

## RESEARCH ARTICLE

# New insights into the role of the pharyngeal jaw apparatus in the sound-producing mechanism of *Haemulon flavolineatum* (Haemulidae)

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**ABSTRACT**

Grunts are fish that are well known to vocalize, but how they produce their grunting sounds has not been clearly identified. In addition to characterizing acoustic signals and hearing in the French grunt *Haemulon flavolineatum*, the present study investigates the sound-production mechanism of this species by means of high-speed X-ray videos and scanning electron microscopy of the pharyngeal jaw apparatus. Vocalizations consist of a series of stridulatory sounds: grunts lasting ~47 ms with a mean period of 155 ms and a dominant frequency of ~700 Hz. Auditory capacity was determined to range from 100 to 600 Hz, with greatest sensitivity at 300 Hz (105.0±11.8 dB re. 1 µPa). This suggests that hearing is not tuned exclusively to detect the sounds of conspecifics. High-speed X-ray videos revealed how pharyngeal jaws move during sound production. Traces of erosion on teeth in the fourth ceratobranchial arch suggest that they are also involved in sound production. The similarity of motor patterns of the upper and lower pharyngeal jaws between food processing and sound production indicates that calling is an exaptation of the food-processing mechanism.

**KEY WORDS:** Haemulidae, Grunt, Sonic mechanism, Pharyngeal jaws, Communication, Exaptation

**INTRODUCTION**

A large number of fishes are known to produce sounds in different social contexts such as agonistic interactions, courtship and competitive feeding (Amorim et al., 2003; Amorim and Hawkins, 2005; Amorim and Neves, 2008; Bertucci et al., 2010; Colleye and Parmentier, 2012; Ladich, 1997; Lobel, 1998; Longrie et al., 2013; Parmentier et al., 2010). Sounds produced by males during courtship interactions for instance can affect the choice of female mate (Amorim et al., 2004; Amorim et al., 2008; Danley et al., 2012; Phillips and Johnston, 2009; Verzijden et al., 2010). The production of sounds does not rely on the same kind of mechanism in all teleost fishes that have evolved a high diversity of sound producing mechanisms (Amorim, 2006; Ladich and Fine, 2006). These mechanisms mostly involve the stridulation of bony structures or the action of muscles deforming the walls of the swimbladder (Ladich and Fine, 2006; Parmentier and Diogo, 2006). Stridulation is a widespread mechanism in fishes (Ladich and Fine, 2006; Moulton, 1958; Salmon et al., 1968) that is based on friction of skeletal

elements such as teeth, fin rays and vertebrae (Burkenroad, 1930; Tavalga, 1971). Stridulation sounds are often composed of a series of rapidly produced and irregular transient pulses, containing a wide range of frequencies (Hawkins, 1993; Fine and Parmentier, in press). In many fishes without obvious distinct sound-producing elements, the sonic mechanism has been (often incorrectly) attributed to sounds that result from the friction of pharyngeal teeth (e.g. Ballantyne and Colgan, 1978; Lanzing, 1974). In this case, sounds would be amplified by the swimbladder or other air-filled cavity (i.e. suprabranchial organ) (Kratochvil, 1985). These assumptions involving the branchial basket are likely based on a study on the white grunt *Haemulon plumieri* in which the upper and lower pharyngeal teeth grate against each other (Burkenroad, 1930). The author noted that the swimbladder acts as a ‘resonator’ because the character of the sound became ‘dry’ and lost its grunt-like quality after swimbladder deflation.

