# RESEARCH ARTICLE

# Sound production mechanism in *Gobius paganellus* (Gobiidae)

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## SUMMARY

**Gobiidae, the largest fish family (>1500 species), has species from at least 10 genera that produce sounds for communication. Studies focused on goby sound production mechanisms have suggested that sounds are produced by the forcible ejection of water through small apertures in the opercles (hydrodynamic mechanism). The present study was a multidisciplinary investigation (morphology, muscle histology, high-speed video, sound analysis and electromyography) of the sound emission mechanism in *Gobius paganellus*, which produces both pulsed and tonal calls. Two populations were used, from Brittany and Venice. In the French population, sounds were accompanied by a suite of coordinated movements of the buccal, branchial and opercular regions. This was not the case in the Venetian population, and thus the direct role of head movements in sound production was rejected. The hydrodynamic mechanism hypothesis was also rejected in *G. paganellus* on the basis of sound oscillogram shape and because sounds are still produced after the opercles and hyohyoid muscles are cut. The use of both electromyography and electron microscopy showed that the levator pectoralis muscle, which originates on the skull and inserts on the dorsal tip of the cleithrum, is involved in sound production. We propose that the contraction of this muscle and associated vibration of the large radials is used to make sounds. In addition, we propose that different sound types (pulsed sounds and tonal calls) could occur because of differences in fish size.**

## INTRODUCTION

Fishes have evolved the largest diversity of sound-generating mechanisms for acoustical communication among vertebrates (Myrberg and Lugli, 2006). However, there exists no commonly accepted classification, mainly because various taxa show many evolutionary convergences. Two general types of sonic mechanisms may occur in fishes. The most consistent group includes the swimbladder mechanisms. The great majority of fish swimbladder muscles are often considered the fastest muscles in vertebrates, and their cycle time (contraction and relaxation) determines sound fundamental frequency (Kastberger, 1981; Fine et al., 2001; Connaughton, 2004; Rice and Bass, 2009; Millot et al., 2011; Parmentier et al., 2011a). However, slow sonic swimbladder muscles have recently been discovered in a carapid fish (Parmentier et al., 2006). They produce sounds using a different principle, namely slowly stretching the swimbladder and allowing it to recoil. A single twitch in *Carapus boraborensis* takes almost 500 ms, and the muscle tetanizes around 10 Hz (Parmentier et al., 2006), whereas toadfish *Opsanus tau* sonic muscle requires about 10 ms for a twitch (Fine et al., 2001). In slow muscle mechanisms, muscle contraction rate determines pulse repetition rate rather than the pulse frequency spectrum (Parmentier et al., 2006). Despite these differences, sonic systems are quite easy to identify because they have distinct sonic muscles associated with the swimbladder. It is not always the case: sounds in tilapia (*Oreochromis niloticus*), pennant bannerfish (*Heniochus chrysostomus*) and pyramid butterflyfish (*Hemitaurichthys polylepis*) also employ the physoclistous

swimbladder, but they do not show any apparent distinct specialized muscle (Longrie et al., 2009; Parmentier et al., 2011b; Boyle et al., 2013).

In another group of mechanisms, sounds result from bone rubbing (Tavolga, 1971), vibration (Barber and Mowbray, 1956; Ladich, 1989; Fine et al., 1997; Parmentier et al., 2010a), teeth collision (Parmentier et al., 2007) or tendon plucking (Kratochvil, 1985). However, many mechanisms are still unexplained, even in well-studied vocalizing taxa such as cobitids, cyprinids and gobiids.

Among the sound-producing fish, Gobiidae are one of the four most studied families with sound production documented in at least 21 species belonging to 10 different genera (Table 1). Within this large and diverse family, drumming, stridulatory and tonal sounds are emitted by the male as a part of the breeding and aggressive behavioural repertoire (Torricelli et al., 1990; Lugli et al., 1997; Lugli and Torricelli, 1999; Malavasi et al., 2003; Myrberg and Lugli, 2006; Amorim and Neves, 2007). With few exceptions, goby calls can be grouped into three major sound types: (1) pulsed sounds that consist of pulse trains repeated at a slow rate and that can be amplitude modulated; (2) tonal sounds characterized by faster pulse repetition rates, where pulses fuse resulting in a sinusoidal-like waveform; and (3) complex sounds that are made of a combination of the two (Lugli et al., 1997). Few studies have focused on the sound production mechanism and it is not even clear at present whether a single or multiple mechanisms are employed by this teleost family. This important and large family of soniferous fishes may offer a great opportunity for comparative and phylogenetic studies

Table 1. Gobiidae vocal species

| Species | Reference |
|---|---|
| *Periophthalmodon septemradiatus* | Polgar et al., 2011 |
| *Padogobius nigricans* | Lugli et al., 1996; Lugli et al., 1997; Bass and McKibben, 2003; Malavasi et al., 2008 |
| *Padogobius martensii* | Torricelli and Romani, 1986; Torricelli et al., 1990; Lugli et al., 1995; Lugli et al., 1997; Bass and McKibben, 2003; Lugli et al., 2003; Malavasi et al., 2008 |
| *Padogobius bonelli* | Malavasi et al., 2008 |
| *Knipowitschia panizzae* | Lugli and Torricelli, 1999; Malavasi et al., 2008 |
| *Knipowitschia punctatissima* | Lugli et al., 1995; Lugli et al., 1997; Bass and McKibben, 2003; Malavasi et al., 2008 |
| *Gobius paganellus* | Malavasi et al., 2008 |
| *Gobius cobitis* | Malavasi et al., 2008 |
| *Gobius niger* | Kinzer, 1961; Malavasi et al., 2008 |
| *Gobius cruentatus* | Sebastianutto et al., 2008 |
| *Zosterisessor ophiocephalus* | Malavasi et al., 2003; Malavasi et al., 2008 |
| *Pomatoschistus minutus* | Lindström and Lugli, 2000; Malavasi et al., 2008 |
| *Pomatoschistus marmoratus* | Ladich and Kratochvil, 1989; Lugli and Torricelli, 1999; Malavasi et al., 2008 |
| *Pomatoschistus canestrinii* | Lugli and Torricelli, 1999; Malavasi et al., 2008; Malavasi et al., 2009 |
| *Pomatoschistus pictus* | Amorim and Neves, 2007; Amorim and Neves, 2008; Amorim et al., 2013 |
| *Neogobius melanostomus* | Protasov et al., 1965; Rollo et al., 2007 |
| *Bathygobius soporator* | Tavolga, 1958 |
| *Bathygobius curacao* | Stadler, 2002 |
| *Bathygobius fuscus* | Zheng and Takemura, 1989 |
| *Odontobutis obscura* | Takemura, 1984 |
| *Gobiosoma bosc* | Mok, 1981 |

on the acoustic communication within a Teleost group. Therefore, an investigation of the sound emission mechanism(s) in gobies is urgently needed.

