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MORPHOLOGICAL INNOVATIONS AND EVOLUTIONARY TRANSITIONSIN BOXFISH ACOUSTIC COMMUNICATION

Eric Parmentier[1], Louise Eche[1], Céline Greeven[1], Marine Banse[1], Frédéric Bertucci[2], Marc Thiry[3], Xavier Raick[1,4], Terry J. Donaldson[5], David Lecchini[6]

[1]Laboratory of Functional and Evolutionary Morphology, Freshwater and Oceanic Science Unit of Research, University of Liège, Liège 4000, Belgium
[2]UMR MARBEC, University of Montpellier, CNRS, IFREMER, IRD, Sète 34200, France
[3]Laboratory of Cellular and Tissular Biology, GIGA-Neurosciences, Cell Biology L3, University of Liège, Liège 4000, Belgium
[4]K. Lisa Yang Center for Conservation Bioacoustics, Cornell University, Ithaca, NY 14850, USA
[5]University of Guam Marine Laboratory/Guam, EPSCoR, UOG Station, Mangilao, Guam 96921, USA
[6]EPHE-UPVD-CNRS, USR 3278 CRIOBE, PSL University, Moorea 98728, French Polynesia

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Abstract

Ostraciid boxfishes comprise two subfamilies: the Atlantic Lactophrysinae and the Pacific Ostraciinae. Although all species are reported to produce sounds, key acoustic and anatomical data remain missing, particularly for Atlantic taxa. This study compares the sound-producing mechanisms in both subfamilies and examines the anatomy of a representative from the sister family Aracanidae to infer the evolutionary history of acoustic communication. Atlantic species possess a novel structure, the sphaera sonica, composed of paired globular connective masses surrounded by fast-contracting sonic muscles with multidirectional fibres. Muscle contraction displaces these masses against the swim bladder fenestra, generating sound. In contrast, Pacific species lack this mass; their sonic muscles are divided into distinct extrinsic and intrinsic layers positioned around the fenestra. Aracanidae show no sonic system, but anterior body muscles might be homologous to those found in boxfishes. These observations suggest that the Lactophrysinae system represents a more ancestral configuration. The transition to the Ostraciinae condition involves reduction of the globular mass and increased muscular differentiation, probably enhancing control over bladder deformation and enabling more complex acoustic signalling. This study highlights how anatomical innovations in muscle arrangement have contributed to the diversification of acoustic mechanisms within the Ostraciidae.

Introduction

Ostraciidae, commonly known as boxfish, derive their name from their distinctively shaped body, which is cubic or tetrahedral in nature. These fascinating creatures possess a bony shell or carapace composed of thick hexagonal modified conjoined plates that almost entirely encase their bodies (Tyler 1963). Given that the rigidity of the carapace prohibits any bending of the body, boxfish have lost the ability to perform undulatory swimming and depend solely on their caudal peduncle and fins for locomotion (Hove *et al.* 2001). Boxfishes (Ostracioidea) comprise 37 nominal species distributed across two families: Aracanidae and Ostraciidae. Evolution within this clade seems to be marked by a significant stiffening of the locomotor apparatus, particularly through the fusion of vertebral pieces with each other and with the neurocranium, with an increasing rigidification of the carapace (Tyler 1963, 1980). From this perspective, the Aracanidae, primarily inhabiting deeper waters than the Ostraciidae, appear less specialized and presumably more primitive than the Ostraciidae, which are predominantly found in shallow waters (Tyler 1980, Klassen 1995). The Ostraciidae family is divided into two subfamilies. Lactophrysinae are found in the Atlantic and include *Acanthostracion* and *Lactophrys* species, whereas Ostraciinae are found in the Indo-Pacific and include species of three genera: *Ostracion*, *Lactoria*, and *Tetrosomus* (Santini *et al.* 2013a). Based on osteological comparisons, the anatomy of the lactophrysin subfamily is more generalized than that of ostraciins. The family Ostraciidae is thought to have diverged into two evolutionary lines. Lactophrysins, which are anatomically closer to their ancestral form, were successful only in the Atlantic, probably representing a relict distribution (Tyler 1980).

Sorensen (1884) indicates that, according to Shaw's Encyclopedia (1804), *Lactophrys trigonus* may emit growls following the sudden expulsion of air through the branchial orifices. Different studies have then reported the ability to produce sounds in different species, but the sounds were not always precisely described. *Acanthostracion quadricornis* produced clicks and grunts when manually stimulated in an aquarium, whereas *Lactophrys triqueter* and *Lactophrys bicaudalis* produced weak grunts and squeaks when held in the hand (Fish and Mowbray 1970). Growling noises of the tropical Atlantic *Lactophrys trigonus* were attributed by Bridge (1904) to the air bladder and associated muscles, but the author did not provide further descriptions of the sound-producing system. However, some scattered descriptions can be found. In *L. triqueter*, Sorensen (1884) reports that the swim bladder is thick and fibrous but presents a dorsorostral rounded area transformed into a gelatinous zone. This area is connected to various muscle fibres, described as a 'hollow ball of muscles' (Tyler 1963, Winterbottom 1974). Without providing an explanation of the function, Sorensen (1884) suggests that these structures could be linked to the sound-production mechanism.

In the Pacific species, sounds were first reported in *Ostracion meleagris*, which produced a heavy low vibration when the fish was hand held (Fish 1948). Additionally, Lobel (1996) identified three distinct sounds produced by *Ostracion meleagris* during spawning, competition, and agonistic behaviour. Parmentier *et al.* (2019) also showed that *Ostracion cubicus* and *O. meleagris* can produce two sounds in a sequence when held by hand. The ability to produce two types of sounds in sequence is exceptional, similar to the capabilities seen in *Pterois* species (Parmentier *et al.* 2024), and is likely to require a specific morphology. *Ostracion* species can generate long (>60 s), faint hums that most probably correspond to the vibration recorded by Fish (1948). These sounds are made of more than hundreds of regularly produced pulses (hums) that are occasionally interspersed with loud clicks (Parmentier *et al.* 2019). Notably, two distinct pairs of sonic muscles were identified, each probably serving a unique sound-producing function. Extrinsic vertically oriented muscles originate from the vertebral column and attach to the swim bladder. Below these muscles, longitudinal intrinsic muscles cover the swim bladder, enabling the second distinct sound-producing mechanism (Parmentier *et al.* 2019).

Sound production has also been reported in the thornback cowfish, *Lactoria fornasini*, in which hums would serve for synchronizing spawning (Moyer 1979). Unfortunately, Moyer did not record the sounds. The cowfish *Lactoria cornuta* produces two kinds of sounds, also called hums and clicks, when hand held (Parmentier *et al.* 2021). Hums are characterized by a series of low-amplitude pulses that persist over extended periods, implying their production by fatigue-resistant muscles. In contrast, clicks are shorter in duration but possess a higher amplitude than hums, suggesting that they arise from more forceful contractions (Parmentier *et al.* 2021). Interestingly, similar to *O. cubicus* and *O. meleagris*, *L. cornuta* also possesses two pairs of sonic muscles associated with the swim bladder, indicating a similarity in the sound-production mechanism across these genera.

In this study, we aim to investigate how anatomical and acoustic traits involved in sound production vary across major lineages of boxfishes (Ostraciidae) and their relatives (Aracanidae). Given that Ostraciidae and Aracanidae are sister groups and that Aracanidae exhibit less fused skeletal structures, we ask whether boxfish sound-producing mechanisms have evolved from generalist muscular and skeletal configurations. We also test whether the variation in sonic muscle organization between Atlantic and Indo-Pacific Ostraciidae corresponds to distinct evolutionary trajectories. We suggest that derived morphologies, such as the differentiation of sonic muscles, will be associated with a broader acoustic capacity. Our comparative approach integrates anatomical, histological, and acoustic data to assess evolutionary scenarios for the emergence and diversification of sound-production mechanisms in boxfishes. In the present study, we recorded the hand-held disturbance sounds produced by different boxfish species from the Atlantic and Indo-Pacific Oceans. Our main objective is to describe the sounds and the sound-producing apparatus in Atlantic Ostraciidae. This dataset will be compared with data from Indo-Pacific Ostraciidae and a representative species from the Aracanidae to investigate the evolutionary change of the sound-production mechanism in Ostraciidae.

Materials and methods

The experiments were approved by the ethical commission of the University of Liège (case 1759).

Indo-Pacific species

Five specimens [total length (TL) 3–5.1 cm] of *O. solorensis* were purchased from an aquarium store, stocked separately in tanks at 26°C ± 1°C, and were maintained on a 12 h–12 h light–dark cycle. The sex of the specimens was not determined. The tanks (65 cm × 35 cm × 45 cm) were equipped with external filters, internal heaters, and bubblers for water oxygenation. Fish were fed with mussels three times a week.

Additional specimens of Pacific species were collected in December 2021 and July 2022 by snorkelling or scuba diving in coral reef areas of Guam and the Seychelles, respectively, between 2 and 20 m depth. Fish were caught by chasing them with a hand net at night. A single specimen of *Lactoria cornuta* (TL 13.7 cm) was collected in Guam. Specimens of *O. meleagris* (*N* = 3, TL 10.5–12 cm) were collected in the Seychelles. Specimens of *O. cubicus* were collected in both Guam (*N* = 2, TL 6.3 and 10.1 cm) and the Seychelles (*N* = 3, TL:13–14.5 cm).