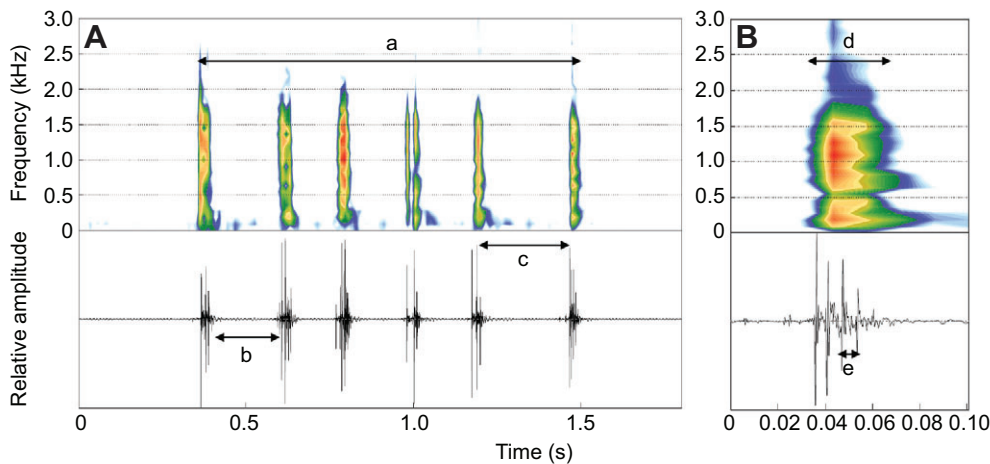
In teleosts, the pharyngeal jaw apparatus is derived from gill arch elements and muscles. It is found at the level of the branchial basket. In perciforms, the lower pharyngeal jaw is composed of tooth plates that are fused to ceratobranchial 5, whereas the upper pharyngeal jaw is made of tooth plates fused to pharyngobranchials 2–4. The motion of the pharyngeal jaw apparatus (PJA) is controlled by a complex series of muscles inserted on the different elements (reviewed by Vandewalle et al., 2000). Pharyngeal jaws have become important for food processing, leading to an increase in their size and to a reduction of buccal teeth, which became dedicated to food catching in higher teleosts (Vandewalle et al., 2000). A further series of anatomical specializations, resulting in the fine control of prey manipulation and processing, made the PJA a key innovation in the evolution and success of certain families. In cichlids and pomacentrids, for example, pharyngeal teeth show a large morphological plasticity according to the specific trophic regime (e.g. Greenwood, 1973; Kornfield and Smith, 2000; Liem and Sanderson, 1986). Although the literature has focused mainly on the anatomy and function of PJA in feeding behavior (Gidmark et al., 2014; Lauder, 1983; Vandewalle et al., 2000; Wainwright, 1989a), studies focusing on the possible role of pharyngeal jaws in sound production remain rare (e.g. Kratochvil, 1985; Lanzing, 1974). Moreover, no direct investigations of the mechanism and implication of the different muscles and/or bones have been conducted in this particular context. An understanding of the sound-production mechanisms involving pharyngeal jaws would significantly add to the knowledge and evolution of sound production in fishes.

Haemulids are commonly called ‘grunts’ because of the numerous representatives of the family showing the aptitude to produce grating sounds when they are held both in and out of the water, i.e. in a distress situation (Burkenroad, 1930; Moulton, 1958; Tavalga, 1965). Such stridulatory sounds are likely produced by means of pharyngeal jaws as observed by Burkenroad (Burkenroad, 1930) in the white

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**Fig. 1. Analysis of the sounds produced by the French grunt *Haemulon flavolineatum*.** Sonagrams (top) and oscillograms (bottom) of a series of grunts (A) and the last grunt of the series (B). Colors indicate relative sound intensity (blue=low and red=high). Some of the measured variables are represented: series duration (a), inter-grunt interval (b), grunt period (c), grunt duration (d), inter-pulse interval (e).

grunt *Haemulon plumieri* Lacepède 1801. This observation was later confirmed by Moulton (Moulton, 1958) in *Haemulon sciurus* Shaw 1803 and the French grunt *Haemulon flavolineatum* Desmarest 1823. However, the hypothesis that the mechanism responsible is that of upper pharyngeal teeth grating against the lower ones has never been experimentally verified and described. The present study uses a multidisciplinary approach to study acoustic communication in *H. flavolineatum*. An analysis of the acoustic signals produced in a distress situation was first performed before investigating the auditory abilities by means of auditory-evoked potential (AEP) technique. High-speed X-ray video recordings were then performed in order to provide a description of the sound-producing mechanism focusing on the kinematics of the pharyngeal jaw apparatus. Finally, a microstructure analysis of pharyngeal teeth using scanning electron microscopy (SEM) and energy-dispersive X-ray microanalysis was performed. This study aims to reveal new insights on sound production by means of pharyngeal jaws in fishes.

