected by the other recorders using the waveform (envelope and time of the highest energy peak) of the reference synchronization signal. This procedure permitted recognition and selection of the same /Kwa/ sound in all recording devices (total N=3338 selected /Kwa/ in each recording chain). Hydrophones record the received sound pressure level (RL) of a sound; the RL is the result of transmission losses (TL) of the sound pressure level (SPL) emitted by the fish [i.e. source level (SL); RL=SL−TL]. As TL is dependent on the distance between the sound source (i.e. fish) and the receiver (hydrophone), we considered the RL of the same /Kwa/, as recorded by the five recording chains, as a proxy for the proximity of the fish to the hydrophone. A higher RL recorded by the hydrophone inside the aquarium means that the sound source (i.e. fish emitting that specific /Kwa/) was closer to the internal hydrophone than to the external one. This indicates that the /Kwa/ was emitted by the fish inside the experimental aquarium. As the used recording chains were characterized by different properties (i.e. sensitivity, gain and dynamics), an inter-chain calibration was carried out. At the end of the experiment, all hydrophones, tied in a cluster, were lowered underwater in STARESO harbour to record the same environmental noise from the same position. In this case, TL differences between chains are negligible and, therefore, eventual differences in RL are only due to different chain characteristics. In the analysis phase, 3000 snapshots of 1 s each were extracted and their power spectral densities were calculated assuming gain=0 dB and dynamic=1 V. The median for each recording chain was calculated and plotted (Fig. S3). The absolute difference in the median power spectral density of each recording chain could then be calculated. This comparative calibration has an error of ±3 dB. A global sensitivity factor was finally calculated and applied during the subsequent calculation of the /Kwa/ RL, using a custom-built code in MATLAB. The script allowed calculation of the RL [dB re.

1 µPa, SPL root mean square (rms)] of the /Kwa/ from the first to the last pulse in the 400–1300 Hz bandwidth. Among the large number of initially selected /Kwa/ (*N*=3338), we retained only those which met the following conditions: (i) low ambient noise (i.e. not overlapping with acoustic energy from *Alpheus* spp. snaps and/or boat noise) and (ii) a good pseudo-harmonic structure (more than four pseudo-harmonics). Of the initial number, 156 /Kwa/ met these conditions; their RL was estimated and compared between all recording chains.

Regarding SNC2, all collected video recordings were observed and listened to. All occurrences of /Kwa/ emitted in clear association with behavioural units performed by the experimental fish were noted. The acoustic track was then extracted from the .mp4 video using Adobe Premiere, and all the above-mentioned occurrences were selected using RAVEN Pro version 1.4. The acoustic tracks extracted from the video were subsequently synchronized with the track recorded by the hydrophone inside the experimental tank (H2a–ZOOM H1) and with the track recorded by the external BCB datalogger (placed at ca. 1.5 m distance from the aquaria). The RL of these /Kwa/ were estimated and compared following the procedure described above.