In different goby species, the sound characteristics suggest the involvement of the swim bladder and muscles acting on it (Lugli et al., 1995; Lugli and Torricelli, 1999; Lindström and Lugli, 2000), but these muscles are not known from anatomical investigations, i.e they are not muscles directly associated with the swimbladder. Moreover, some gobiid species do not have a swimbladder. In *Padogobius nigricans*, Lugli and colleagues (Lugli et al., 1996) proposed that sound production could be due to the contraction of specialized muscles inserting on the pectoral girdles, but the muscles were not identified and the mechanism has not been demonstrated. In *Odontobutis obscura*, sounds could be produced by the rubbing teeth of the upper and lower phraryngeal jaws (Takemura, 1984). The most complete studies on the subject concerned the mechanism in *Bathygobius curacao* in which hydrodynamic sounds would result from the forcible ejection of water through gill opening (Tavolga, 1958; Stadler, 2002). This hypothesis was based on three points: (1) similarities between the fish calls and sounds from ejecting water through a pipette; (2) behavioural motor patterns occurring during sound production forced water through gill openings; and (3) the lack of specialized features associated with sound production, eliminating stridulatory and swim bladder mechanisms. Despite these previous hypotheses, the sound emission mechanism in this Teleost group remains unknown.

The present paper aimed to fill this gap, by investigating, through a combination of morphological and experimental analyses, the sound emission mechanism in the rock goby *Gobius paganellus*. We tested the different mechanisms previously proposed using a multidisciplinary approach: morphology, muscle histology, high-speed video films, sound analysis and electromyography (EMG).

*Gobius paganellus* is a relatively large goby found along the North-Eastern Atlantic, from Scotland to Senegal, the Mediterranean Sea and the Black Sea. It is a common inshore and intertidal species, occurring under stones and in pools on sheltered rocky shores with much weed cover (Miller, 1986). It reproduces in winter and early spring, with a peak in February and March, when water temperatures are lowest (Azevedo and Simas, 2000). Sounds were recently described in specimens inhabiting the Venice lagoon (Malavasi et al., 2008). According to Malavasi and colleagues (Malavasi et al., 2008), *G. paganellus* calls are tonal sounds lasting *ca.* 350 ms, composed of 30 pulses with a pulse rate of 90 Hz, and the dominant frequency is around 300 Hz. Our study was based on two populations from Venice (Italy) and Brittany (France).

## MATERIALS AND METHODS

*Gobius paganellus* Linnaeus 1758 specimens were caught during February and March 2010 and 2011 in Brittany, France. They were caught by hand during the low tide in front of Saint-Malo and in Roscoff, and transported to the laboratory (Liège, Belgium). These specimens (total length, TL 56−105 mm, N=18) were kept in two tanks (150×40×45 cm) with gravel on the bottom and fragments of terracotta bowls on a 12 h:12 h light:dark cycle. They were offered mussels once a day *ad libitum*. The environmental temperature was 20°C and the salinity was between 32 and 34‰.

Adult individuals (N=10) were also captured from the Venice Lagoon (TL 85–150 mm) in October 2010 and then transported back to the laboratory and maintained in holding (150 l) tanks provided with filtered salt water. Temperature varied between 20 and 22°C, and the photoperiod followed natural conditions (11 h:13 h light:dark). Each tank was covered with *ca.* 50 mm of sand on the bottom and provided with an artificial shelter made of an opaque plastic pipe cut in half (35×40 mm). A second batch (N=5) was also caught in December 2012 and sent to the Aquarium-Museum of Liège (Belgium) for EMG.

In community tanks, aggressive fish turned black with a yellow band on the anterior dorsal fin when defending a small territory. These fish were selected for further experiments dealing with high-speed camera and sound recordings. The procedure we used to obtain sounds was performed according to previous studies (Lugli and Torricelli, 1999; Amorim and Neves, 2007; Malavasi et al., 2009). Fish were isolated in small tanks (59×29×33 cm) divided into two compartments of equal size by means of double opaque removable partitions. Each tank was provided with a layer of sand on the bottom, one water filter, and terracotta bowls to be used as nest

sites. In each compartment, a male was left to acclimatize for a minimum of 3 days before it was used in trials. Sounds were recorded at the point at which a second fish was added to the tank. This study dealt with the sound-producing mechanism and not with ethology; we did not try to determine the sex of the added fish or to qualify the sonic message, the aim was just to obtain sounds.

### Video during sound production
The fish were first filmed at 24 frames s$^{-1}$ with a video camera (Legrifia FS 19, Canon) coupled with a hydrophone (High Tech. Inc., Long Beach, MS, USA) having a flat response of 20 Hz to 20 kHz and a nominal calibration of $-164$ dBV μPa$^{-1}$. Some fish were also recorded with an Orca hydrophone (sensitivity $-186$ dB re. 1 V μPa$^{-1}$) connected to a Tascam HD-P2 stereo audio recorder (Wiesbaden, Germany).

### High-speed video during sound production
The fish were filmed at 200 frames s$^{-1}$ with a Redlake MotionPro high-speed camera (resolution 1280×1024 pixels; San Diego, CA, USA). This camera was connected to a computer (video chart: Asus v9280S, San Diego, CA, USA), making it possible to visualize the fish's movements in real time. This imagery system was synchronized with a hydrophone BK 8106 (sensitivity $-173$ dB re. 1 V μPa$^{-1}$, flat frequency response between 7 Hz and 80 kHz; Naerum, Denmark) coupled to a Nexus conditioning amplifier (type 2690; Naerum through a data acquisition box (Midas, DA Module, BNC Breakout Box, Cambridge, MA, USA). The Midas program (version 2.2.0.7; Redlake) was used for data acquisition and to follow the fish movements in an $x$–$y$ coordinate system. A buffer memory with a capacity of 2 GB was used to record the data after visualization. Lateral and dorsal views were recorded. Lighting was provided with a halogen lamp (OSRAM, 1000 W/230 V) situated 1.5 m behind the camera.

Videos allowed observation of the general movements of the fish during sound production. However, multiple landmarks were used to follow the sound production movements of the fish more precisely. In lateral view, these landmarks were: (1) the rostral end of the upper jaw, (2) the rostral end of the lower jaw, (3) the rostro-ventral end of the urohyal, (4) the neurocranium and (5) the anterior end of the dorsal fin. In dorsal view, four landmarks were used: (1) the rostral end of the upper jaw, (2) the left and (3) right margins of the opercles, and the anterior end of the dorsal fin.

Sounds were digitized at 44.1 kHz (16 bit resolution) and analysed with Avisoft-SASLab Pro version 4.33 software (1024 point Hanning window fast Fourier transform, FFT). The following features were measured from sound recordings: sound duration (duration from the beginning of the first pulse to the end of the last pulse, in ms), number of pulses in a series, pulse duration (i.e. the time interval between the onset of one pulse and its end), pulse interval (i.e. the time interval between the end of one pulse and the beginning of the next pulse) and pulse period (measured as the average peak-to-peak interval between consecutive pulse units in a series). The fundamental frequency corresponds to the lowest frequency component in a harmonic sound and the dominant frequency is the highest amplitude frequency component in either a broad-band or harmonic sound. Temporal features were measured from oscillograms whereas frequency (Hz) was obtained from power spectra (filter bandwidth 117 Hz, FFT size 512 points, time overlap 96.87% and a flat top window). The resonant frequency of each tank was calculated with the equation of Akamatsu et al. (Akamatsu et al., 2002). The frequencies obtained were cut with a low-pass 2346 Hz filter for the experimental tank.

### Morphology
Nine *G. paganellus* (TL 105–140 mm) specimens were killed with MS-222 (500 mg l$^{-1}$) and fixed in 7% formaldehyde for dissection and *in toto* staining. Three specimens were stained with Alizarin according to a previous method (Taylor and Van Dyke, 1985) in order to visualize osseous structures. These three specimens and intact fish were dissected and examined with a Wild M10 binocular microscope (Leica Camera, Leica, Wetzlar, Germany) equipped with a camera lucida. The nomenclature used to designate parts of the musculature is based on previous studies (Winterbottom, 1974; Adriaens et al., 1993).