## RESULTS

### Description of sounds

Sounds produced in distress situations consisted of grunts uttered alone or in series of  $2.2 \pm 2$  grunts (mean  $\pm$  s.d.; min–max=1–24) (Fig. 1). Grunts lasted  $47 \pm 11$  ms, with a period of  $155 \pm 31$  ms and were composed of  $6 \pm 2$  pulses ( $N=198$  sounds from eight individuals). Grunts had a dominant frequency of  $718 \pm 180$  Hz (Fig. 2) and were produced with an intensity of  $154 \pm 4$  dB re.  $1 \mu\text{Pa}$  ( $N=68$  sounds from four individuals) recorded at  $\sim 10$  cm. The dominant frequency of grunts ( $N=9$ ,  $r=0.90$ ,  $P < 10^{-3}$ ), the grunt period ( $N=9$ ,  $r=0.90$ ,  $P < 10^{-3}$ ) and the number of pulses per grunt ( $N=9$ ,  $r=0.58$ ,  $P=0.017$ ) were all negatively correlated with total specimen length (Fig. 3).

Sounds recorded during food processing ( $N=14$  from three individuals) showed a significantly shorter duration of  $27 \pm 9$  ms (Wilcoxon test,  $Z_{8,4}=-7.2$ ,  $P < 10^{-3}$ ) and a significantly shorter grunt period of  $108 \pm 30$  ms (Wilcoxon test,  $Z_{8,4}=-3$ ,  $P=3.10^{-3}$ ) than distress calls. Dominant frequency ( $536 \pm 148$  Hz) and number of pulses within a grunt ( $5 \pm 2$ ) did not vary significantly.

### Hearing abilities

Mean thresholds established using the AEP technique showed that individuals were most sensitive to low frequencies (100–600 Hz) and they did not detect frequencies higher than 1200 Hz. The most sensitive frequency was 300 Hz ( $105.0 \pm 11.8$  dB re.  $1 \mu\text{Pa}$ ). There was a 40–45 dB difference in threshold level between the frequency of greatest sensitivity and lowest sensitivity (600–1200 Hz) (Fig. 4).

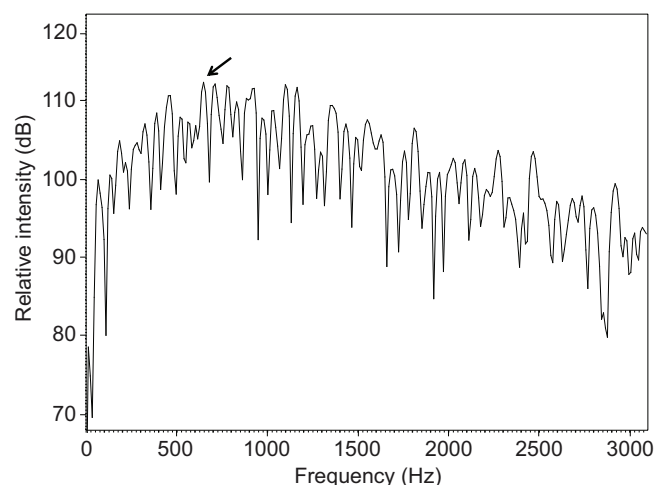
No artefacts were detected at high sound levels when a dead fish control was run.