RESULTS

Gross morphology of the sonic apparatus

In all *Scorpaena* spp., the structures potentially involved in sound production are located at the level of the abdominal region within the hypaxial musculature. These fish do not possess a swimbladder. Before fixation, sonic muscles are characterized by a pale yellow coloration, which contrasts with the white coloration of the surrounding hypaxial musculature. They originate on the exoccipital bone and insert on the anterior part of the backbone, from the sixth to the ninth vertebrae. The complete apparatus consists of bilaterally symmetric muscular bundles (three muscular bundles on each side), connected to four long tendons, which insert on lateral branches of the haemal arch (vertebrae VI and VII) or on haemal spines (vertebrae VIII and IX) underneath different vertebral bodies (Fig. 2). The first two muscular bundles, the medial bundle (α) and the intermediate bundle (β), pass above Baudelot's ligament, which connects the basioccipital bone with the upper medial side of the supracleithrum from the pectoral girdle. These two bundles, which are relatively narrow, are connected to two tendons, the medial (α) and intermediate (β) tendon, respectively. Tendons pass between ribs and epineurals before inserting on the distal portion of the ventral processes of vertebrae VI and VII, respectively. The lateral bundle (δ) is larger than α and β and it is characterized by a more unusual configuration because it is divided into two parts by Baudelot's ligament: the rostral part (δA) originates on the skull and inserts on Baudelot's ligament; the caudal part (δB) originates on Baudelot's ligament and is connected to two tendons (lateral tendons δ1 and δ2), which insert on the haemal spine underneath vertebrae VIII and IX. Although the most common configuration of this apparatus presents four tendons on each side, the number of tendons can vary between species, and between and within individuals (Table 1). Furthermore, different individuals of *S. porcus*, *S. scrofa* and *S. notata* are characterized by different numbers of tendons on each side of the body (Table 1). The length of the α medial and β intermediate tendons (standardized for fish size, i.e. tendon length/fish length) does not vary between species, while the length of the δ1 and δ2 lateral tendons does (α: H=3.13, P>0.05; β: H=2.2, P>0.05; δ1: H=8.8, P=0.032 and δ2: H=9.0, P=0.01) (Fig. 3C,D). In particular, the δ1 lateral tendon is significantly longer in *S. scrofa* than in *S. porcus* and *S. notata* (*S. scrofa* versus *S. porcus*, U=9, P>0.007;
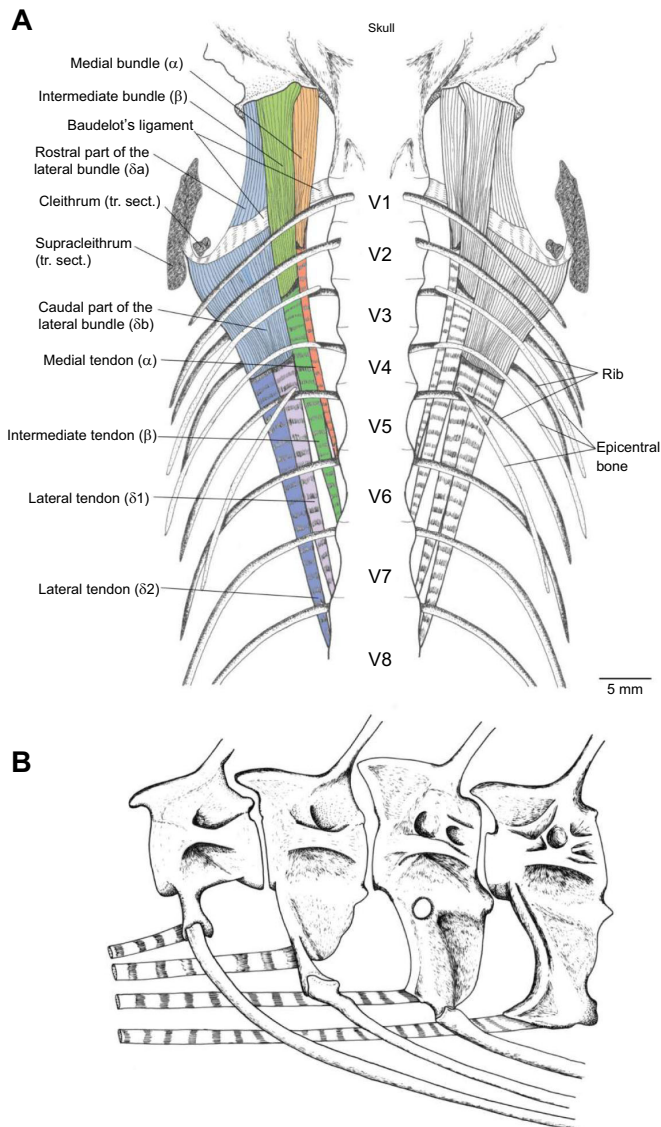
Fig. 2. Schematic diagram of the sound-producing apparatus in *Scorpaena porcus*. (A) Dorsal view; (B) lateral view. Note that tendons do not insert on the vertebral bodies but on haemal spines that are underneath the vertebral bodies as shown in B.

S. scrofa versus *S. notata*, $U=12$, $P=0.048$), while the δ2 lateral tendon is significantly longer in *S. scrofa* than in *S. porcus* ($U=9$, $P>0.001$) (Fig. 3D).

Histological and functional comparison of sonic and epaxial musculature

Scorpaena porcus epaxial and sonic muscles are clearly distinguishable at the microscopic level because of the proportion of myofibrils and sarcoplasmic reticulum (Fig. 4A,B). In cross-sections, 95% of the epaxial muscle area is occupied by myofibrils. In the sonic muscle, only 58% of the area is occupied by myofibrils and the sarcoplasmic reticulum is significantly wider ($t=7.16$, $P<0.001$). Furthermore, the epaxial fibres are significantly thicker (49 ± 14 μm, $N=12$) than those of the sonic muscle (35 ± 6 μm, $N=12$; $t=-21.33$, $P<0.001$) (Fig. 4C). Although histological preparations were examined for one species only (*S. porcus*), it can reasonably be assumed that *S. notata* and *S. scrofa* are characterized by similar histological features of the sonic muscle; in several teleost taxa, it

has indeed been shown that closely related species show similar histological configurations of fast-contracting sonic muscles (e.g. doradids: Boyle et al., 2015; holocentrids: Parmentier et al., 2011). Epaxial and sonic muscles respond differently to the same electrical stimulation. The sonic muscle responds with a series of cyclical pulsations with peak periods of 20 ± 1 ms. The same stimulation does not induce a similar response in the epaxial muscle, which appears incapable of sustaining the rhythm, showing a chaotic response (Fig. 4D,E).

Acoustic and visual validation in SNC

Out of 156 /Kwa/ sounds recorded during SNC1 and compared between hydrophones, 11 were recorded with higher RL by the hydrophones located inside the experimental aquaria (Table 2). This indicates that 7% of the analysed /Kwa/ recorded during SNC1 was emitted by the *Scorpaena* spp. placed inside the experimental setup (Fig. 5A,B). These 11 /Kwa/ were all emitted during the fourth day of recordings, i.e. when all partitions separating the fish were removed and fish were allowed to interact physically. The remaining 145 /Kwa/ were probably emitted by fish inhabiting the harbour.