In the final set of experiments, mutation was performed to confirm the sonic mechanism. Fish were first isolated and their sounds were recorded with the previously described Orca hydrophone. Calling fish were then anaesthetized with MS-222. In the first set, the opercles and hyohyoideus muscles were cut to prevent the fish from retaining water in its buccal cavity. In the second set, the basihyal was cut to prevent its contact with the oral valve. Fish were recorded 2 days after the operations.

After glutaraldehyde fixation, muscle samples were dehydrated in an ethanol–propylene oxide series and embedded in epoxy resin (SPI-PON 812). The cellular ultrastructure was examined on ultrathin sections (60–80 nm) stained with uranyl acetate and lead citrate. The sections were viewed with a JEOL JEM 100SX transmission electron microscope under an 80 kV accelerating voltage.

### EMG
For EMG, bipolar electrodes were fashioned from Formvar insulated nichrome wire (37 μm outer diameter, 25 μm core diameter; Clark Electromedical Instruments, Harvard Apparatus, Holliston, MA, USA). Two wires were inserted into a 27.5 gauge hypodermic needle, ~1 mm of insulation from the tip of the wires was removed, and the tips were bent to form hooks. A fish was anaesthetized with MS-222 (100 mg l$^{-1}$) and EMG electrodes were inserted through the skin into the right and left levator pectoralis muscle. A suture was placed in the spinous dorsal fin of the fish, looped around the electrodes and secured with cyanoacrylate glue to keep the electrodes secure. The fish was allowed to recover from anaesthesia in the aquarium for ~30 min until a second intruder fish was introduced to elicit sound production.

EMG signals were amplified 10,000 times with an AM-Systems (Sequim, MA, USA) model 1700 differential amplifier with a band-pass (100–10,000 Hz) and 50 Hz notch filter. Output from the differential amplifier was digitized at 44.1 kHz with a USB sound card (Creative model SB0270, Creative Labs, Singapore) using Adobe Audition 2.0 software (Adobe, San Jose, CA, USA). Sounds were recorded as described above, and line output from the stereo audio recorder was fed into one of the channels of the USB sound card at the beginning and end of recording trials to allow synchronization based on the visual comparisons of sound files with the EMG files in Adobe Audition.

## RESULTS
### Call features
*Gobius paganellus* sounds were first described by Malavasi and colleagues (Malavasi et al., 2008). In *G. paganellus* that were collected on the French coast, pulsed calls lasted between 127 and 313 ms (mean ± s.d. 192±52.5 ms, $N$=31) and were made of 6–22 pulses (11±5 pulses, $N$=31). The amplitude of the pulses progressively increased during the first two-thirds of the call before decreasing. Pulses lasted between 19.0 and 22.4 ms and the pulse period ranged from 33 to 139 Hz (60±23 Hz, $N$=31). Sounds were
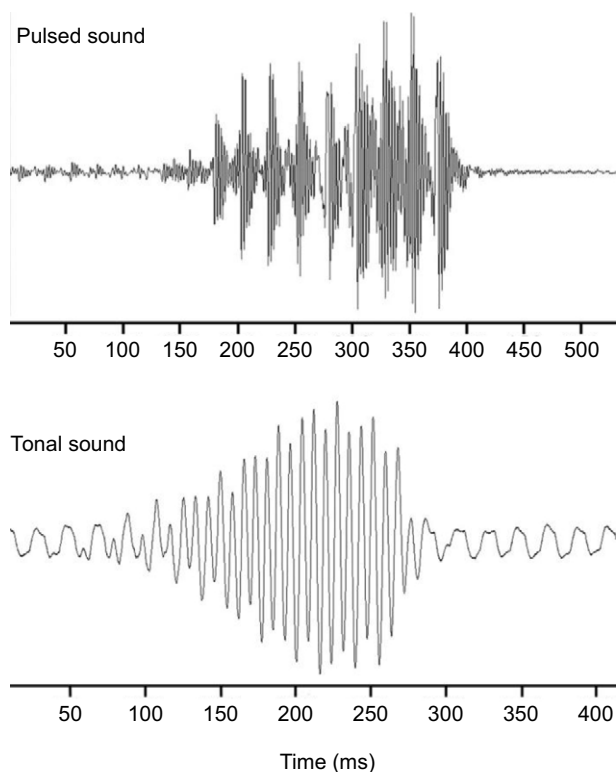
Fig. 1. Oscillograms of tonal and pulsed sounds in *Gobius paganellus*.

harmonic and the fundamental frequency (95–280 Hz) was not necessarily the main frequency, which can be the first or the second harmonic in some cases.

In Italy, most of the calls were tonal. Calls lasted between 113 and 643 ms (344±109 ms, $N$=124) and were made of 15–62 pulses (31±7 pulses, $N$=124). Pulse period ranged from 60 to 143 Hz (93±17 Hz, $N$=124) and fundamental frequency from 61 to 220 Hz.

Italian and French calls were significantly different (Mann–Whitney test, $P$<0.05) for all characteristics: pulse duration, pulse number, pulse period and fundamental frequency. Generally, French sounds were more pulsatile than Venetian sounds, but occasionally French sounds appeared tonal. Conversely, most Italian sounds were tonal, but a few pulsatile calls were also recorded. The presence of pulsed and tonal sounds in both populations shows fish are able to modulate their signals (Fig. 1).

### Kinematics
In the French population, the description of the movements observed was based on seven different high-speed videos, from six different fish. Prior to movements directly linked to sound production, *G. paganellus* showed a preparation phase. Before making sound, the fish stopped swimming and lay on the bottom of the tank, resting its body on its fused pelvic fins and on the anal fin. The preparation phase consists of head elevation (at a 20 deg angle), mouth closure and spreading of the pectoral fins.

During sound production, two kinds of rhythmic movements were observed and they were closely correlated with the oscillogram shape (Figs 2, 3). Each positive peak of the oscillogram corresponded to simultaneous (1) elevation of the head, (2) shortening of the distance between the dorsal fin and the head, (3) elevation of the closed upper and lower buccal jaws, (4) elevation of the anterior part of the branchial basket, (5) dilatation of the opercular cavity and (6) erection of the dorsal fin (Fig. 2A,C). Each negative peak of the oscillogram corresponded to simultaneous (1) lowering of the head, (2) increasing distance between the dorsal fin and the head, (3) lowering of the closed upper and lower buccal jaws (corresponding to protraction of the jaws, gliding on the ethmoid region), (4) lowering of the anterior part of the branchial basket and (5) compression of the opercular cavity and lowering of the dorsal fin (Fig. 2B,C).

In these rhythmic movements, the cycle is performed in four images, which corresponds to 20 ms at 200 frames s$^{-1}$. This result fits perfectly with the pulse period of the call (Fig. 3).

These nodding movements were not observed in five fish from Venice. In these fish, we just observed the movements associated with the preliminary phase. This observation could be linked with the important differences between calls: more pulsatile calls in the French population and more tonal calls in the Italian population.

### Head osteology
To explain the kinematics results, it was necessary to compare the head anatomy. We did not find differences between French and Venetian populations.