### High-speed X-ray video recordings during sound production

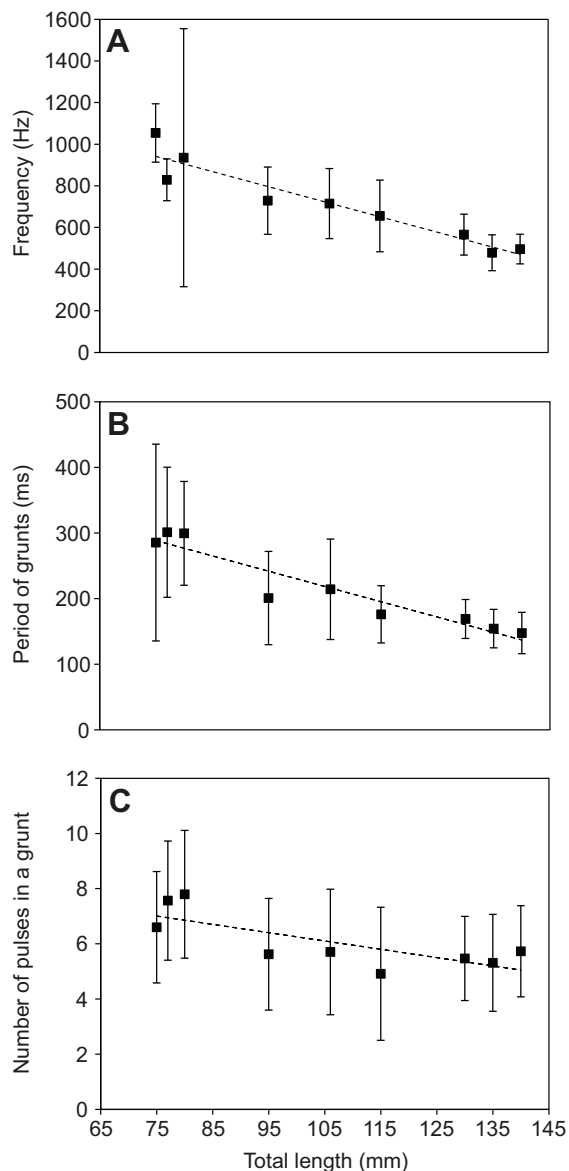
Observations of pharyngeal jaw motion using cineradiography revealed that the upper pharyngeal jaw (UPJ) undergoes a larger excursion during sound production than the lower pharyngeal jaw (LPJ; Fig. 5). At the start of the movement (Fig. 5, point 0), the UPJ moved anteriorly before moving posteriorly and ventrally until they met the LPJ (Fig. 5, point 1). The LPJ movement was more restricted and started with a slight anti-clockwise rotation before meeting the UPJ. The two jaws then moved posteriorly and dorsally after the UPJ also rotated anti-clockwise in order to be parallel to the LPJ (Fig. 5, point 2). Along this course, the UPJ moved faster and rasped against the LPJ. This rasping movement peaked when the course of the UPJ continued whereas the LPJ started to move anteriorly downward (Fig. 5, point 3). After separation, both jaws moved ventrally and anteriorly to return to rest (Fig. 5, point 4). The average time of contact (rasping) between upper and lower jaws was  $43 \pm 9$  ms, which is consistent with the previously measured duration of a grunt.

### Structure of pharyngeal teeth and associated musculature

As already observed by Wainwright (Wainwright, 1989a; Wainwright, 1989b), secondary electron imaging (SE imaging) in



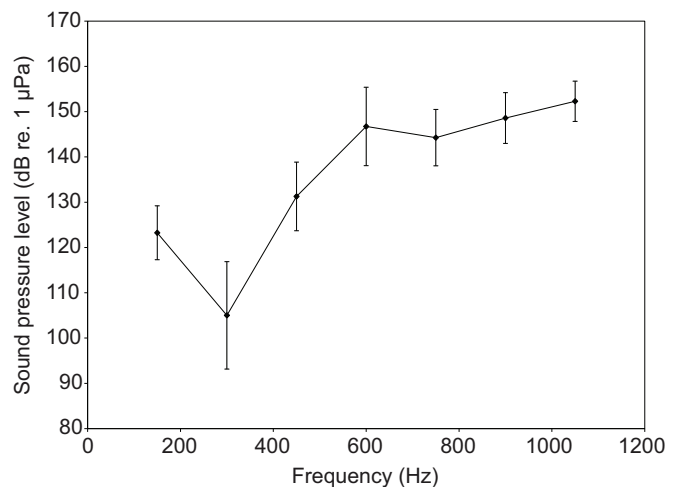
**Fig. 2. Logarithmic power spectrum of a grunt produced by *H. flavolineatum*.** Arrow indicates dominant frequency (650 Hz in this example).