Atlantic species

Two field missions were conducted in Guadeloupe: the first from February to June 2020 and the second in February 2023. The collection of specimens was carried out using a combination of methods. Fish were captured with a net during snorkelling sessions at various sites along the Guadeloupean coast; others were purchased from professional fishermen or obtained from the Aquarium de la Guadeloupe (https://www.aquariumdelaguadeloupe.com). From these field trips, 19 *Acanthostracion polygonius* (TL 16–27 cm), two *Acanthostracion quadricornis* (TL 25 cm), four *L. bicaudalis* (TL 15–18 cm), one *Lactophrys trigonus* (TL

22 cm), and 22 *Lactophrys triqueter* (TL: 9–20 cm) were captured. Sexes were not determined.

After capture, the specimens were either kept in large nets to be recorded *in situ* or transported to the Marine Biology Laboratory of the University of the Antilles in Guadeloupe, where they were placed in three tanks (one large, 65 cm × 35 cm × 45 cm; and two small, 40 cm × 30 cm × 30 cm). Only one fish was placed per aquarium owing to toxins released, particularly by *L. triqueter*, which could cause the death of other individuals, and also owing to potential aggressive behaviours, because these species are usually solitary. The tanks were equipped with aerators for oxygenation, and stones and coral fragments were provided as shelters. The water was regularly replaced with fresh seawater supplemented with coral debris, molluscs, and sponges, collected under a nearby pontoon or at the collection sites. The photoperiod was 12 h–12 h, and the water was maintained at room temperature (~26°C). The individuals were fed daily with food pellets for reef fish.

Sound recording

Recording conditions varied depending on whether the specimens were recorded *in situ* or in tanks. Whenever possible, recordings in the natural environment were prioritized to minimize the influence of the tank on the sounds (Akamatsu *et al.* 2002, Boyle et al. 2014, Banse *et al.* 2023). When this was not feasible, we resorted to recording the fish in aquariums. Sounds were recorded using an HTI-96-min hydrophone (High Tech Inc., Long Beach, CA, USA) flat frequency response range between 2 Hz and 30 kHz (sensitivity: −164.4 dB or 1 V μPa^{-1}) connected to a Tascam recorder (TASCAM DR-05X, Wiesbaden, Germany). The sampling rate was 44.1 kHz. The fish were hand-held 15 cm deep in the water at a distance of 5 cm from the hydrophone (mouth towards the hydrophone). *Ostracion solorensis* were recorded in the same way, with the same equipment, in a tank (100 cm × 50 cm × 50 cm). Although handling has a level of artificiality, it does provoke fish to produce sounds as if they were captured by a predator (Oliveira *et al.* 2014). This recording method was chosen because it elicits sounds from the same behavioural context and it ensures that sounds are produced at the same distance from the hydrophone, which is also placed in the same part of the tank in order to account for differences in signal loss (Markl 1971, Parmentier *et al.* 2011a, b, Millot and Parmentier 2014). Moreover, this distance also enables fish to remain within the attenuation distance (Akamatsu *et al.* 2002). With this methodology, variations in features within the sounds are attributable to the fish and not to environmental conditions. This paper does not concern fish behaviour.

Sound analysis

Sounds were analysed with AviSoft-SAS Lab Pro v.5.2 software (Avisoft Bioacoustics, Glienicke, Germany). Recordings in small tanks induce potential artefacts because of reflections and tank resonance, and an estimated minimum resonant frequency of 2250 Hz was calculated following the equation of Akamatsu *et al.* (2002). A low-pass filter (2 kHz) was consequently applied to all recordings. According to the observation that a call is made of trains of successive pulses, the following temporal acoustic variables were measured manually on oscillograms: call duration (time from the beginning of the first pulse to the end of the last pulse, in milliseconds); pulse period (time between the onset of two consecutive pulses, in milliseconds); pulse duration (time from the beginning to the end of a pulse, in milliseconds); and the number of pulses in the sound. For spectral characteristics of sounds, sounds were initially downsampled to 4 kHz, and the dominant frequency of pulses (frequency component with the most energy, in hertz) was measured from power spectra [fast Fourier transform: 512 points, Hamming window].

Anatomy

Six *L. triqueter*, seven *A. polygonius*, and three *O. solorensis* specimens were euthanized in a tricaine methanesulphonate solution (MS 222). Specimens of *L. triqueter* and *A. polygonius* were immediately dissected to sample the muscles attached to the swim bladder and other tissues associated with the sound-producing mechanism. Muscle samples were fixed in a cacodylate buffer solution of glutaraldehyde (1 volume of 25% glutaraldehyde, 4 volumes of 25% cacodylate buffer, and 5 volumes of distilled water) for 24 h, then placed in cacodylate buffer for transmission electron microscopy (TEM). Muscle, swim bladder, and vertebral column samples were fixed in 5% formalin for optical histology. One specimen of *L. triqueter* and one *A. polygonius* were fixed in 5% formalin for 48 h, then transferred in 70% ethyl alcohol for micro-CT scanning.

In TEM preparation, samples were post-fixed for 60 min in 2% osmium tetroxide and dehydrated through graded ethanol–propylene oxide series, then embedded in epoxy resin (SPI-PON 812, SPICHEM). The resin was then polymerized at 60°C for 48 h. Semi-thin (1 µm) and ultra-thin (60–70 nm) sections were cut using a diamond knife (Diatome) mounted in an ultramicrotome (Ultracut S Leica). Semi-thin sections were stained with a Toluidine Blue solution and observed under an Olympus Provis Light Microscope at ×40 magnification to select the areas to be sampled for TEM. Ultra-thin sections were contrasted in the dark for 15 min in uranyl acetate solution and for 15 min in lead citrate solution. For ultrastructural analyses, random fields of these pieces were examined under a Jeol TEM JEM-1400 transmission electron microscope at 80 kV, and random fields were photographed using an 11 megapixel camera system (Quemesa, Olympus). For each muscle, 11 fields were microphotographed at ×1500 magnification to analyse their fine structural organization. Morphometric measurements were performed with iTEM v.5.2 (Olympus, Tokyo, Japan) for the TEM microphotographs and analysed using the program ImageJ v.1.52a.

After formalin fixation, cross-sections were made at the level of the sound-producing apparatus and in the oesophagus of *L. triqueter*. Samples were dehydrated in butanol, decalcified, embedded in paraffin, sectioned serially at 10 µm (Reichert microtome) and stained using Gill III Haematoxylin or Masson's Trichrome. Histological sections were observed with a digital microscope VHX 7000 (Keyence, Osaka, Japan) that allowed measurements on the cross-sections. Five photographs per species of sonic muscles in cross-section were used to measure the area of muscle fibres and myofibrils. A total of 340 sonic muscle fibres were measured per species using Photoshop software (v.14.1). The cell contours and the myofibrils were coloured differently. The number of pixels coloured for each area was then converted to micrometres squared using the scale present on the photographs. In this way, it was possible to infer the surface area occupied by the myofibrils within the muscle cells. Five optical microscopy photographs of the erector dorsalis (muscle of the dorsal fin) from *L. triqueter* ($N = 1$) were used to measure the area of 340 fibres using the semi-automatic measurement tool of the VHX-7000 software (Keyence). Assuming arbitrarily that the muscle fibres are analogous to circles, the diameter is deduced from the measured area.

Micro-CT scannings of an *A. polygonius* and a *Polyplacapros tyleri* specimen were completed using a FleXCT system consisting of a customized UniTOMXL scanner from Tescan XRE (De Samber *et al.* 2021). Images of *A. polygonius* were generated at 70 kV and 1000 µA. This generated 2166 images with a voxel size of 65 µm. Images of *P. tyleri* specimens were generated at 70 kV and 1126 µA. This generated 2563 images with a voxel size of 65 µm. The image reconstruction was performed using the software Panthera from Tescan XRE. Segmentation, visualization, and analysis were performed using the software Aquila™. Three-dimensional images were produced in 16-bit and subsequently converted into 8-bit voxels using ImageJ (Abramoff *et al.* 2014). Micro-CT scanning of an *L. triqueter* specimen was completed using an RX EasyTom with an aluminium filter. Images were generated at 65 kV and 272 µA. This generated 2228 images with a voxel size of 65 µm. Reconstruction was performed using X-Act software from RX Solutions. Segmentation, visualization, and analysis were performed using Dragonfly software (Object Research Systems Inc., Montreal, QC, Canada, 2019). Three-dimensional images were produced in 16-bit and subsequently converted into 8-bit voxels using ImageJ (Abramoff *et al.* 2014). Direct volume renderings (iso-surface reconstruction) were used to visualize the subset of selected voxels of the skeleton, muscle, and swim bladder in AMIRA v.2019.2.

Statistical analysis

Descriptive statistics were realized for each variable of both types of sounds and for muscle features. For each type of sound, the mean and SD were calculated for all the acoustic variables. The morphological features were tested initially for the assumption of normality (Shapiro–Wilk test) and homoscedasticity of variances (Levene's test). The Mann–Whitney *U*-test was used to compare the sonic muscle and erector dorsalis muscle in *L. triqueter* and to compare sonic muscles of *L. triqueter* and *A. polygonius*. Statistical analyses were carried out with R v.3.0.2 or GraphPad (v.5, GraphPad Software, Inc.).

Results

Sound production

The sounds of Ostraciinae can be categorized into two main groups. *Ostracion* and *Lactoria* can produce long trains of low-intensity pulsations (hums), which are comparable to prolonged growls, or short-duration sounds (clicks) composed of one to a few pulses depending on the species, with an amplitude 10–40 times greater than the hums produced by specimens of the same species. Further details are provided below. During this study, we did not systematically record these two kinds of sounds in *Lactophrys* and *Acanthostracion* species. Sounds in *Acanthostracion* were most often short, made of isolated pulses having high amplitude. Sounds in *Lactophrys* species were longer series of pulses having low amplitude.