The analysis of the video recorded during SNC2 revealed three instances in which the emission of the /Kwa/ was clearly associated with a behavioural unit performed by one of the *S. porcus* placed inside the experimental aquarium. These three instances occurred between 23 and 29 min from the time when the second fish was inserted in the aquarium. All three instances begin with the fish performing a 'lateral display' (i.e. bodies aligned parallel to each other, on the bottom and at a close distance; dorsal fins are erect). The (likely) calling fish (always the same in all three instances) rose up from its position on the bottom by propping itself up on its pectoral fins; subsequently, its caudal fin moved from right to left while the head lowered and the /Kwa/ was emitted (see Movie 1). In the first two instances, only one /Kwa/ was emitted, whereas during the third instance, two /Kwa/ were emitted in close repetition. The first /Kwa/ was emitted in association with the first caudal movement (right to left), while the second occurred in association with the second tail movement (left to right). In all instances, the /Kwa/ were recorded with higher received levels by the hydrophone located inside the experimental aquaria (Table 2, Fig. 5C).

DISCUSSION

All *Scorpaena* spp. observed during this study shared the same sonic apparatus at the level of the abdominal region. This apparatus, present in both males and females, consists of 3 bilaterally symmetrical muscular bundles, having 3–5 long tendons, which insert on ventral bony apophyses of the vertebral bodies. Although the electrostimulation experiment cannot be considered as a robust physiological characterization because of its limited sample size, it nevertheless shows that the three muscular bundles responded differently from the epaxial muscle to the same electrical stimulation (Fig. 4D,E). In turn, this suggests that these three muscular bundles have a different function, as confirmed by histological comparison with the epaxial muscle. Muscular bundle fibres are indeed thinner than epaxial fibres and show a significant development of the sarcoplasmic reticulum, where myofibrils cover only 58% of the fibre volume. These characteristics indicate that these three muscular bundles have evolved to sustain quick cycles of contraction and relaxation (Millot et al., 2011; Boyle et al., 2014; reviewed in Parmentier and Diogo, 2006), and set these muscles apart from typical vertebrate locomotory muscles (in which the volume of myofibrils accounts for 90% of the fibre volume; Rome and Lindstedt, 1998). Altogether, the morphological, histological
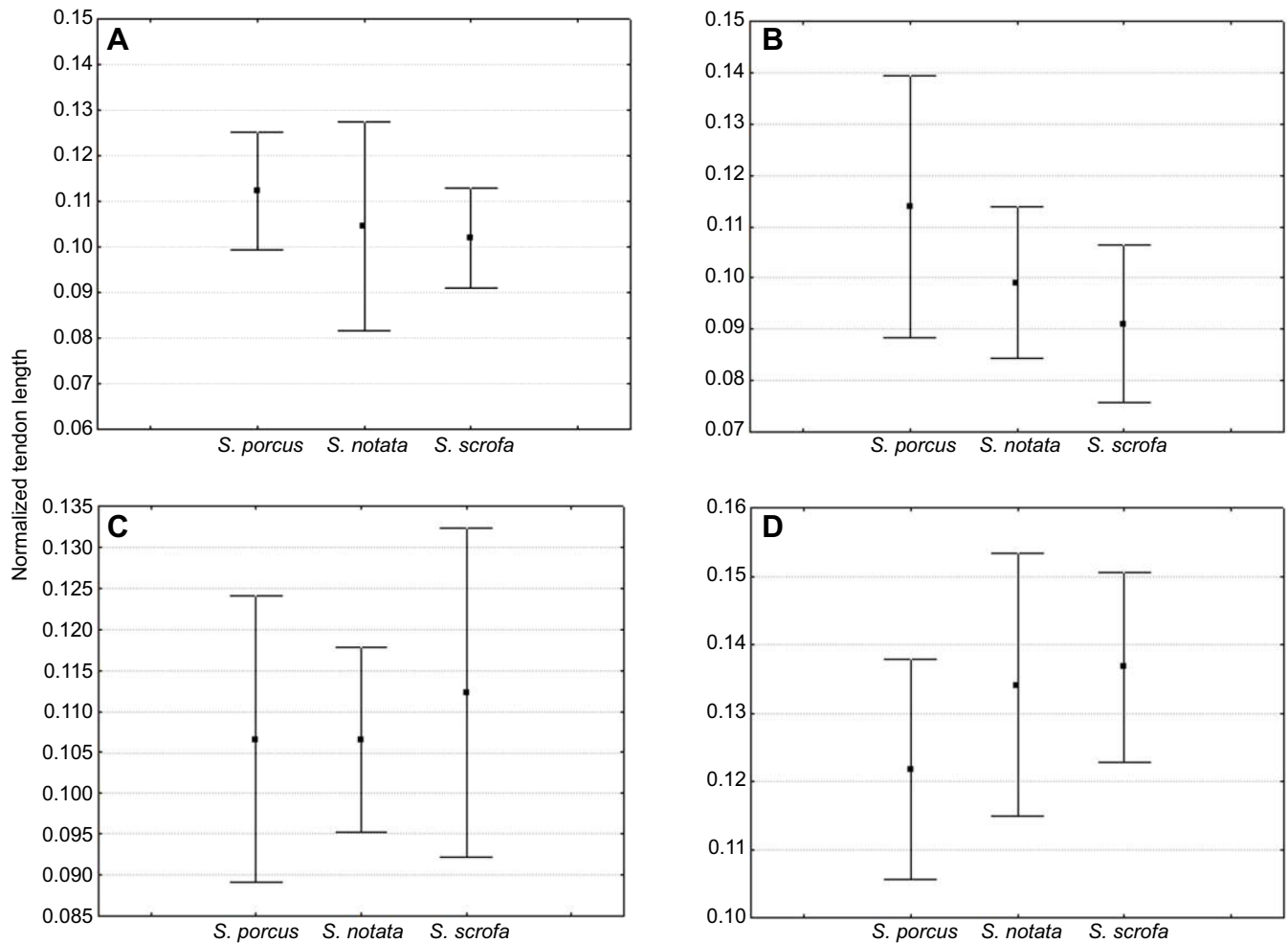
Fig. 3. Sonic apparatus parameters of *S. porcus*, *S. notata* and *S. scrofa*. Length of (A) α medial tendon, (B) β intermediate tendon, (C) δ1 lateral tendon and (D) δ2 lateral tendon. Data (means±s.d.) were normalized to fish standard length (SL, cm).