The cephalic anatomy of the *G. paganellus* head is typical for Perciformes (Vandewalle et al., 1982; Barel, 1983; Liem, 1993). From video films, we focused only on structures that could be involved in movements related to sound production. A complete description of the head skeleton and muscles is given elsewhere (Adriaens et al., 1993) for *Pomatoschistus lozaoni*.

### Suspensorium and opercle
The preoperculum, symplectic and quadrate delineate a fenestra that is covered by a membrane (Fig. 4A). Rostrally, the palatine shape is T-like and its ventral process (articular process) articulates with the maxillary. A (palatine) ligament is attached to the rostral part of the palatine and inserts on the opposite rostral end of the palatine. This ligament allows the upper jaws to slide downward and upward
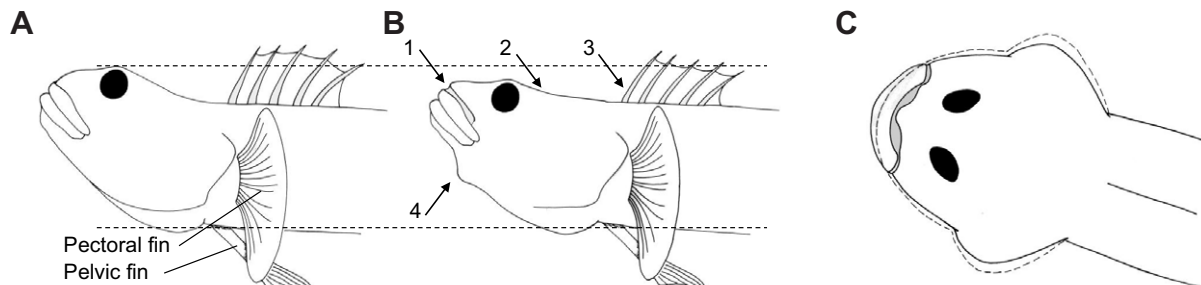


Fig. 2. Lateral (A and B) and dorsal (C) view of head movements during sound production in *G. paganellus*. A and dashed lines in C correspond to head elevation and opercle dilatation. B and solid lines in C correspond to head lowering and opercle constriction. In B, arrows indicate (1) lowering of the closed upper and lower buccal jaws, (2) lowering of the head, (3) recoil of the dorsal fin and (4) lowering of the anterior part of the branchial basket.

along the ethmoid edge. A second (maxillary) ligament inserts on the outer articular process of the maxillary and on the ethmoid bone, limiting the protraction of the upper jaw (Fig. 4A).

The opercle includes the bones usually present in teleosts. However, the suboperculum and the operculum are fused in a single plate that is connected to the interoperculum *via* a ligament. A second ligament connects the interoperculum to the posterior end of the mandible.

### Branchial basket

Observation of the hyoid bars and branchial baskets did not reveal unusual features. The branchial basket morphology of *G. paganellus* is typical of the Gobiidae (Murdy, 1985). The oral cavity is dorso-ventrally depressed, and thus wider than it is high. Basihyals and basibranchials are plate-like and closely applied to the flat buccal cavity roof. The five branchiostegal rays of the hyoid bars outline the ventral part of the buccal cavity.

### Pectoral girdle

The suspension of the pectoral girdle from the skull occurs at the post-temporal bone. The post-temporal consists of a basal plate with two rostrally directed processes (Fig. 4A, Fig. 5A). The dorsal and ventral processes form a fork with a dorsal and a lateral attachment to the skull (Fig. 5). The rostral tip of the dorsal process is flattened and is firmly connected to the epiotic bone *via* a syndesmosis, restricting rotation around a dorsoventral axis (see also Adriaens et al., 1993). The processus ventralis is situated on the lateral side of the skull. This process extends rostrally into a ligament that is attached to the neurocranium at the intercalar bone. At the tip of the cleithrum, a foramen is present through which Baudelot's ligament runs and terminates with an attachment on the medial face of the supracleithrum (Fig. 5B). Together, the forks prevent forward displacement of the post-temporal, which rotates only slightly.

The supracleithrum connects the post-temporal to the cleithral bone that constitutes the main part of the shoulder girdle (Fig. 4A, Fig. 5A). The cleithral bone forms the caudal margin of the branchial cavity. At this level, the transverse section of the body is wider than the buccal cavity width, meaning that the closure of the opercles against the pectoral shoulder completely closes the caudal openings of the buccal cavity, preventing water expulsion. However, ventrally the cleithrum has a small hemispheric depression against which the opercles cannot apply. Thus, it is still possible to have a small water flow.

The four radial bones in *G. paganellus* form the major part of the shoulder plate. These bones are laterally compressed and interconnected by collagenous fibres, thus forming one rigid plate (Adriaens et al., 1993).

### Head musculature

The adductor mandibulae originates on the skull and on the hyomandibula. These muscles include the $A_1$ bundles inserted on the upper jaw, the $A_2$ bundles attached to the mandible, the $A_3$ and $A\omega$ bundles attached to the inner side of the mandible (Fig. 4). The adductor $A_2$ is very thick, inserts on the coronoid process of the dentary and is divided into two bundles: $A_2\alpha$ inserts on the preoperculum and hyomandibular and $A_2\beta$ on the preoperculum and the quadratre. Muscle $A_3$, under $A_2$, is a thin layer of fibres that is inserted dorsally on the metapterygoid and on the inner face of the dentary *via* a tendon. $A\omega$ inserts on the inner face of the dentary and on the $A_3$ bundle tendon.

In *G. paganellus*, $A_1$ is also divided into two distinct bundles. Dorsally, $A_1\alpha$ is inserted on the hyomandibular and on a tendon
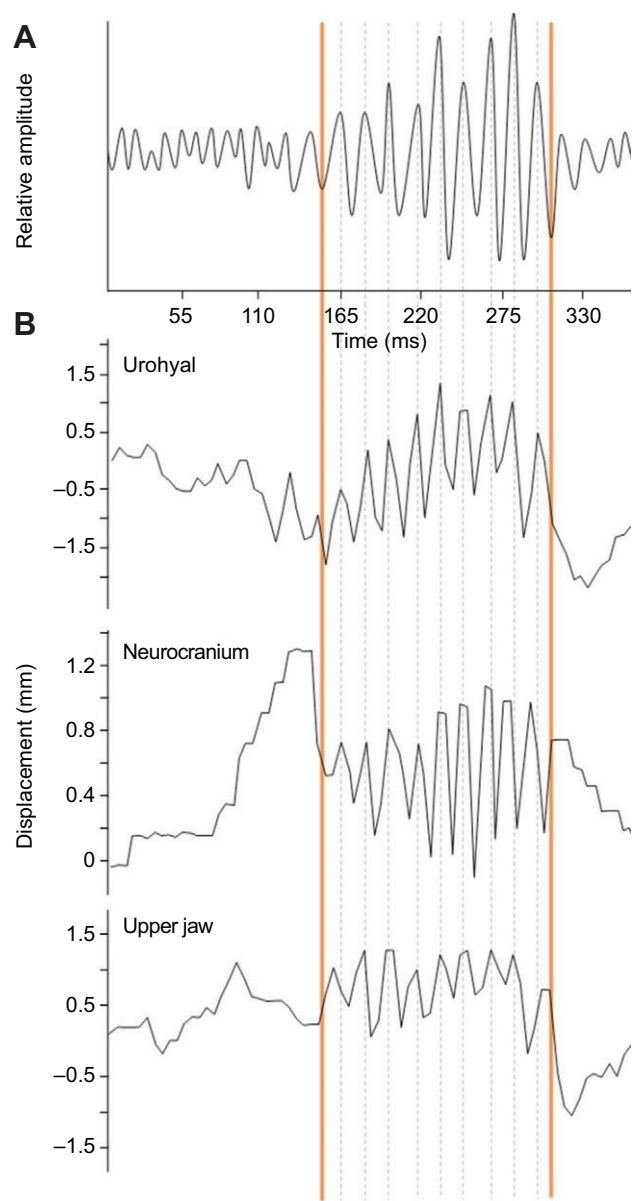


Fig. 3. Oscillogram (A) of the sound in *G. paganellus* with corresponding up and down movements (B) at the level of the urohyal from the branchial basket, the neurocranium and upper jaw. Dashed lines show positive peaks in the oscillogram corresponding to elevation of the urohyal, neurocranium and upper jaw.

that joins the maxillary and the mandible. Ventrally, $A_1\beta$ inserts on the same tendon and on the quadrate and the preoperculum (Fig. 4B).