Ostracion solorensis

Hums and isolated clicks were recorded from juveniles of *O. solorensis* (Fig. 1A). Hums ($N = 5$ specimens and $n = 51$ sounds) consist of trains lasting between 2 and 18 s. Within the pulse trains, pulses are organized into blocks of one to five pulses. The period between blocks was 61 ± 20 ms (mean ± SD), and the period between pulses within a block was 11.5 ± 11 ms. The dominant frequency was 108 ± 32 Hz. Only three clicks were recorded, which is too few for a detailed description; however, this highlights their ability to produce them. Each of the three clicks consisted of a single peak.

Ostracion cubicus

Ostracion cubicus produces hums that can be interspersed with clicks (Fig. 1B). The hums consist of isolated pulses that appear to be emitted at an irregular rate. For the three specimens from the Seychelles, the average period of the hums was 15 ± 20 ms ($n = 69$), and it was 70 ± 16 ms ($n = 89$) for the two specimens recorded in Guam. This significant difference is statistically supported (Mann–Whitney $U = 89$, $P < .001$), but a larger number of specimens should be used to validate the results.

In both populations, clicks can be produced either in isolation or in series of 2–3 units. Clicks have a duration of 85 ± 40 ms ($n = 14$) in the Seychelles and 53 ± 14 ms ($n = 69$) in Guam. For all specimens, the average frequency was ~220 Hz ($n = 67$). The clicks manifest in two forms. The first type is characterized by a regular succession of peaks. The other type is particularly interesting because it seems to illustrate how they are constructed. Some clicks are preceded by pulsations typically emitted during hums, but a gradual decrease in the emission period of the pulsations is observed, alongside an increase in their amplitude (Fig. 1C). At the end of the click, a pattern of regular peak emissions is observed, when it is no longer possible to distinguish individual pulses. In this case, the period between peaks corresponds to the frequency of the click. The amplitude of the clicks is 30 times greater than that of the pulses in the hums.

Ostracion meleagris

Ostracion meleagris exhibits hums that can also be interspersed with clicks (Fig. 1). The hums consist of pulses emitted at an irregular frequency, with periods ranging from 22 to 100 ms (53 ± 16 ms, $n = 158$), without any particular emission patterns. However, a review of the sequences suggests that some pulses might not always be detected, potentially skewing the average period upwards. The clicks consist of a single peak with an average duration of 15 ± 2 ms ($n = 15$) that are not produced regularly. The amplitude of the clicks is six times greater than that of the pulses in the hums. Additionally, a new type of sound was identified. Grunts, lasting 73 ± 15 ms ($n = 8$), consist of ~6.2 ± 1 pulses ($n = 8$) emitted with a period of 13.6 ± 3 ms ($n = 42$), with an average frequency of 80 ± 10 Hz ($n = 8$). The pulsations in the grunts are made of a single peak.

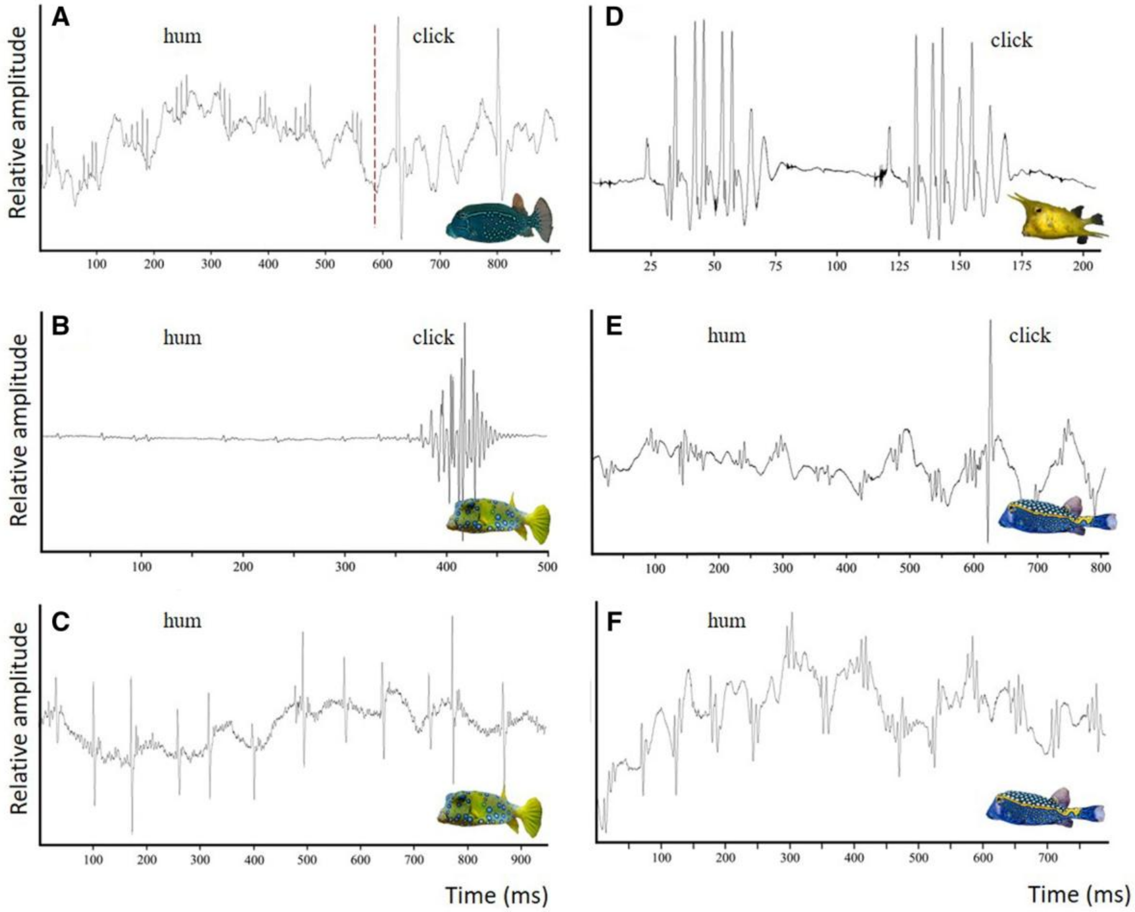

Figure 1. Oscillograms of two types of sounds (hum and click) produced by different Ostraciinae species: *Ostracion solorensis* (A), *Ostracion cubicus* (B, C), *Lactoria cornuta* (D), and *Ostracion meleagris* (E, F). Pulses constituting the hums in *O. solorensis* and *O. meleagris* are made of bursts of peaks and of a single peak in other studied species. Clicks in *L. cornuta* and *O. cubicus* appear to be made of different pulses and of a single pulse in *O. meleagris* and *O. solorensis*. The vertical red dashed line in A separates the hum and clicks.

Lactoria cornuta

In the specimen we have recorded (Fig. 1), sounds lasted between 38 and 51 ms (42 ± 4 ms; *n* = 11) and had a pulse period extending from 3 to 11 ms (6 ± 2 ms, *n* = 71). The dominant frequency ranged between 172 and 194 Hz (186 ± 7 Hz, *n* = 15). The oscillogram allows a clear specific feature to be distinguished. Each sound invariably begins with an isolated pulse, followed by a series of pairs of pulses that are produced with an increasing emission period. This observation helps to explain the previously noted high variability in pulse periods. The period between two pulses in a pair is 4.2 ± 1 ms (*n* = 24), and the period between pairs of pulses is 10 ± 1 ms (*n* = 33).

Lactophrys triqueter

In *L. triqueter*, only two sounds were recorded in a tank from one individual (Fig. 2). These sounds lasted 404 and 318 ms and consisted of 13 and 10 pulses, respectively. The pulse average duration was 19 ± 4 ms (*n* = 23), and the average duration of the pulse period was 32 ± 3 ms (*n* = 21). The dominant frequency was 31 Hz in the short sound and 62 Hz in the long sound. However, this second sound was a harmonic sound with the fundamental frequency being 31 Hz. Each pulse was made of two main peaks, the first one being of higher amplitude than the second one.

Lactophrys bicaudalis

In *L. bicaudalis*, a total of 49 sounds were recorded in a tank from three individuals. The duration of the sounds and the number of pulses within the sounds varied widely, ranging from 46 ms to 13 s and from 1 to 97 pulses, respectively. These pulses had an average duration of 69 ± 22 ms (*n* = 567) and an average dominant frequency of 45 Hz. The average duration of the pulse period was 142 ± 71 ms (*n* = 336) during the production of long trains of pulses (Fig. 2). However, the same types of pulses could be produced at a more spaced interval with a pulse period of 369 ± 139 ms (*n* = 111). In this case, it was possible to distinguish the different pulses clearly. They consisted of four peaks organized in groups of two, with a period of 21.7 ± 4 ms (*n* = 21).