and electrophysiological data indicate that the apparatus described here for the first time in *Scorpaena* spp. has evolved to produce sounds.

In other Scorpaeniformes, such as the sculpin *Myoxocephalus scorpius*, the presence of sonic muscles originating on the skull and inserting onto a lateral vertebral element has been reported (Barber and Mowbray, 1956). Furthermore, a similar sound-producing structure, based on a pair of sonic muscles originating on the occipital portion of the cranium and connected to two or more tendons attaching on different parapophysis, was described by Hallacher (1974) in 82 species of rockfishes (Sebastidae species, Scorpaeniformes). Three main differences can be found between the sonic apparatus of Sebastidae species described by Hallacher (1974) and that of *Scorpaena* spp. described here. *Scorpaena* spp. possess three pairs of muscular bundles (rather than a single muscle), the swimbladder was absent in all specimens observed as part of this study, and the sonic system of *Scorpaena* spp. was characterized by a higher number of tendons than for that of Sebastidae described by Hallacher (1974). These morphological differences could explain the difference between the /Kwa/ acoustic structure and that of Sebastidae sounds reported in the literature. Several studies have documented a wide range of sound types emitted by *Sebastes* spp. (Miyagawa and Takemura, 1986; Nichols, 2005; Širović and Demer, 2009; Širović et al., 2009; Zhang et al., 2015), which could correspond to the high variability of sonic apparatus configurations

reported by Hallacher (1974). All sounds are characterized by rapidly dampened pulses with low-frequency content, i.e. below 1000 Hz (Miyagawa and Takemura, 1986; Zhang et al., 2015). These studies suggest that single and simultaneous twitches of the bilateral sonic muscles attached to the swim bladder produce a single sound pulse consisting of 2–3 cycles, where the peak frequency of the call is determined by the rate of muscle contraction (Miyagawa and Takemura, 1986; Zhang et al., 2015).

In contrast, the period of muscle contraction associated with the /Kwa/ sound (i.e. 80–100 Hz corresponding to a pulse period of 9–13 ms; Di Iorio et al., 2018) is not responsible for the peak frequency of the call, which is in the range 746±135 Hz (Di Iorio et al., 2018). Di Iorio et al. (2018) suggested that the acoustic structure of the /Kwa/ implies that each muscle twitch produces multiple vibrations, causing the radiated sound to have a different peak frequency from the muscle contraction rate. The /Kwa/ peak frequency corresponds to the cycle period within each single pulse. As the muscle cannot contract at a speed higher than ca. 200 contractions per second (Rome and Lindstedt, 1998; Connaughton, 2004), direct muscular control would be unable to generate sounds with such spectral content. A frequency multiplier that produces multiple vibrations for each contraction is therefore required (Bradbury and Vehrencamp, 1998). Several small animals resort to different kinds of frequency multiplier; for example, in arthropods (which possess hard exoskeletons), external 'comb-like'
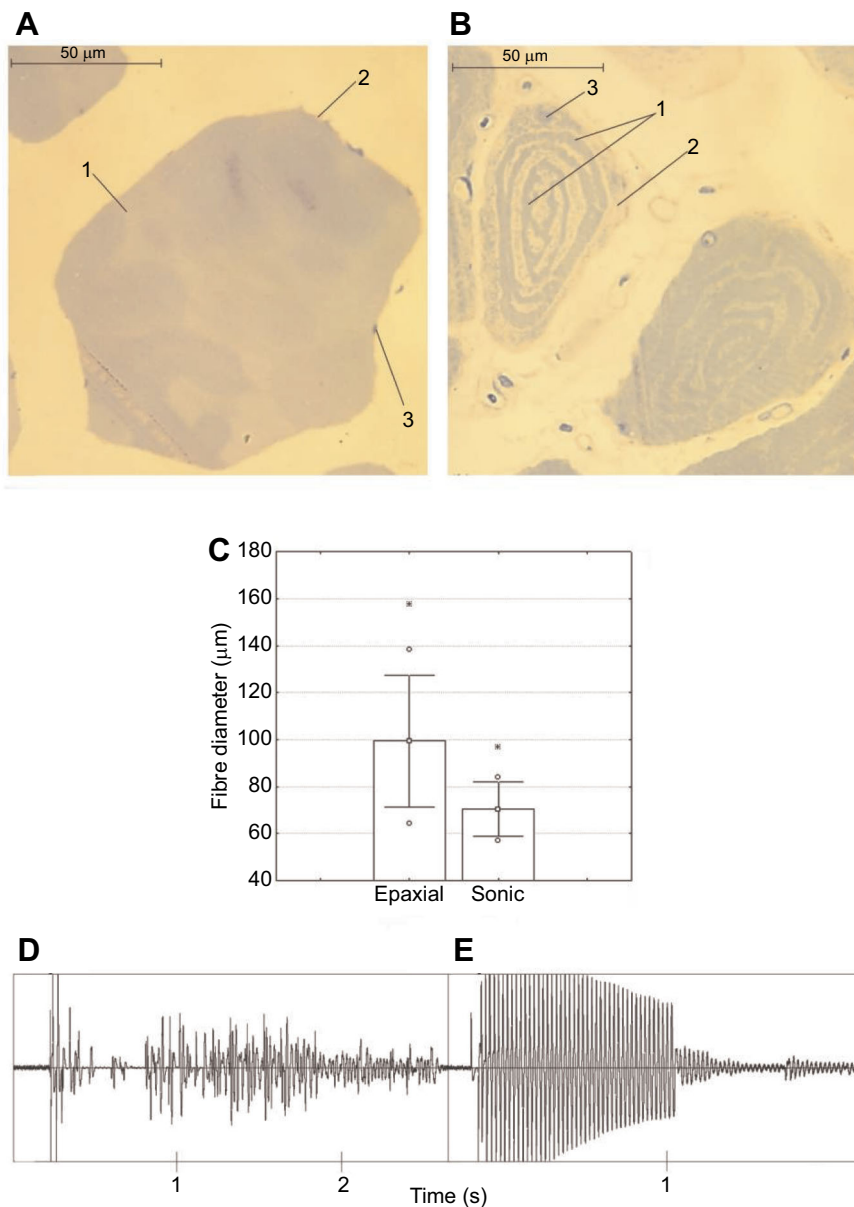
Fig. 4. Histological and functional comparison of epaxial and sonic muscles of _S. porcus_. (A,B) Transverse section of (A) epaxial and (B) sonic muscle fibres (observed at with an optic microscope at ×40 magnification). 1, myofibrils; 2, sarcoplasmic reticulum; 3, nucleus. (C) Epaxial and sonic muscle fibre diameter. (D,E) Waveform of the response of epaxial (D) and sonic (E) muscle to the same electric stimulation (7 ms stimulation at 1 V repeated every 25 ms).