The levator arcus palatini originates behind the orbit roof on the inner face of the sphenotic. It is almost entirely covered by $A_2\alpha$ and $A_1\alpha$ fibres and inserts on the hyomandibular and the metapterygoid. Its antagonist, the adductor arcus palatini, is a long muscle inserted medially on a major part of the parasphenoid and externally on the inner sides of all suspensorium bones except the preoperuclum and symplectic. It covers the entire orbit floor.

The dilatator operculi originates on the posterior crest of the sphenotic, runs between the upper parts of the hyomandibular and the preoperculum, and attaches to the outer side of the operculum. The levator operculi is in an original position. It has oblique fibres

**A**

Ascending process (Pa)
Ectopterygoid
Palatine ligament
Metapterygoid
Hyomandibula
Dorsal process (Pt)
Maxillary ligament
Palatine
Ventral process (Pt)
Post-temporal
Articular process (Pa)
Premaxilla
Operculum
Suboperculum
Maxilla
Dentary
Symplectic
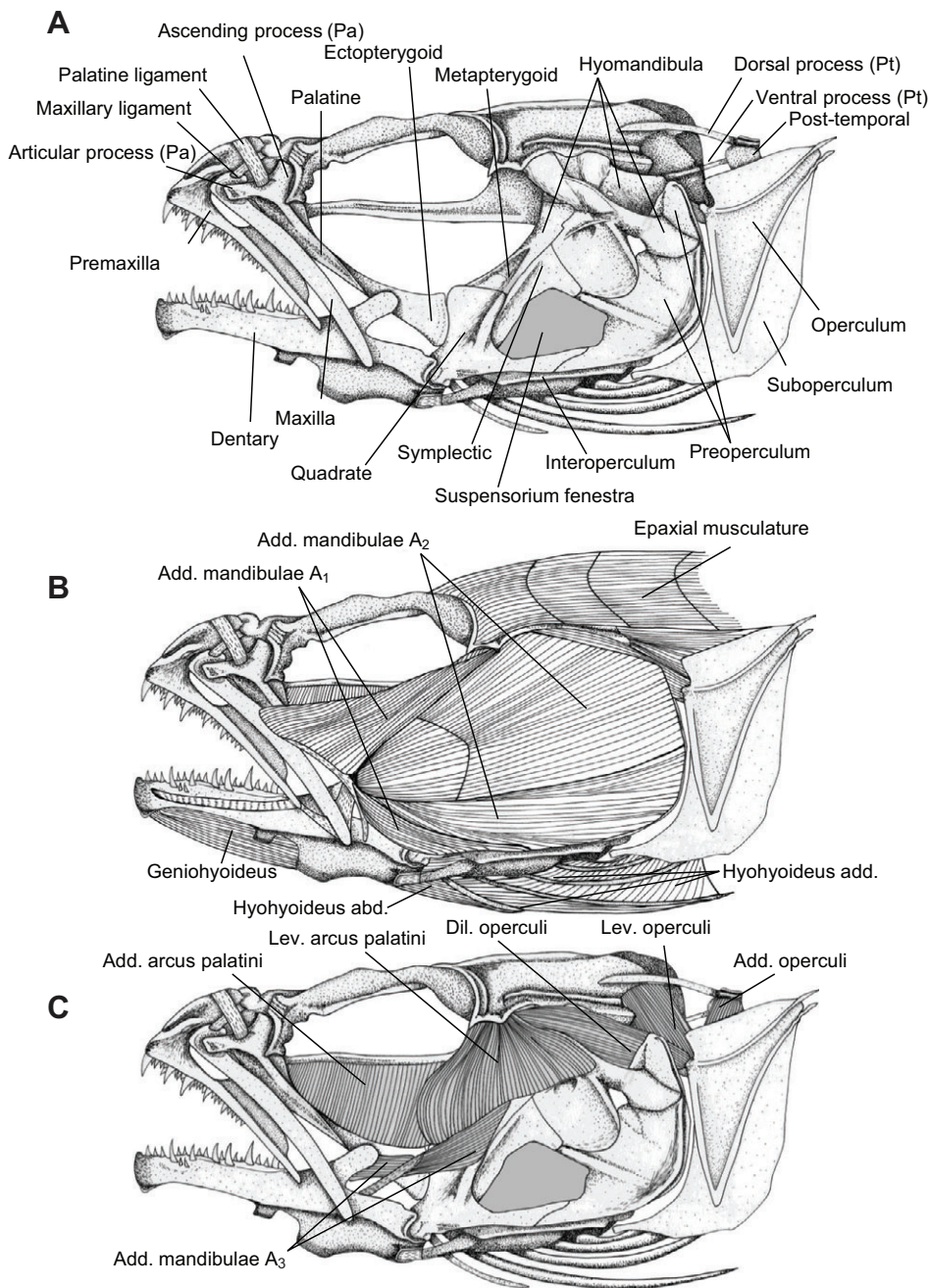Preoperculum
Quadrate
Interoperculum
Suspensorium fenestra

Fig. 4. Left lateral view of the head anatomy in *G. paganellus*. (A) Cephalic skull, (B) superficial muscles, (C) deep muscles after removal of adductor muscles $A_2$ and $A_1$. Pa, palatine; Pt, post-temporal; Add., adductor; Dil., dilatator; Lev., levator.

**B**

Add. mandibulae $A_2$
Add. mandibulae $A_1$
Epaxial musculature
Geniohyoideus
Hyohyoideus add.
Hyohyoideus abd.

**C**

Lev. arcus palatini
Dil. operculi
Lev. operculi
Add. arcus palatini
Add. operculi
Add. mandibulae $A_3$

that are inserted on the upper inner crest of the operculum and on the post-temporal plate of the shoulder girdle. The adductor operculi is divided into two bundles. Part α inserts on the occipital region, at the level of the epiotic and on the inner dorsal face of the operculum. Part β is deeper and inserts on the epiotic at the level of hyomandibular–operculum articulation: some fibres are on the inner face of the rostral part of the operculum and others at the level of the articular condyle of the hyomandibular.