Acanthostracion polygonius

In *A. polygonius*, a total of 60 sounds were recorded in the field from *N* = 3 individuals in an open environment (Fig. 3). The sounds consisted of two to four pulses, each having an average duration of 14 ± 2 ms. The pulse period was 17 ± 2 ms, and the average dominant frequency was 221 ± 32 Hz. Each pulse was composed of four main peaks. The first pulse was of particular interest because it was similar across different sounds, whether they consisted of two or four pulses. In this pulse, the first three

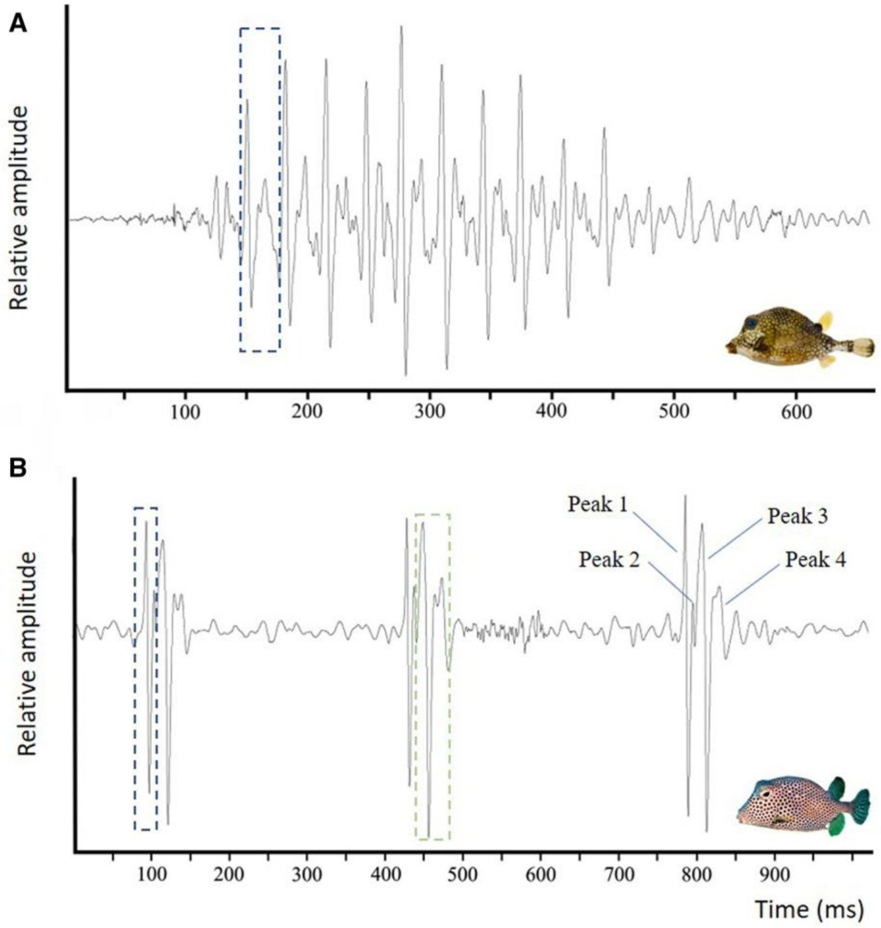

Figure 2. Oscillograms of sounds produced by and *Lactophrys triqueter* (A) and *Lactophrys bicaudalis* (B). The dotted rectangle delineates a single pulse.

peaks showed decreasing amplitude, before the fourth peak exhibited more energy. When present, the third pulse was similar to the second, whereas the peaks of the fourth pulse were always of lower intensity. The period between the peaks (ranging from 4.6 to 6.3 ms) appeared to correspond to the dominant frequency.

A second type of sound, a grunt (Fig. 3), lasting 70 ± 21 ms (*n* = 11) consisted of short trains of two to three pulses with an average duration of 13 ± 2 ms (*n* = 27). The average duration of the pulse period was 36 ± 8 ms (*n* = 17), and the average dominant frequency was 85 ± 2 Hz (*n* = 28). Each pulse was composed of two main peaks separated by peaks of lesser amplitude.

Acanthostracion quadricornis

In *A. quadricronis*, a total of 15 sounds were recorded in a tank from *N* = 3 individuals. Similar to *A. polygonius*, these sounds consisted of two to four pulses (Fig. 3) with an average duration of 16 ± 4 ms. The pulse period was 22 ± 4 ms, and the average dominant frequency was 141 ± 54 Hz. The first pulse displayed the same organization as in *A. polygonius*, with a decrease in the amplitude of the peaks before the fourth, which was more prominent.

Anatomy

Ostraciinae

The sound-producing apparatus of three species of Octraciinae has already been described (Parmentier *et al.* 2019, 2021), allowing us to provide a summary of the observations here. Observation of *O. solorensis* corroborates previous findings. In a dorsal view, *Ostracion* species exhibits a T-shaped swim bladder, whereas it appears heart-shaped in *Lactoria* species (Supporting Information, Video 1). At the anterior part of the T, there is a large swim bladder fenestra, the median part of which is located below the vertebral column (Fig. 4). On either side of the vertebral column, the swim bladder fenestra is covered by an intrinsic longitudinal sonic muscle, which originates anteriorly to the fenestra on the swim bladder and inserts at the fenestra caudal side. This intrinsic sonic muscle is overlaid with an extrinsic sonic muscle, which is perpendicular to the first one (Fig. 4). The latter originates from the fused neural spines of the anterior vertebrae and inserts on the lateral margin of the swim bladder fenestra.

Lactophrysinae

The sound-producing apparatus of the Lactophrysinae is described here for the first time, based on *L. triqueter* (Fig. 4). This apparatus is unique among teleost fishes, and based on its exceptional organization, we name it the sphaera sonica. It comprises elements from the swim bladder, sonic muscles, and skeletal parts. First, similar to *Ostracion*, the swim bladder possesses a dorsal fenestra, which is topped by the vertebral column. Rostrally, the swim bladder is firmly connected to the fused ventrolateral processes of abdominal vertebrae 4 and 5 and to the vertebral column at the

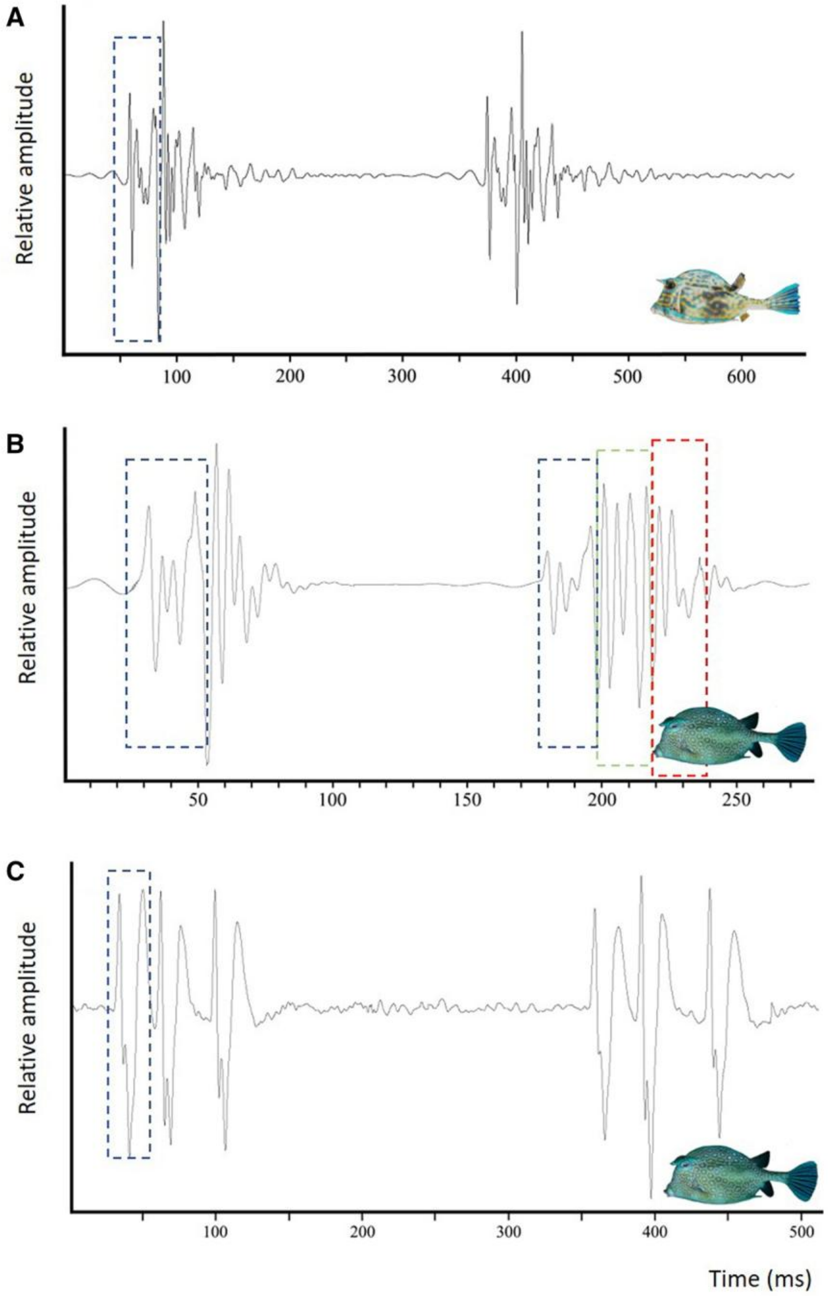

Figure 3. Oscillograms of sounds produced by *Acanthostracion quadricornis* (A) and *Acanthostracion polygonius* (B, C). The different dashed rectangles delineate different pulses.

rear of the swim bladder fenestra (Fig. 4). These different points of insertion on the skeleton prevent its displacement. Second, dorsally, two globular, translucent masses are situated above the swim bladder fenestra, one on either side of the vertebral column. Third, each mass is surrounded by an extrinsic sonic muscle, whose fibres exhibit different orientations and insertions. These muscular fibres attach to the vertebral column and the lateral processes of the fourth and fifth vertebrae, surround the globular mass, and insert on the lateral edge of the fenestra.