**Fig. 3. Correlation of acoustic features of *H. flavolineatum* with total body length.** (A) Dominant frequency of grunts (linear regression equation:  $y = -7.25x + 1485.60$ ). (B) Period of grunts ( $y = -2.33x + 462.93$ ). (C) Number of pulses in a grunt ( $y = -0.03x + 9.26$ ). Values are individual means  $\pm$  s.d.

SEM confirmed that teeth were present on upper pharyngeal jaw (i.e. pharyngobranchials 2–4) and lower pharyngeal jaw (i.e. ceratobranchial 5). However, they were also well developed on ceratobranchial 4 (CB4) (Fig. 6). Teeth were conical with a sharp tip, bent towards the inside part of the branchial basket. On the UPJ, teeth were uniformly distributed on the tooth plates. Medial teeth were the largest and their size decreased from the center to the periphery (Fig. 6A). Sizes of teeth of the LPJ also decreased from the center to the periphery (Fig. 6B). Ceratobranchial 4 had teeth on its posterior half. They were not evenly distributed but formed groups of 2–9 teeth carried by button-like gill rakers. The size difference between central and lateral teeth was less obvious here (Fig. 6C). The enamel of most external and the thinnest teeth in the upper and lower pharyngeal jaws, and in ceratobranchial 4 showed erosion (Fig. 7).

The elemental X-ray microanalyses of the enamel at the tip of the different teeth (Table 1) were performed on bulk samples and



**Fig. 4. Audiogram of *H. flavolineatum*.** No response was obtained for the 1200 Hz stimuli for any of the intensities tested. Values are means  $\pm$  s.d.

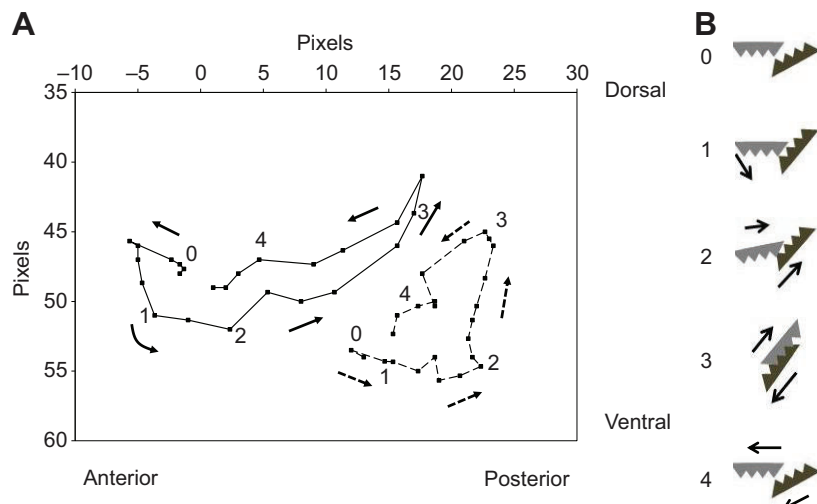
polished slices in a ESEM-FEG XL30 working in low- and high-vacuum conditions, respectively. They revealed a significantly higher proportion of calcium (ANOVA,  $F_{2,26}=3.24$ ,  $P=0.05$ ) in teeth of the UPJ than in teeth of the LPJ and CB4 (Fisher's LSD *post hoc* test,  $P<0.05$ ). A higher level of magnesium was found in teeth of the LPJ (ANOVA,  $F_{2,26}=3.92$ ,  $P=0.03$ ; Fisher's LSD *post hoc* test,  $P<0.05$ ). A significantly higher level of fluorine (ANOVA,  $F_{2,26}=3.89$ ,  $P=0.03$ ) was found in teeth of CB4 compared with teeth of the UPJ (Fisher's LSD *post hoc* test,  $P=0.01$ ). No significant differences in phosphorus levels were found.