structures can be dragged over sharp edges with a single muscle contraction, causing sounds with high-frequency content (Bradbury and Vehrencamp, 1998). A typical example of frequency multipliers are the strings found in all chordophones (i.e. stringed instruments), which vibrate at frequencies that are dependent only on string properties (length, weight, linear density, tension) and not on the initial frequency that excited them, i.e. the rate at which the strings were plucked (Bradbury and Vehrencamp, 1998). Our hypothesis is that each muscle contraction provokes perpendicular vibrations of the tendons. As in every chordophone, it is possible that at certain locations along the tendon, the various reflected waves cancel each other out (nodes), while the tendon moves further in other locations (antinodes). Such stable patterns of large and low vibration amplitude are called standing waves, and the frequencies that generate them are called natural modes of the string. In one-dimensional systems such as a tendon or a string, the natural modes are likely to constitute all or parts of a harmonic or pseudo-harmonic series (overtones) (Bradbury and Vehrencamp, 1998). We suggest that each of the 3–5 tendons found in the sonic mechanism of _Scorpaena_ spp.

acts as a frequency multiplier of the muscle bundle contractions and that resonant properties of the tendons determine the peak frequency of the /Kwa/, the frequency spectra and the pseudo-harmonic profile, which characterize this peculiar fish sound. In _Scorpaena_ spp., it is possible that each of the three muscular bundles on each side of the midline contracts synchronously and/or alternately, as shown in the Northern searobin (_Prionotus carolinu_s; Connaughton, 2004). This potential for alternate contraction of each muscular bundle, together with the variability in the length and number of tendons found between and within species, could explain the high variability of /Kwa/ acoustic features reported by Di Iorio et al. (2018). Moreover, the case of the lateral bundle (δ) is particularly intriguing, because its separation into a rostral part and a caudal part could easily explain the important call variability. The caudal part could produce vibrations in the lateral tendons (δ1 and δ2); contraction of the rostral part could modify the tendon tension and thus the frequency during sound production. Further studies are required to determine the function of the Baudelot's ligament, which could also produce vibrations of the pectoral girdle.