### Muscles attached to the shoulder girdle apparatus

The epaxial musculature corresponds to bundles that are dorsal to the lateral septum. In *G. paganellus*, epaxial muscle bundles are inserted dorsally on the neurocranium, behind the level of the orbits (Fig. 4B). Ventral to the epaxial muscle, the levator pectoralis muscle

is easily distinguishable and is separated into two bundles: pars lateralis and pars medialis (Fig. 5). The musculus levator pectoralis pars lateralis originates from the caudal margin of the pterotic bone of the neurocranium and inserts on the rostral margin of the cleithral bone. The musculus levator pectoralis pars medialis originates more ventrally, at the exoccipital bone, and is attached to the medial side of the supracleithral bone and the rostral side of the cleithral bone. On the cleithral bone, the insertion of the pars medialis is dorsal to pars lateralis, meaning the two parts cross.

### Testing of previous hypotheses

In conclusion, the head anatomy of *G. paganellus* does not possess unusual features associated with the sound-producing mechanism. However, movement analysis and the sound structure suggest several hypotheses for the sonic mechanism.
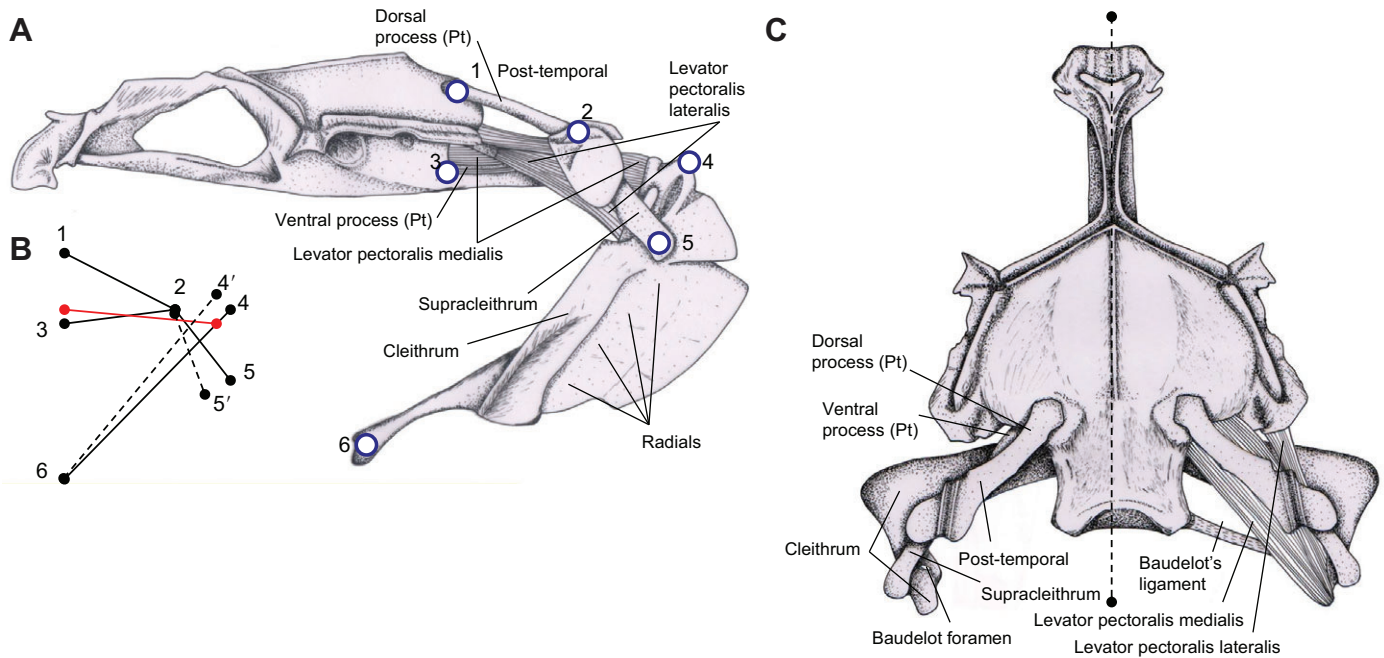
Fig. 5. (A) Left lateral view of the neurocranium, pectoral girdle and sonic muscles in *G. paganellus*. (B) Schematic lateral view of the pivot points of the pectoral shoulder; numbers refer to those in A; dashed lines correspond to the movements of the pectoral bones at the time of contraction. (C) Dorsal view of the neurocranium and pectoral girdle (left) and with sonic muscles indicated (right). Pt, post-temporal.

## Hypothesis 1

The positive peaks of the calls (Fig. 3) correspond to a set of movements that provokes compression of the oral cavity and an increase in internal buccal pressure. Conversely, the negative peaks of the calls are linked to a low pressure phase due to the dilatation of mouth volume. These modifications are possible because the opercles appear to be closed, maintaining a constant volume of water. We first tested the role of the membranous fenestra in the suspensorium (Fig. 4A). This tissue is able to deform and could act like a drumming membrane with back-in-forth movements as pressure alternates in relation to the alternation between high and low levels. In this experiment, different parts of the fish head were cut to allow the rapid outflow of water during nodding movements. In two fish, the left and right opercles were cut to prevent their closure against the pectoral girdle. In addition, the hyohyoideus adductor and hyohyoideus abductor were also cut (Fig. 4C), allowing the ventral outflow of water. Fish were still able to make the same sounds, rejecting the hypothesis of suspensorium drumming.

## Hypothesis 2

A hydrodynamic sound could be due to the forcible ejection of water through the gill opening (Tavolga, 1958; Stadler, 2002). Our first set of cuts of the opercles also allowed rejection of this hypothesis. Moreover, the shape of the oscillograms (Figs 1, 3) is not in agreement with this hypothesis. The constant ejection of water should correspond to decaying pulses within the call because more water is present at the time of the first nodding movements than later. In *G. paganellus*, the amplitude of the peaks rises consistently to a maximum before decaying.

## Hypothesis 3

During hand manipulation of the fish, we noted that the oral valves were well developed at the level of the upper and lower jaws. Moreover, these membranes were in close contact with the rostral part of the tongue, i.e. the basihyal. During nodding, the basihyal should follow the movement of the urohyal and undergo up and down movements that could vibrate the membrane like a guitar string. The basihyal was cut to prevent its communication with the membrane. This cutting did not prevent sound production, rejecting the third hypothesis.

## Hypothesis 4

At this level, none of the hypotheses seemed to satisfy the description of the sonic mechanism in *G. paganellus*. We have, however, noticed that the morphology of *G. paganellus* and different *Cottus* sp. is really similar, mainly because they have many parallels in their way of life. They all live mainly on the bottom between rocks, are not good swimmers and establish cavities for nesting. Moreover, nodding movements were observed during sound production in different cottid species (Ladich, 1989; Ladich, 1990; Whang and Janssen, 1994). Although it has not been described in detail, the sound-producing mechanism in Cottidae involves the pectoral girdle (Barber and Mowbray, 1956; Ladich, 1989). Hypothesis 4 concerned a possible parallel between the cottid and gobiid mechanisms. Dissections (Fig. 5), transmission electronic microscopy (TEM) and EMG were used to study the shoulder and associated structures.