In the sphaera sonica, the uncommon features are the globular masses. Consequently, we have used optical and electron microscopy to understand their origin and nature (Fig. 5). Given that the swim bladder is derived from the oesophagus, the latter was used to make comparisons. Examinations showed that the globular masses are evaginations from a portion of the swim bladder tissue. In teleosts, the swim bladder is typically divided into two major structures: the tunica externa and the tunica interna. In *L. triqueter*, the tunica interna consists of the mucosa, which consists of an endodermal epithelium and a lamina propria made up of loose connective tissue, nerves, and blood vessels. The tunica externa consists of two layers. The outermost, the dense submucosa, is the thickest layer and is composed of densely packed collagen fibres that are mainly perpendicular to the body axis (Fig. 5). It surrounds a second very thin layer within the submucosa, here termed the loose submucosa. However, we cannot exclude the possibility that this second part originates from the lamina

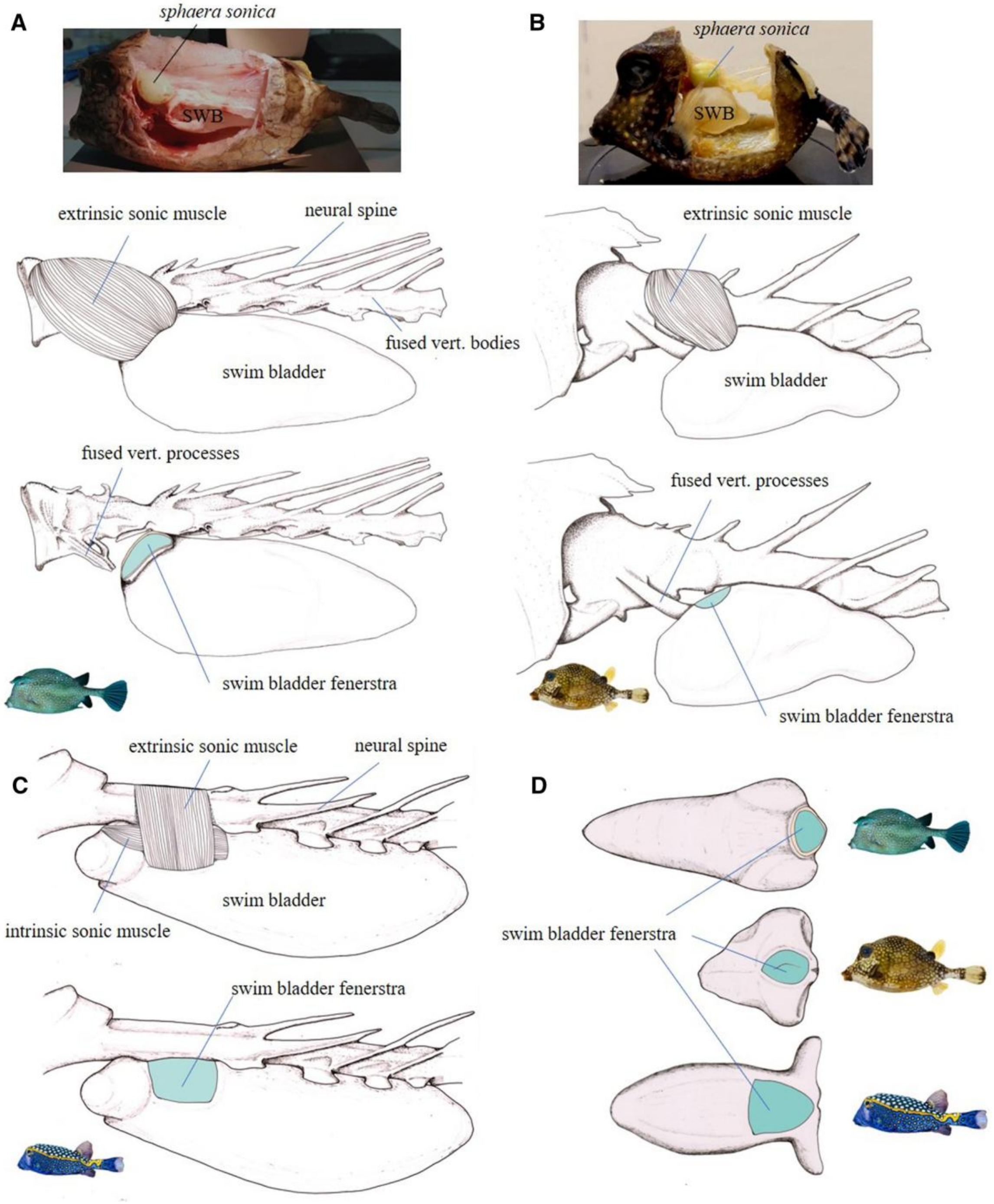

Figure 4. Sound-producing mechanisms in different Ostraciidae: *Lactorphrys triqueter* (A), *Acanthostracion polygonius* (B), and *Ostracion meleagris* (C). D, dorsal view of the swim bladder in different Ostraciidae, showing the swim bladder fenestra.

propria. At the level of the swim bladder fenestra that is deprived of the thick submucosa, the thin loose submucosa layer evaginates and increases in volume to form the two globular masses. Each mass is made of a connective tissue with a predominance of ground substance (few cells and few fibres) and includes collagen and elastic fibres, connective cells, and blood vessels. The fibres do not show a particular orientation and specific organization. The globular masses are externally delimited by a fibrous envelope. The tissues of the oesophagus are similar to those of the swim bladder, except that the former also includes the muscularis mucosae.

The sonic muscle of the sphaera sonica shows multiple layers of muscle fibres, whose organization varies but it can be categorized broadly into three main layers (Fig. 5). The outermost layer consists of fibres that are perpendicular to the axis of symmetry, encircling two layers of fibres that are parallel to the axis of symmetry. The outer layer of these parallel fibres is denser than the inner layer, which lies adjacent to the globular mass.

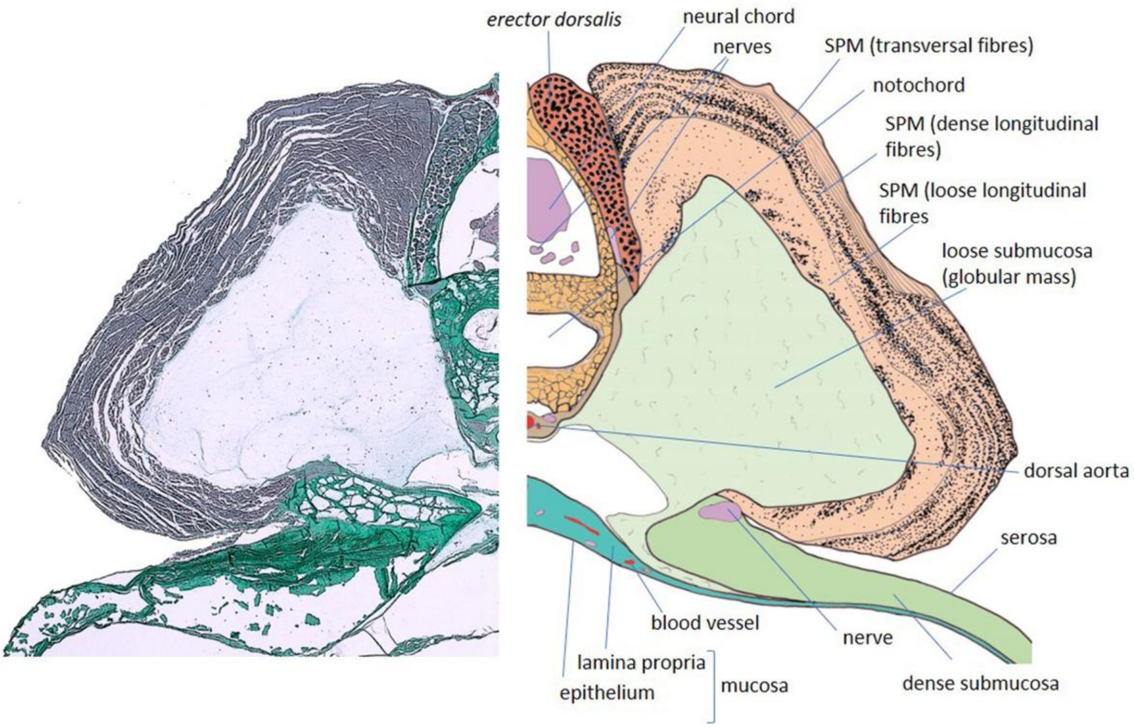

Figure 5. Cross-section (left) and corresponding schematic view (right) depicting the sphaera sonica, Masson's Trichrome staining. Abbreviation: SPM, sound-producing muscle.

Table 1. Morphometric data from different muscles in the longitudinal section in *Lactophrys triqueter*, *Acanthostracion polygonius Lactoria cornuta*.

Parameter	*Lactophrys triqueter*		*Acanthostracion polygonius*	*Lactoria cornuta*		
	Erector dorsalis	**Sonic muscle**	**Sonic muscle**	**Intrinsic sonic muscle**	**Extrinsic sonic muscle**	**Epaxial**
Myofibril diameter (µm)	179±6 (n=340)	52 ± 20 (n = 340)	65 ± 8 (n = 340)	74 ± 17 (n = 18)	340 ± 103 (n = 18)	376 ± 79 (n = 18)
Sarcoplasm ratio (% surface cell)		37 ± 1 (n = 340)	21 ± 0.5 (n = 340)	14.5 ± 9 (n = 20)	12.6 ± 9 (n = 18)	2.6 ± 1 (n = 8)
Sarcomere length (µm)		1.71±0.33 (n=340)	1.9±0.07 (n=340)	1.7±0.13 (n=104)	1.83±0.15 (n=96)	1.7±0.08 (n=103)
Sarcomere width (µm)		0.58±0.2 (n=340)	0.52±0.2 (n=340)	0.35±0.08 (n=104)	0.27±0.07 (n=102)	0.66 ± 0.02 (n = 98)

Data for *L. cornuta* were extracted from Parmentier *et al.* (2021).