Table 2. Received levels (dB re. 1 µPa SPL rms) during SNC1 and SNC2

		Aquarium 1				Aquarium 2			
		Internal hydrophone 1		Internal hydrophone 2		Internal hydrophone 3		Internal hydrophone 4	
Analysis	External hydrophone RL	RL	Difference	RL	Difference	RL	Difference	RL	Difference
SNC1	93.3	99.6	**+6.3**	95.1	+1.8	97.5	**+4.2**	95.7	+2.4
SNC1	96.7	103.6	**+6.9**	93.4	−3.3	101.3	**+4.6**	100.5	**+3.8**
SNC1	90.2	97.3	**+7.1**	94.4	**+4.2**	95.2	**+5**	92	+1.8
SNC1	96.2	99.5	**+3.3**	100.8	**+4.6**	98.8	+2.6	96.8	+0.6
SNC1	94.1	101.2	**+7.1**	98.2	**+4.1**	98.4	**+4.3**	100.9	**+6.8**
SNC1	94.8	101.6	**+6.8**	98	**+3.2**	99.4	**+4.6**	100.4	**+5.6**
SNC1	95.8	102.2	**+6.4**	98	+2.2	100.2	**+4.4**	100.3	**+4.5**
SNC1	95.7	100	**+4.3**	96.5	+0.8	98	+2.3	98.2	+2.5
SNC1	95.6	101.1	**+5.5**	98.3	+2.7	99.1	**+3.5**	99.8	**+4.2**
SNC1	94.8	101.2	**+6.4**	97.6	+2.8	99.3	**+4.5**	98.6	**+3.8**
SNC1	90.3	96.9	**+6.6**	95.8	**+5.5**	92.9	+2.6	90.4	+0.1
SNC2	93.3	97.3	**+4**						
SNC2	96.3	100.3	**+4**						
SNC2	99.8	105.8	**+6**						

SNC1, semi-natural conditions; SNC2, semi-natural conditions with video recording; SPL, sound pressure level; rms, root mean square. For the internal hydrophones (located inside the experimental aquaria), both the received level (RL) and the difference from the external hydrophone RL (i.e. control) are shown (bold indicates differences >3 dB).

Sound-producing mechanisms with long tendons present advantages. Metabolic energy can be saved because tendons store and return elastic strain energy, tendons can recoil elastically much faster than muscles and tendon elasticity enhances force (Alexander, 2002; Roberts and Azizi, 2011). In the case of sound production with a high number of pulses, muscles do not need to exert force but only to sustain the rhythm. A reduction in the number of sarcomere (i.e. a shorter muscle) and an elongation of the tendon would allow more energy to be saved.

The lowest frequency of a tensed string is given by the speed of waves on the string:

$$c_{\text{wave}} = \sqrt{\frac{T}{m/L}} = \sqrt{\frac{P}{\rho}}, \quad (1)$$

where T is string tension (in N), m is string mass, L is string length, P is stress (in Pa) and ρ is volumetric mass (Fletcher, 1964). The lowest frequency ($f=c_{\text{wave}}/2$) corresponds to a wavelength that matches the length of the string, occurring when $L=\lambda_{\text{wave}}/2L$. For the /Kwa/ sound, $L\approx2$ cm and $f\approx800$ Hz, meaning that $c_{\text{wave}}=32$ m s^{-1}. Such a speed occurs when the stress P is equal to 1 MPa, which is reasonable in vertebrate tendons (Alexander, 2002).

Scorpaenidae spp. are a monophyletic group of sedentary, solitary and benthic species often characterized by an overlapping distribution (La Mesa et al., 2005; Turan et al., 2009; Saju et al., 2014). From an evolutionary perspective, species sharing the same acoustic space would be favoured by all morphological and physiological modifications that permit differentiation of their call characteristics or temporal dynamics from those of the other species (acoustic niche hypothesis; Krause, 1987); at the same time, the range of signal variability is limited by similar environmental constraints (acoustic adaptation hypothesis; Morton, 1975) and, in related taxa, also by similar morphological and phylogenetic constraints. The term 'new adaptive zone' refers to 'a set of ecological niches that may be occupied by a group of species that exploit the same resources in a similar manner after the acquisition of morphological and/or physiological characteristics' (Parmentier and Fine, 2016). In parallel, we suggest that morphological specialization of the sound-producing apparatus should favour the appearance of a new soundscape window. In this context, all

Scorpaena spp. examined during this study (*S. porcus*, *S. scrofa* and *S. notata*) share the same kind of sound-producing mechanisms that allow them to communicate in an 'unusual' acoustic space (i.e. higher frequency niche). Scorpaenidae are benthic, sedentary and territorial fish, and *S. porcus* and *S. scrofa* in particular are ubiquitous inhabitants of the pivotal coastal environment of *Posidonia oceanica* meadows (Ferri et al., 2012; Matić-Skoko et al., 2015). We hypothesized that the /Kwa/ sound might have evolved as a result of environmental constraints imposed by the meadows itself, which, with their dense vegetation, limit the effective range of visual communication, especially for sedentary and benthic species.