## TEM

In levator pectoralis muscle, cells are rounded and vary in size (mean ± s.d. diameter 58.8±20 μm, *N*=77). In the same bundles, some cells can be five times bigger than adjacent cells. In the muscle cells, three zones can be easily distinguished (Fig. 6): (1) at the periphery, there is first a large sarcoplasmic band beneath the sarcolemma that contains numerous mitochondria; (2) at the level of the myofibrils, the outer zone has a radial architecture and is flanked by linear arrangements of sarcotubules; and (3) the arrangement is less structured in the centre of the cell, which may
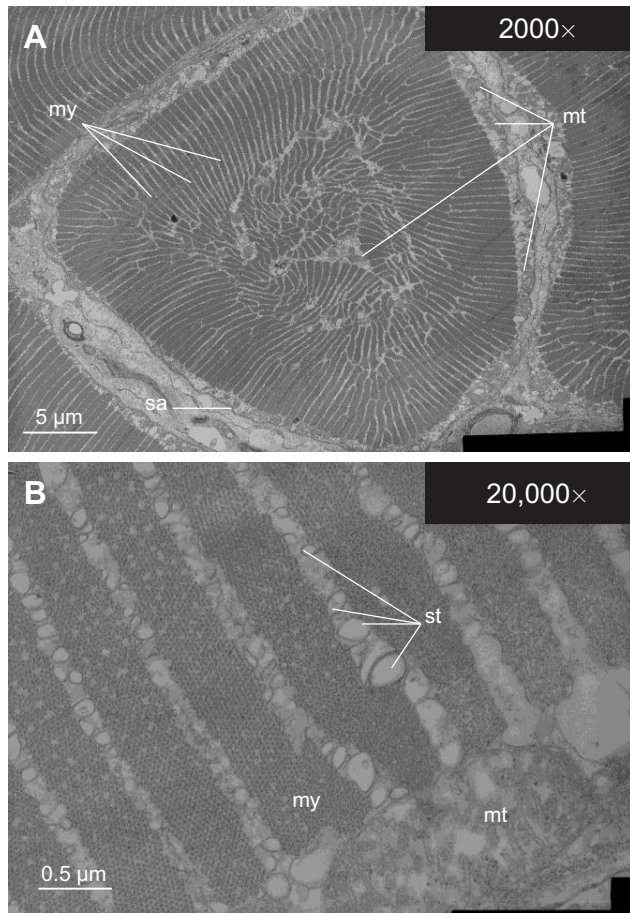
Fig. 6. Transmission electron micrograph cross-sections of sonic muscle (levator pectoralis) in *G. paganellus*. (B) Enlargement of the radially arranged myofibrils in the periphery. Cross-section of sonic muscle fibre shows three zones (see Results). mt, mitochondria; my, myofibril; sa, sarcolemma; st, sarcotubule.

present a central core that is more or less developed. Moreover, mitochondria are also found in this third zone whereas this was not the case in the second (Fig. 6).

In the epaxial musculature, white fibres are classical. They are polygonal in cross-section, compact, larger than sonic fibres, do not show radial arrangement of myofibrils and do not have a sarcoplasmic layer beneath the sarcolemma.

## EMG

Based on data provided by TEM analysis, EMG experiments were conducted on the levator pectoralis muscle, and more precisely on its medial part, which is larger than the lateral part. A pair of electrodes was placed in right and left levator pectoralis muscle. EMG recordings revealed burst activity coincident with sound emission (Fig. 7). Peak-to-peak duration in EMG showed a period of $8.1\pm0.7$ ms ($N=83$ from five bursts), which corresponds perfectly with the fundamental frequency of the calls. Therefore, the pulse contraction rate determines the sound frequency in *G. paganellus*. The amplitude of the EMG was not constant during the call, increasing to a plateau and, in some cases, decreasing towards the end of the call. A short delay of 1 ms was recorded between the left and right electrodes, but we cannot explain it. Also, differences between the two graph shapes are probably due to the electrode position or to difference in connectivity of the electrodes.

## DISCUSSION

Differences between fish population vocal repertoires have already been described (Fine, 1978; Parmentier et al., 2005; Phillips and Johnston, 2008; Parmentier et al., 2009; Parmentier et al., 2011a; Tellechea et al., 2011). In the case of *G. paganellus*, both populations were able to make pulsed and tonal sounds but we observed more pulsed sounds from fish of the tidal zone in France and more tonal sounds in the Italian population. These differences are evidenced by the statistically different sound properties found between the two populations. However, the size range of the two sets of individuals recorded differed, with the Venetian fish on average being larger than the French ones. This size difference does not allow us to draw conclusions on a real geographic difference between the two populations; rather, differences in terms of ontogenetic stage or growth rate between the two groups of individuals recorded may reflect the differences in sound properties. In the context of the present paper, it is important to highlight the continuum between a pure tonal sound and a more pulsed sound that characterize the general feature of the call of this species across its geographic range.
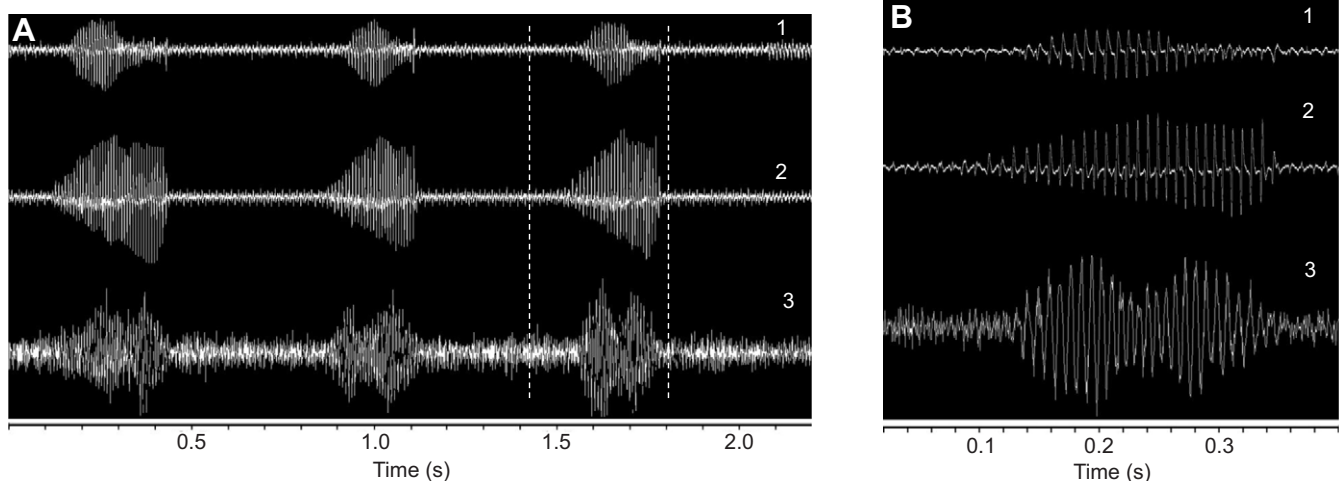


Fig. 7. Electromyography (EMG) bursts corresponding to the contractions of the right (1) and left (2) levator pectoralis pars medialis during sound production (3) in *G. paganellus* (total length 140 mm). Bursts in B correspond to the signals found in between the dashed lines in A.

It implies that the signals can be modulated in terms of pulse packing. According to Crawford (Crawford, 1997), tonal sounds result from pulsed sounds with no inter-pulse intervals. In *Padogobius bonelli*, males are also able to produce drumming (pulsed) and tonal sounds (Lugli et al., 1995). Malavasi and colleagues (Malavasi et al., 2008) suggest that the tonal sound originates from an initial drumming structure within the clade of the larger species belonging to the genus *Gobius* and *Padogobius*. Pulse period in the French population is 16 ms whereas it is 10.7 ms in the Italian one, suggesting the shorter period may be related to the production of tonal sounds.