In cross-section, the area of the fibres of the erector dorsalis is 10 times larger than the fibres of the sonic muscle (Wilcoxon–Mann–Whitney: $W = 113885$, $P < .0001$, $n_{\text{sonic fibres}} = 340$, $n_{\text{dorsalis fibres}} = 340$; Table 1). Sonic muscles show the classic organization of skeletal striated muscles. Triads are present at the Z-lines of the sarcomeres. The sarcomeres of *L. triqueter* measure on average 1.71 ± 0.33 µm in length ($n_{\text{sarcomeres}} = 235$) and 0.58 ± 0.11 µm in width ($n_{\text{sarcomeres}} = 291$). Within the sonic muscles, fibres show a high proportion of sarcoplasm relative to the cell surface area (37% ± 0.92%) ($n_{\text{fibres}} = 340$). However, the organization of the myofibrils appears disorganized within the muscle cells, as evidenced by the observation of various orientations. Mitochondria are predominantly found at the periphery of the muscle fibres and, less frequently, between the myofibrils. The sonic muscle does not show a central core or sarcoplasmic peripheral ring.

The sonic apparatus in *A. polygonius* has the same general organizational structure as in *L. triqueter*. However, the sphaerica sonica occupies a more dorsorostral position, cantilevered over a triangular-shaped swim bladder. The sarcomeres of the sonic fibres in *A. polygonius* are statistically longer and narrower than those in *L. triqueter* (width, Wilcoxon–Mann–Whitney: $W = 34421$, $P < .001$, $n_{\text{sarcomeres}} = 291$; length, Wilcoxon–Mann–Whitney: $W = 38729$, $P < .001$, $n_{\text{sarcomeres}} = 235$). There is statistically less sarcoplasm in the myocytes of *A. polygonius* (73 ± 29 µm^2) in comparison to *L. triqueter* (128 ± 78 µm^2) (Wilcoxon–Mann–Whitney: $W = 30607$, $P < .001$, $n_{\text{cells}} = 340$). Given that the cell area is similar between the two species (Wilcoxon–Mann–Whtiney: $W = 58705$, $P = .67$, $n_{\text{cells}} = 340$), the sarcoplasm is proportionally less significant in *A. polygonius* (Wilcoxon–Mann–Whtiney: $W = 8611.5$, $P < .0001$, $n_{\text{cells}} = 340$).

Aracanidae vs. Ostraciidae

In aracanid *P. tyleri*, there is no swim bladder fenestra and no apparent sound-producing muscle. Given that *P. tyleri* does not display any sound-producing apparatus, we have decided to investigate further the description of the rostrodorsal body muscles to make comparisons between taxa and determine whether it might be possible to identify muscles that could be homologous to the sonic muscles of Ostraciidae (Fig. 6). In *P. tyleri*, the epaxial muscle originates on the posterior part of the skull and extends along the body to insert on the caudal fin. In the abdominal cavity, several muscles are present beneath the epaxial muscle. The obliquus superior is the most lateral, originating from the first vertebrae, and also inserts on the caudal fin. The epaxial and obliquus superior muscles vary inversely in proportions along the body, with the epaxial muscle having a larger cross-sectional area anteriorly and the obliquus superior being more prominent posteriorly. As a result, the obliquus superior has a larger insertion surface on the caudal fin. Dorsally, between the two epaxial muscles, the anterior supracarinales muscles originate on the supraoccipital and insert on the first neural spines. Medially to the obliquus superior, fibres from the anterior dorsal fin muscle (erector dorsalis) originate on different vertebral bodies, starting from vertebra 1, and insert on skeletal pieces of the dorsal fin. Interestingly, the rostral fibres of the erector dorsalis are covered by two small muscle bundles (spinalis 1 and 2) that originate on the basioccipital and on vertebra 1 and insert at the base of the neural spines of vertebrae 2 and 3, respectively. The anterior supracarinales and the two spinalis are not found in Ostraciidae.

In Lactophrysinae, the epaxial muscle originates at the back of the neurocranium and extends to the ural complex of the caudal fin. The obliquus superior muscle is positioned further back than in *P. tyleri*. It originates on the pterygiophores of the anal fin, and its insertion on the caudal fin is larger than that of the epaxial muscle. Muscle fibres from the erector dorsalis insert on vertebrae 1 and 2 and are, consequently, beneath the sphaera sonica.

In Ostraciinae, the epaxial muscle also originates at the back of the neurocranium and extends to the ural complex of the caudal fin. The anterior part of the obliquus superior muscle is pushed towards the back of the fish body and forms a thin band that originates from the anterior end of the most lateral anal fin pterygiophore and connective tissue in contact with the swim bladder. The most anterior fibres of the erector dorsalis insert laterally on the vertebral column at the level of the extrinsic sonic muscle.

A comparison of species from the three taxa reveals modifications in the epaxial and obliquus superior muscles (Fig. 7). Following a line positioning Aracanidae, then Lactophrysinae, and finally Ostraciinae, it can be observed that the epaxial muscles are proportionally thicker in Ostraciinae, with a concurrently larger insertion surface on the caudal complex. Conversely, the obliquus superior muscle shows a proportional reduction in size, a retraction of its origin towards the caudal part of the body, and a decrease in insertion surface on the caudal fin. In comparison to Aracanidae, the fibres of the erector dorsalis in Ostraciidae insert more caudally on the vertebral column. The two small muscles that were found only in *P. tyleri* are ideal candidates for hypothesizing the origin of sonic muscles.

Discussion

This study provides several original insights. In particular, the descriptions of sounds alongside the anatomy of the sound-producing apparatus highlight a new type of mechanism, not only in Lactophrysinae but also for all teleosts. Additionally, anatomical comparisons across different species offer the opportunity to hypothesize about the evolutionary history of acoustic communication in Ostraciidae.

Mechanism

The sphaera sonica, corresponding to the 'hollow ball of muscles' briefly described by Tyler (1963), in fact consists of a globular mass evaginating from the swim bladder wall surrounded by muscles, except on its ventral surface, which rests on the swim bladder fenestra. The globular masses are dominated by the ground substance, whose constituents include proteoglycans that act as polyanions, attracting water and providing resistance to compression. The ground substance is also composed of mucopolysaccharides (Meyer and Rapport 1951) made up of glycosaminoglycans, which help to maintain the homeostasis of cells and fibrous elements

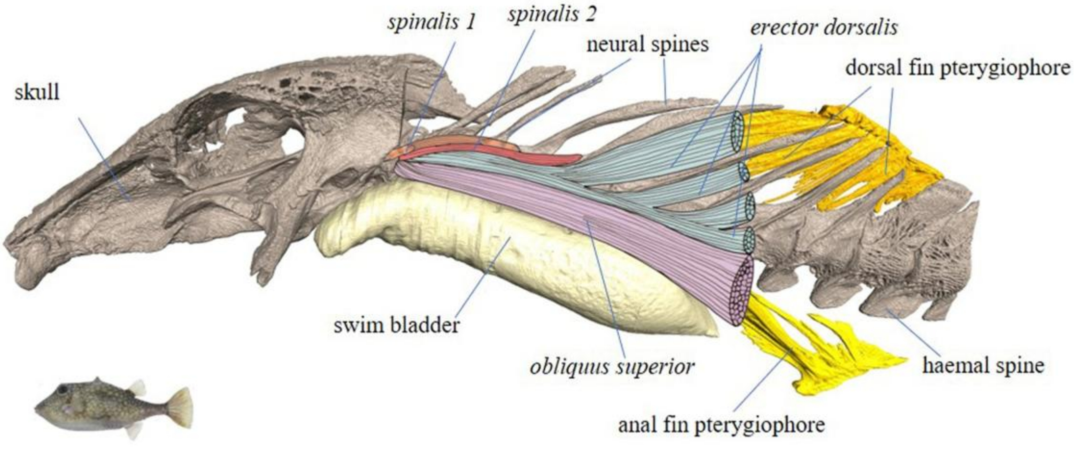

Figure 6. Left lateral view of the anterior part of the body in *Polyplacapros tyleri*, showing the skull, rostral vertebrae, and associated dorsal muscle. The epaxial muscle and anterior supracarinales are not shown. Erector dorsalis and obliquus superior are cut at the posterior end.