The description of the different pharyngeal muscles (Fig. 8) can be found in Wainwright (Wainwright, 1989b) who based his study on nine Haemulid species: *Anisotremus virginicus*, *Haemulon aurolineatum*, *H. flavolineatum*, *H. sciurus*, *H. plumieri*, *H. chrysargyreum*, *H. bonairiense*, *H. carbonarium* and *H. macrostomum*. We agree with this description except at the level of the protractor pectoralis. Wainwright (Wainwright, 1989b) indicated that 'the protractor pectoralis muscle runs from its origin on the skull to insert primarily by a thick tendon on the distal tip of ceratobranchial 5. This muscle also inserts in the connective tissue sheet that runs between the pectoral girdle and ceratobranchial 5'. According to our observations, we can add that a small branch of the tendon is also found between the protractor pectoralis tendon and epibranchial 4, meaning that the contraction of the protractor pectoralis should also induce the backward movement of epibranchial 4 (and consequently of the UPJ) and should improve the meeting ability of the upper and lower pharyngeal jaws.

## DISCUSSION

### Sounds and auditory capacities

Similar to other vocal Haemulids, the sounds of *H. flavolineatum* consist of grunts emitted in series with most energy below 1 kHz (Fish and Mowbray, 1970; Moulton, 1958). This corresponds to their hearing ability because this species do not detect sounds beyond 1050 Hz. However, the discrepancy between the dominant frequency of sounds and the frequency of greatest auditory sensitivity indicates that hearing abilities are not tuned exclusively to detect sounds of conspecifics. This could also be true for *Haemulon sciurus* in which auditory frequency is between 50 and 1200 Hz (Tavolga and Wodinsky, 1963; Tavolga and Wodinsky, 1965).



**Fig. 5. Two-dimensional movement of the pharyngeal jaws of *H. flavolineatum* during sound production.**

(A) Data were collected from video tracking the center of the barium sulphate contrasted pharyngeal jaws. Upper jaw, solid line; lower jaw, dashed line. Left lateral view. Units are relative distance, in pixels, from a reference point (center of otolith). Arrows indicate the course of upper (solid line) and lower (dashed line) pharyngeal jaws. Numbers adjacent to points indicate homologous position in time. (B) The position and movements of upper (gray) and lower (black) jaws schematized for each of the points 0–4 in A. Images are separated by 5 ms.

Most studies confirm that Haemulids are more sensitive at low frequencies (below 300–400 Hz). McFarland and Hillis (McFarland and Hillis, 1982) suggest that these sounds are used for night foraging in order to locate other group members. Haemulids also aggregate for spawning (Heyman and Kjerfve, 2008; Trott et al., 2010). Sounds could therefore be used to synchronize social activities and ensure foraging or reproductive success (Amorim et al., 2003; Rowe and Hutchings, 2006). However, we cannot exclude the possibility that sounds are involved in interspecific communication. Currently, except for in the distress situation, little is known about the use of sound in the genus *Haemulon*. As in many other fish species, negative correlations exist between some acoustic features and body size of the individuals (e.g. Bertucci et al., 2012; Colley et al., 2009; Crawford et al., 1997). It suggests that acoustic signals carry information on the sender's size. Further field recordings are needed to appreciate the diversity of acoustic signals in this fish. Identifying the different social roles of these sounds and how conspecific individuals use them (e.g. as alarm calls or aggregation signals) need to be experimentally tested in behavioral experiments.