Fish sounds are generally considered to be species specific (Amorim, 2006; Mann et al., 2016), where the diversity of sound features between species is generally greater than the variation within species. One of the main evolutionary advantages of sympatric species producing species-specific sounds would be the maintenance of sexual isolation (Bradbury and Vehrencamp, 1998) or the identification of a specific partner. Within the framework of this study, it was not possible to label the /Kwa/ as a courtship or agonistic sound. However, the behavioural observation carried out during SNC2 shows that the /Kwa/ sound was emitted with a reduced latency after the insertion of the second individual into the aquarium. Accordingly, during SNC1, all /Kwa/ emitted by the experimental animals were recorded immediately after the fish were allowed to interact physically in the same aquarium. An interesting comparison can be made with *Sebastes* spp., which do not show sexual dimorphism of the sonic apparatus and for which territorial or agonistic communication has been reported in more than one species (Hallacher, 1974; Nichols, 2005; Miyagawa and Takemura, 1986). Nichols (2005) reported sound production in *Sebastes nebulosus*, *S. atrovirens*, *S.carnatus*, *S. chrysomelas*, *S. caurinus* and *S. maliger*. A considerable overlap between agonistic call characteristics was found. The author concluded that these agonistic sounds are designed for short-range communication and that they are not species specific (Nichols, 2005). These observations support the hypothesis that the /Kwa/ sound might be important during territorial defence and potentially emitted by both sexes. The majority of fish sounds associated with territorial defence are described in intra-specific interactions between males. However, inter-specific vocalization has
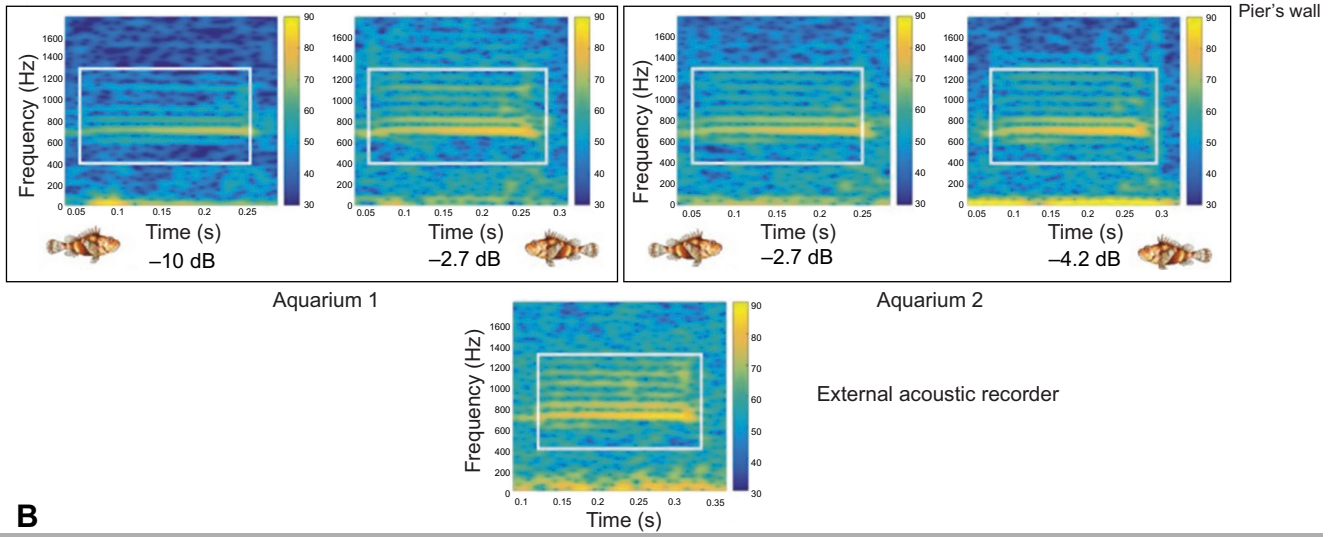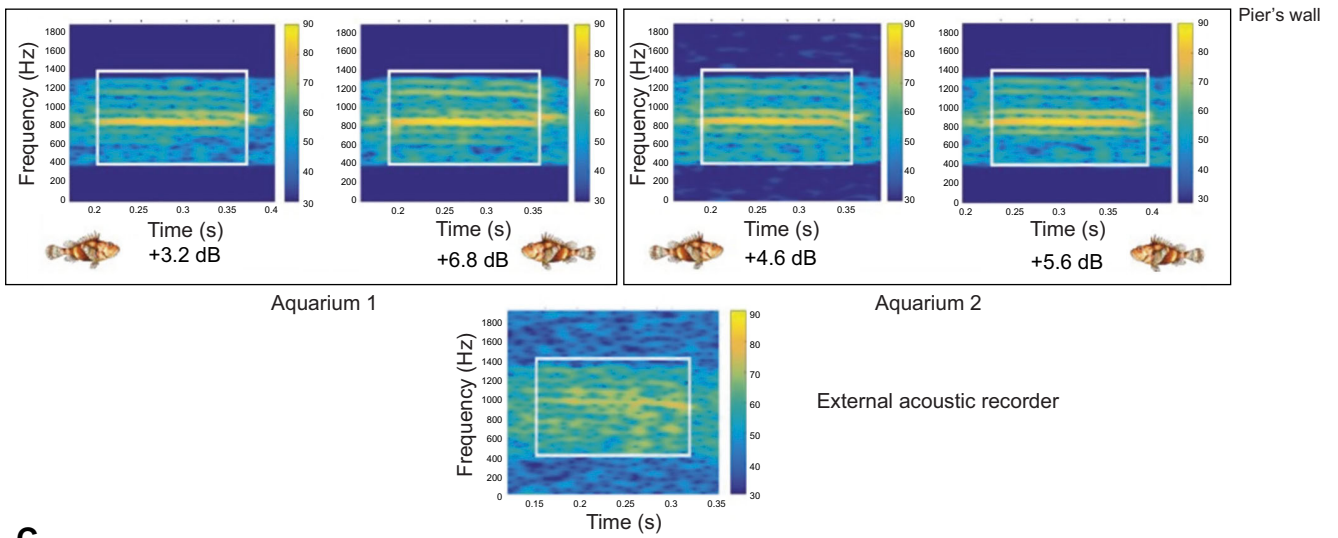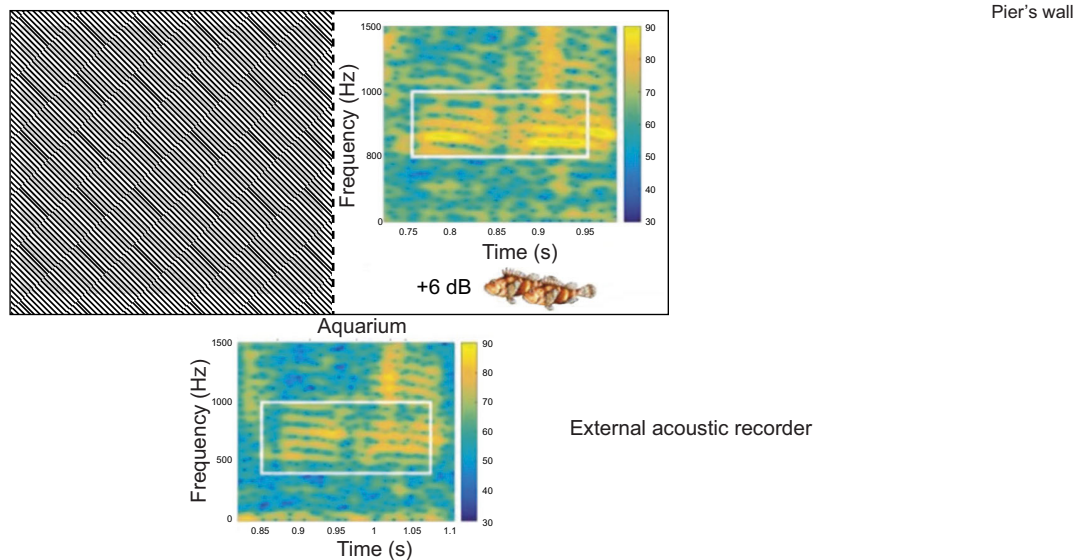
Fig. 5. See next page for legend.