Differences were also present in head movements. In the study of the French population, the high-speed camera recording clearly showed the movements were not restricted to the head nodding and opercular movements previously mentioned (Ladich and Kratochvil, 1989; Torricelli et al., 1990; Amorim and Neves, 2007) but also involved more complicated patterns because the pulses were associated with up and down movement of the buccal jaws and branchial basket (Fig. 2). Moreover, the kinematic analysis indicated a relationship between the movement and the pulse construction (Fig. 3). From a morpho-functional point of view, this mechanism is very unusual and does not correspond to head movements usually associated with respiration or feeding. For example, the lowering of the mandible or the elevation of the head is always accompanied by mouth opening in teleost fishes (Liem, 1978; Lauder, 1980; Drucker and Jensen, 1991; Vandewalle et al., 1995). The head skeleton, ligament and the muscles of *G. paganellus* do not indicate special characters dealing with jaw movements (Figs 4, 5). Therefore, the new motor pattern we describe is probably due to neuronal motor patterns. Moreover, the Italian fish clearly show that all these movements are not required for sound production in *G. paganellus*. Consequently, nodding movements should be considered as a by-product of visual displays that accompany sound production. Moreover, some nodding movements can be made without sound production (E.P., unpublished observations), reinforcing the hypothesis of visual displays. The association of sounds and visual display has also been observed in other species: *Dascyllus flavicaudus* often tie some swimming motion to sound production, but can also make signal jumps (courtship movements) without calling (Parmentier et al., 2010b).

Tavolga (Tavolga, 1958) suggested a mechanism of sound emission caused by rapid ejection of strong jets of water through the opercula during downward thrusts of the head. Stadler (Stadler, 2002) supported this hypothesis in *Bathygobius curacao*. In this mechanism, a volume of water is taken into the mouth, the buccal and branchial basket are then closed and the water is expelled through a small aperture due to oral compression at the opercles (Stadler, 2002). This mechanism cannot apply to *G. paganellus* for at least three reasons. (1) Expelling water through the opercles ought to produce sounds with pulses showing decreasing amplitude because the water volume in the mouth decreases regularly. The oscillogram does not show this pattern: the amplitude of the pulses increases before decreasing at the end of the call. The same oscillogram pattern was also found in other goby species (*Padogobius*, *Gobius*, *Zosterisessor*, *Pomatoschistus*, *Knipowitschia*) (Malavasi et al., 2008). Lugli and colleagues (Lugli et al., 1996) did not observe strong fluxes of water during sound production in *Padogobius nigricans*. (2) The hydrodynamic sound resulting from water ejection implies a small aperture. However, *G. paganellus* was still able to make sounds after cutting of the opercle (Fig. 4A) and the hyohyoideus (Fig. 4B) between the branchiostegal rays. With this operation, the fish cannot control the water volume in the mouth and no longer possess a small opening.

Therefore, sounds are not due to forcible ejection of water through the gill opening. (3) High-speed camera data indicate many cycles of movements, not just one. We did not study *B. curacao* and cannot reject the hydrodynamic mechanism in this species.

According to our results, sounds in *G. paganellus* are not hydrodynamic and differences between the two populations indicate that head movements are probably not involved. However, the dissection of *G. paganellus* showed the skeleton and muscle morphologies are closely related to those from fishes from the Cottidae family. The two taxa are, however, not phylogenetically close (Thacker and Hardman, 2005; Li et al., 2009) and these similarities should be considered as adaptive convergences, Cottidae and Gobiidae being mainly demersal species. For example, the suspensorium fenestra (Fig. 4A), the large radials (Fig. 2A) and the dorso-ventral depressed body are unusual characters found in species of both families. Data from two different cottid species (*Myoxocephalus octodecimspinosus* and *Cottus gobio*) support the hypothesis that sound production in Cottidae results from the contraction of muscles originating on the skull and inserting on the pectoral girdle (Barber and Mowbray, 1956; Ladich, 1989). Two different kinds of observation support the hypothesis the goby sounds result from the contraction of the levator pectoralis. This muscle has many characteristics of fast contracting muscles (Ono and Poss, 1982; Bass and Marchaterre, 1989; Fine et al., 1993; Loesser et al., 1997; Ladich, 2001; Boyle et al., 2013): mitochondria in the core, numerous peripheral mitochondria, ribbon-like disposition of myofibrils and well-developed sarcoplasmic reticulum tubules. Sonic diameter is quite high on average (54 μm) in comparison to that of other sonic muscles (Parmentier and Diogo, 2006) but is also highly variable (from 20 to 94 μm). Finally EMG showed the relationship between sound production and the levator pectoral contraction.

The sound-producing muscle has been identified but the mechanism remains to be explained. The preparation phase was usually attributed to the thrust of the head but corresponds to the erection of the pelvic fins. In the erect position, the shoulder girdle does not rely on the substrate, allowing the cleithrum (indicated by lines 4–6 in Fig. 5B) to manoeuvre freely. The schematic drawing in Fig. 2B indicates roughly the movement during sound production. The contraction of the levator pectoralis should pull the upper part of the cleithrum forward and cause forward displacement of the distal part of the supracleithrum. This movement is possible because the osseous fork, formed by the dorsal and ventral processes of the post-temporal, forms two anchoring points on the skull, prevents forward movements of the post-temporal and can be used as a fulcrum for displacement of the cleithrum (Adriaens et al., 1993). However, we did not observe any ridges on the bones as in some catfish species (Schachner and Schaller, 1981; Fine et al., 1997; Parmentier et al., 2010a), indicating *G. paganellus* calls are not stridulatory sounds.

The occurrence of sounds in some gobiid species deprived of a swimbladder indicates this structure is not required for low-frequency, non-stridulatory sounds (Lugli et al., 1996). Sounds may be generated by the periodic contraction of the levator pectoralis muscle that generates the vibration of the pectoral girdle and, possibly, of the large radials that are greatly enlarged in this family compared with generalized teleosts (Adriaens et al., 1993). Furthermore, it could explain why in most vocal gobies, pectoral fins are abducted during calls. Large radials could act like the membrane of a loud speaker or help in directing the calls. In the longhorn sculpin *Myoxocephalus octodecimspinous*, the same kind of mechanism involving the cranioclavicular muscle and pectoral

girdle also allows the emission of low-frequency (<150 Hz) sounds (Barber and Mowbray, 1956; Fish and Mowbray, 1970). In levator pectoralis, the crossing of pars medialis and pars lateralis should provide a greater resulting force and increase the power of the muscle. However, EMG techniques were not applied to pars lateralis and the role of this muscle remains to be confirmed.

According to Malavasi and colleagues (Malavasi et al., 2008), sound duration is strongly predicted by the mean body size of each species, suggesting a constraint related to morphology and the emission mechanism. The pectoral based mechanism is coherent with this relationship because a similar correlation was also observed in *Ictalurus punctatus*, which makes sounds using the pectoral girdle (Fine et al., 1999). Further studies are, however, required to better understand the sound-producing mechanism in Gobiidae and the vibratory characteristics of the pectoral girdles and radials. Different experiments are also definitively required to understand the transition from pulsed to tonal sounds.

## AUTHOR CONTRIBUTIONS

E.P. conceived and designed the experiments; E.P., Y.-E.C., L.S. and S.M. performed dissections, and sonic and video recordings; E.P. and S.M. analyzed the data; L.K. and K.B. performed electromyography; E.P. and S.M. wrote the paper.

## COMPETING INTERESTS

No competing interests declared.

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