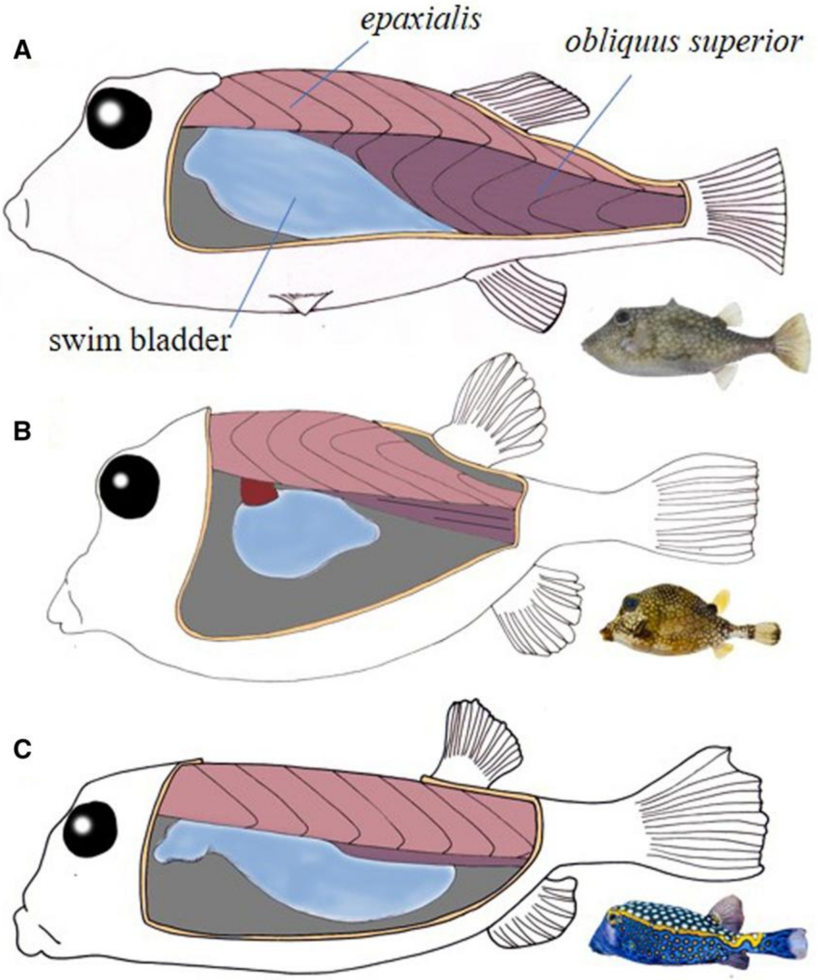

Figure 7. Left lateral view of the anterior part of the dorsal body muscles in *Polyplacapros tyleri* (A), *Lactophrys triqueter* (B), *and Ostracion meleagris* (C), showing the differences in dorsal body muscle proportions.

(Rodén 1980). Additionally, collagen contributes rigidity (Linsenmayer 1991, Gelse et al. 2003), while elastic fibres enhance the tissue elasticity (Ross 1973, Mecham and Heuser 1991). Together, these components confirm the ability of the globular masses to resist compression while remaining deformable (Fig. 8; Supporting Information, Video 2). The ground substance dominates the globular masses, confirming that they are designed to resist compression and are deformable.

Having circular fibres in a muscle is rare. The orientation of the fibres in the sonic muscles of the sphaera sonica is reminiscent of those in the orbicularis oculi, which is involved in eyelid closure. In Lactophrysinae, the contraction of the sonic muscles must result in a reduction of their surface area; consequently, they push the globular masses into the swim bladder. The relaxation of the sonic muscles, in conjunction with the elasticity of the tissues and the internal pressure of the swim bladder, then causes the balls to return to their initial position (Fig. 8; Supporting Information, Video 2).

The pulses from the Lactophrysinae sounds seem to consist of two parts consistently, with the peaks being narrower in the first part (Fig. 2). We hypothesize that these two parts correspond to the back-and-forth movements of the globular masses within the bladder. However, additional manipulations, including an approach using electromyography, are necessary to understand the mechanism of sound production in detail. According to the forced response hypothesis (Fine 2012, Fine and Parmentier 2022), the contraction speed of the muscle dictates the dominant frequency by vibrating the swim bladder. This forced response is possible only if the fish possesses fast sonic muscles (Fine and Parmentier 2015, Parmentier and Fine 2016). Consequently, in different species within Serrasalmidae (Millot *et al.* 2011, Banse *et al.* 2021), Sciaenidae (Sprague *et al.* 2000, Parmentier *et al.* 2014), or Batrachoididae (Fine *et al.* 2001, Rice and Bass 2009), the fundamental frequency can be inferred from the period at which pulses are produced. Interestingly, the muscles of *A. polygonius* and *L. triqueter* exhibit characteristics typical of fast muscles, i.e. smaller diameters of the fibres and fewer myofibrils in comparison to the reference muscle (erector dorsalis), alongside a proportionally more developed sarcoplasmic reticulum and a large number of mitochondria, which gives the muscle the capacity to sustain contraction efforts (Lindstedt *et al.* 1998, Parmentier *et al.* 2021).

In *L. triqueter*, there is a correspondence between the period and the recorded frequency. In *L. bicaudalis*, the pulses consist of

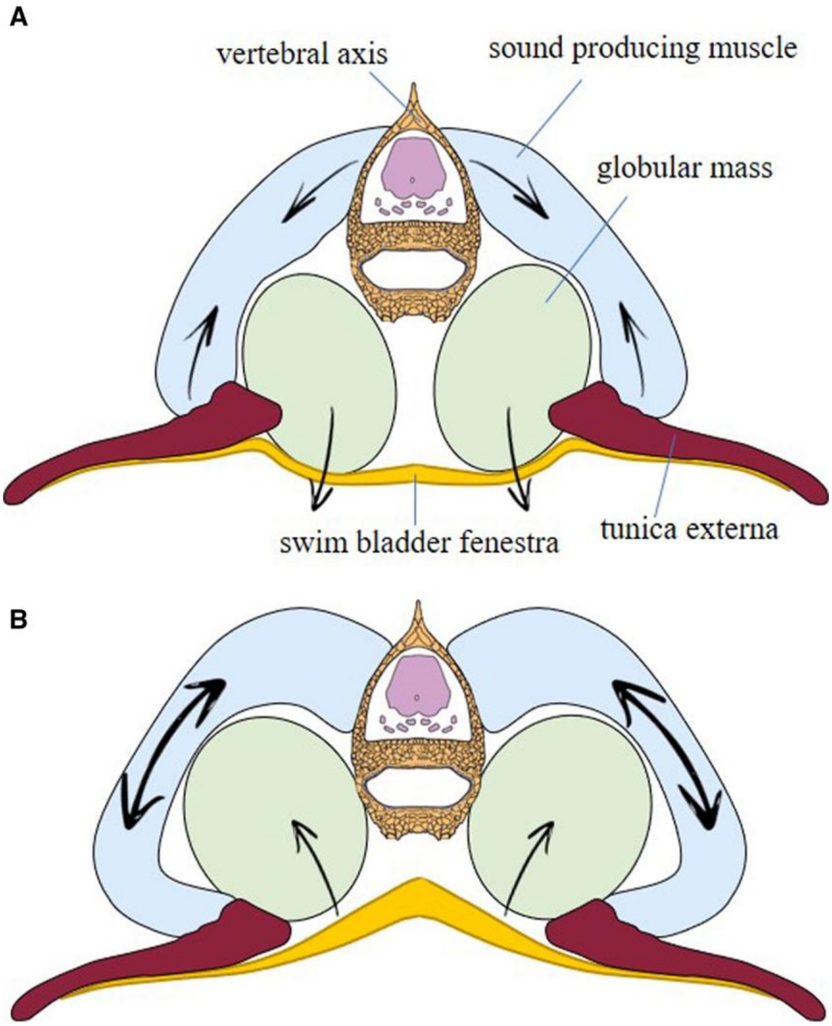

Figure 8. Schematic transversal view, showing the movement of the globular masses inside the swim bladder during (A) muscle contraction (single arrows) and (B) relaxation (double arrows).

four peaks organized in pairs (Fig. 2). The recorded frequency (30–to 45 Hz) matches the period between the two groups of peaks. *Lactophrys* species are thus in concordance with the forced response hypothesis. However, understanding the mechanism in the two species of *Acanthostracion*, where the dominant frequency does not correspond to the period, is more complicated. In *A. polygonius*, the frequency could correspond to the period at which the peaks are emitted. In this case, the dominant frequency would not correspond to the pulse period but to the peak period within the pulse. This suggests that the movement of the globular mass does not occur in a single motion but could be the result of contraction bursts before a single relaxation. Electromyographic studies will be necessary to understand the mechanism further.

All Ostraciinae recorded in this study and previously have been capable of producing hums and clicks, but there seem to be differences depending on the species. Initially, it was proposed that each sonic muscle was responsible for a type of sound, a hum or a click (Parmentier *et al.* 2019). However, new data suggest that the pattern is somewhat more complex. In *L. cornuta* and *O. cubicus*, hums are simple peaks produced in series, whereas in *O. meleagris* and *O. solerensis* the hums are organized by the production of pulse trains, each made of two to five peaks. It seems that hums result either from individual, regularly spaced contractions of the sonic muscles (*O. cubicus* and *L. cornuta*) or they are the product of bursts of contraction trains (*O. meleagris* and *O. solerensis*). The mechanics would therefore be identical among Ostraciinae species, but the neurophysiology underlying their activation dictates the observed differences in sounds. Lastly, the same types of observations were made in a large-scale study based on sounds produced by many different species of Holocentridae. Their comparisons supported that sounds were made using the same type of mechanism that was, however, activated differently (Banse *et al.* 2024).

In comparing extrinsic and intrinsic sonic muscles, it has been suggested that extrinsic sonic muscles are capable of producing fast, strong, and short contractions corresponding to clicks (the shortest sounds with the greatest amplitude), whereas intrinsic sonic muscles should be able to sustain longer contractions and are thought to generate hums. In *O. meleagris* and *O. solorensis*, clicks are constructed from a single peak, similar to the pulses of hums, indicating that they might depend on a single type of muscle.

The construction of clicks appears more complex in *L. cornuta* and *O. cubicus*, where clicks are composed of many pulses. In *O. cubicus*, data from the present study additionally suggest a relationship between the pulses of hums and those of clicks. Clicks seem to be produced by an acceleration and an increase in the power of the pulses of hums. Based on the configuration of clicks in *Lactoria*, clicks could be produced in the same manner. These new findings indicate that, in these two species at least, the production of both types of sounds results not from the contraction of one muscle or the other but from coordinated action between the two.