Fig. 5. Schematic representation of the semi-natural condition (SNC) recording setup and analysis. In each situation, the spectrogram of the same /Kwa/, as recorded by the hydrophones inside the experimental aquaria (top) and by the external acoustic recorder (control, bottom), is shown. The dashed line indicates the location of the partitions to control visual and tactile communication between the two individuals. The difference in received levels (dB re. 1 µPa) between each internal hydrophone and the external datalogger is indicated. (A) Example in which the /Kwa/ was recorded with higher received levels by the external recorder. (B) Example in which the /Kwa/ was recorded with higher received levels by the internal hydrophones. (C) Two /Kwa/ associated with a lateral display (see Materials and Methods; SNC2: 3rd occurrence in Movie 1). These two /Kwa/ were recorded with higher received levels by the internal hydrophone.

been reported (Ladich, 1997; Lagardère et al., 2005; Parmentier et al., 2010b). The mormyrid *Gnathonemus petersii* utters 'click' sounds against *Gymnotidea* spp. (Rigley and Marshall, 1973; Ladich, 1997), while sunfish produce 'rasping' sounds when defending their territory against co-generics (Ballantyne and Colgan, 1978; Ladich, 1997). Furthermore, territorial sounds can also occur during competitive feeding, as shown in grey gurnards (Amorim et al., 2004). In the light of the results from this study, it can be hypothesized that different Scorpaenidae sharing the same environment might obtain a fitness-related advantage by resorting to inter-specific communication in order to defend their feeding territory from intruders, either co-specific or co-generic. In this sense, further studies are required to elucidate the relationship between sonic mechanisms and neural patterning variability among species. It will also be interesting to investigate whether the /Kwa/ inter-specific variability is greater than the intra-specific variability; if this is the case, it would be important to evaluate whether different species can can perceive and discriminate con-specific and hetero-specific signals, as demonstrated in *Stegastes* spp. (reviewed in Myrberg and Lugli, 2006).

In conclusion, this study used an interdisciplinary approach for investigating the identity of fish species emitting the most abundant and particular fish sound dominating the soundscape of *P. oceanica* meadows, the /Kwa/. Morphological, histological and electrophysiological characterization of the sound-producing apparatus, interpreted with visual and acoustic evidence recorded in semi-natural conditions permitted the identification of *Scorpaena* spp. as the species complex emitting the /Kwa/ sound.

Author contributions
Conceptualization: M.B., L.D.I., C.G., E.P.; Methodology: M.B., J.S., L.D.I., C.G., E.P.; Software: L.D.I., C.G.; Validation: M.B., J.S., L.D.I., C.G., E.P.; Formal analysis: M.B., J.S., C.G., E.P.; Investigation: M.B., J.S., E.P.; Resources: P.L., S.G., E.P.; Data curation: M.B., J.S., L.D.I.; Writing - original draft: M.B.; Writing - review & editing: M.B., J.S., L.D.I., C.G., P.L., S.G., E.P.; Visualization: M.B., J.S.; Supervision: M.B., L.D.I., E.P.; Project administration: E.P.; Funding acquisition: M.B., L.D.I., E.P.

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