Although there are parallels in the construction of sounds, there is no concordance with the phylogeny, because *O. solorensis* is phylogenetically closer to *O. cubicus* than to other species, for example. This again shows that once a mechanism is established, modifications in the organization of motor patterns in muscles can lead to different types of sounds.

Evolution

The relationship between Aracanidae and Ostraciidae has been discussed for several decades based on skeletal and muscular morphology (Winterbottom 1974, Tyler 1980, Winterbottom and Tyler 1983) and, more recently, using molecular data (Alfaro et al. 2007, Santini *et al.* 2013a, 2013b). Comparative osteological and molecular studies consistently position Aracanidae as the sister group to Ostraciidae. As sister taxa, these two families share a common ancestor, from which they diverged independently. Although the ancestral state cannot be observed directly, the morphological condition exhibited by Aracanidae, characterized by less specialized features, such as unfused vertebrae and loosely connected bony plates, appears closer to this hypothesized ancestral condition in comparison to the highly derived, specialized morphologies observed in Ostraciidae (Tyler 1963). Consequently, the anatomical features of Aracanidae provide valuable insights into the likely primitive conditions from which the specialized traits of Ostraciidae have evolved. This perspective is reinforced by observations of more specialized structures in Ostraciidae, such as the development of a more rigid, box-like carapace that provides substantial advantages in swimming by minimizing vortices and drag and by improving manoeuvrability (Bartol *et al.* 2005, Van Wassenbergh *et al.* 2015). This rigid structure is formed by the fusion of bony plates, a specialized adaptation that differs from the more generalized form seen in Aracanidae (Klassen 1995). Furthermore, various osteological (Tyler 1980, Klassen 1995) and molecular studies (Santini *et al.* 2013a) have clearly supported the division of Ostraciidae into two groups, the first comprising two genera found in the Atlantic and the second including three genera inhabiting the Indo-Pacific. Given that Aracanidae are not found in the Atlantic, it is tempting to posit the origin of Ostraciidae in the Pacific, before an invasion of the Atlantic. The lactophrysins, being anatomically closer to their ancestral form, are considered representatives of an ancestral form (Tyler 1980) that would have disappeared from the Indo-Pacific.

Textbooks on evolution abound with examples of structures that have evolved between lineages. Having a generally less rigid body, the body musculature also appears more generalized in Aracanidae, with a greater number of muscle bundles. The more rigid body in Ostraciidae is marked by a stiffening of the vertebral column, a reduction in the diversity of swimming muscles, and the development of dorsal epaxial musculature at the expense of the obliquus superior. The observation of *P. tyleri* is particularly interesting because of the presence of two small muscle bundles (spinalis 1 and 2) found in the anterior region of the swim bladder. Their position and relationship to the vertebral column and the erector dorsalis make them ideal candidates as homologous of the sonic muscles in Ostraciidae. We suggest that these muscles have lost their function in swimming to instead participate in sound production, illustrating a new type of exaptation (Parmentier *et al.* 2017). This, however, implies significant modifications, such as the transition of insertion sites on the swim bladder. Comparisons with other Aracanidae and ontogenetic studies should provide evidence supporting this hypothesis. Furthermore, it is complicated to understand the reasons behind the development of a swim bladder fenestra, a structure that seems to have evolved by convergent evolution in many unrelated groups. This structure is found in many sound-producing fishes (Parmentier *et al.* 2003, 2016) and should help in the deformation of the swim bladder during sound production. However, it does not seem to be a prerequisite for generating sound from the swim bladder (Fine *et al.* 2001, Mok *et al.* 2011, Parmentier *et al.* 2011a, Boyle *et al.* 2014, Millot and Parmentier 2014). In the development of a sound-production mechanism in boxfishes, it would therefore not be impossible to have had a stage without a swim bladder fenestra.

According to Tyler (1980), the Lactophrysinae are morphologically closer to the ancestral form of the Ostraciidae. Building on this hypothesis, we propose that the evolution of the sound-producing system within Ostraciidae might have involved a progressive transformation of the sphaera sonica. Specifically, this transformation could have entailed the reduction of the globular mass and the differentiation of surrounding muscle fibres into two distinct layers: an external layer giving rise to the extrinsic sonic muscles and an internal layer giving rise to the intrinsic sonic muscles observed in Indo-Pacific Ostraciinae. This muscular specialization might have enabled more refined control over swim bladder deformation and contributed to the emergence of a more diverse acoustic repertoire (Fig. 9).

These observations support a broader evolutionary scenario, in which sonic muscles in Ostraciidae evolved through exaptation (Parmentier *et al.* 2017) from generalized dorsal musculature already present in the common ancestor with Aracanidae. The formation of the globular connective mass (sphaera sonica) at the swim bladder fenestra, and its association with fast-contracting muscles, might have constituted an early innovation that later diverged structurally and functionally within the family. In this context, the evolution of distinct extrinsic and intrinsic muscles in Ostraciinae represents a derived condition, whereas the single-layered muscle system observed in Lactophrysinae is likely to retain more ancestral features (Fig. 9).

Although this study focuses primarily on anatomical mechanisms, the differences observed between the two subfamilies might also have important ecological and behavioural implications. In Indo-Pacific Ostraciinae, the presence of two muscle layers [supporting both rapid contractions (for clicks) and sustained activity (for hums)] suggests a greater capacity for acoustic variation. Such complexity in signal production might facilitate social behaviours,

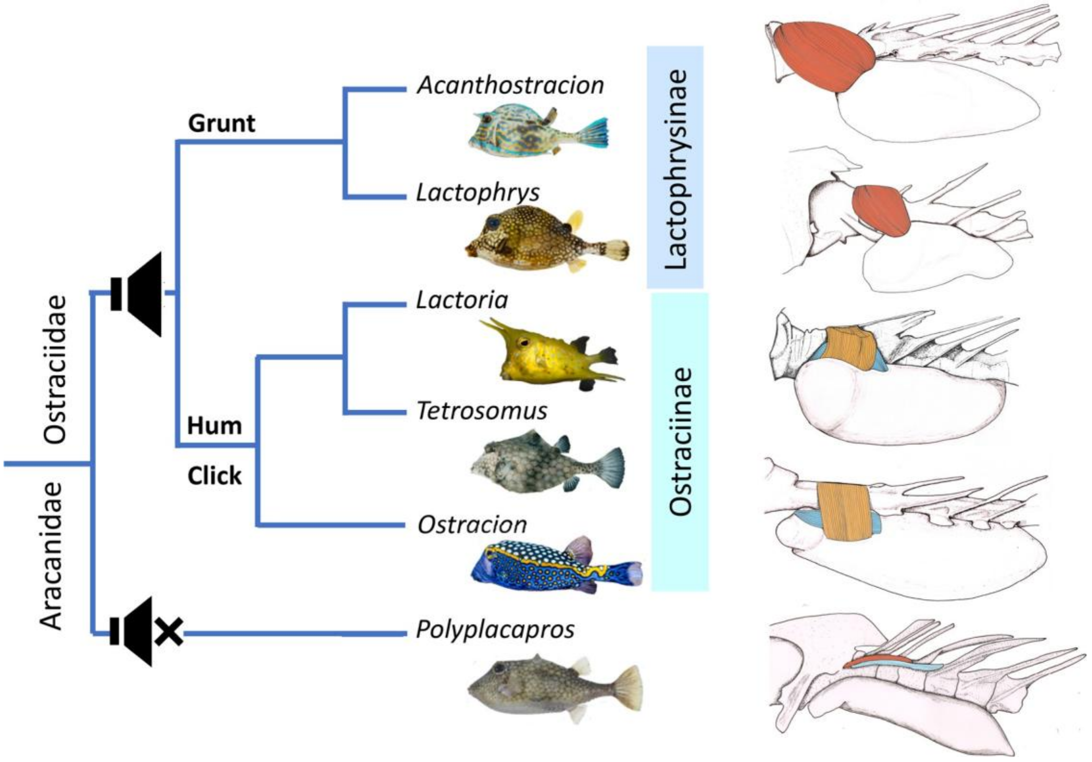

Figure 9. Phylogenetic relationships among boxfishes (modified from Santini *et al.* 2013a, 2013b), illustrating the distribution of mute and sound-producing taxa. Differences in the types of sounds produced by members of the subfamilies Lactophrysinae and Ostraciinae are indicated. Variations in sound-producing mechanisms are mapped onto the tree, supporting the evolutionary interpretation of acoustic function and anatomical specialization across taxa.

such as territorial defence and reproductive interactions, as observed in *O. meleagris* and *L. cornuta* (Moyer 1979, 1984, Lobel 1996). In contrast, Atlantic Lactophrysinae, possessing a simpler mechanism, appear to produce fewer and less diverse sounds (Fig. 9), which could reflect different ecological constraints or selective pressures in Atlantic environments. Further behavioural and neurophysiological studies will be essential to clarify the ecological significance and selective drivers of these anatomical differences.

Overall, the evolutionary trajectory from Aracanidae to Ostraciidae exemplifies how both structural simplification and functional innovation can act synergistically in shaping new communication systems. The transition from generalized dorsal musculature to specialized sonic muscles, coupled with increasing morphological rigidity and complexity in the swim bladder apparatus, illustrates the diverse pathways through which novel functional traits emerge and diversify within a lineage.

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SUPPLEMENTARY DATA

Supplementary data is available at *Biological Journal of the Linnean Society* online.

CONFLICT OF INTEREST

None declared.

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DATA AVAILABILITY STATEMENT

The data underlying this article will be shared on reasonable request to the corresponding author.

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