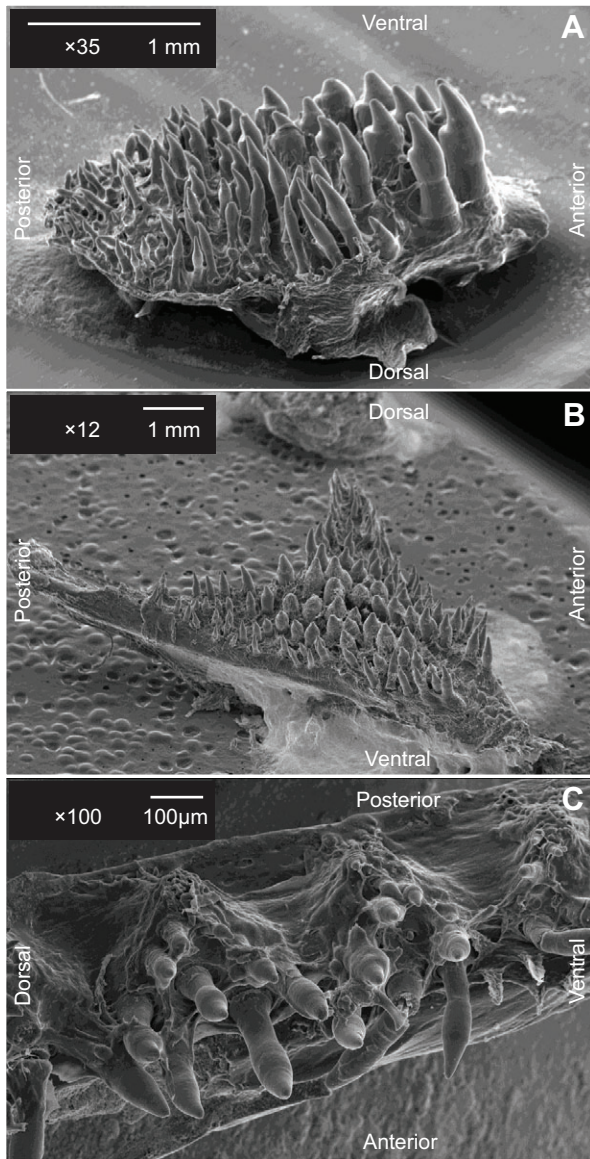
### Sound-production mechanism

Results of our high-speed X-ray video recordings allowed the visualization of the mechanism suggested by Burkenroad (Burkenroad, 1930). Grunts are indeed the result of the teeth scratching between the UPJ and the LPJ (ceratobranchial 5). The teeth of ceratobranchial 4 are also involved in the mechanism by scratching against the most external teeth of the UPJ. Fourth ceratobranchials are usually not part of the LPJ, mainly because they do not have teeth in other species. According to Wainwright (Wainwright, 1989b), simultaneous contraction of transversus ventralis anterior that unites left and right ceratobranchials 4 and transversus ventralis posterior that unites left and right ceratobranchials 5 should allow these two bones to act as a single-toothed element. Moreover, the width of the virtual plate composed of ceratobranchials 4 and 5 corresponds roughly to that of the UPJ. The description of the movements of the pharyngeal jaws during sound production highlights the fact that it corresponds to the cyclic jaw movement patterns made during food processing. During feeding, this mechanical action crushes food and brings it to the oesophagus (Wainwright, 1989a; Wainwright, 1989b). The description of the movements allowing pharyngeal transport (Wainwright, 2005) applies here. At the start of each cycle the UPJ

moves posteriorly and ventrally until it meets the LPJ. During the recovery stroke the UPJ moves dorsally before also recovering anteriorly, so that the overall cycle does not involve the jaw exactly retracing its path. LPJ motion is more restricted than UPJ movement. The LPJ cycle involves posterior retraction that peaks before the UPJ reaches its most posterior and ventral position. The similarity of sounds produced during food processing and in distress situation confirms the hypothesis that the kinematic patterns are similar. There are, however, some differences because the duration and period of distress calls are longer than food-processing sounds. This suggests that the same mechanism could be used but at different speeds. Electromyography on the pharyngeal muscles should be performed to confirm the similarity of the motor patterns. Many sounds are emitted during feeding events in fishes (Ladich and Fine, 2006; Lagardère and Mallekh, 2000; Longrie et al., 2009; Phillips, 1989; Scholz and Ladich, 2006) but they are (*a priori*) unlikely to be involved in communication: they are generally only a byproduct of the food processing. The mechanism of sound production could be an example of exaptation. This term refers to a functional character previously shaped by natural selection for a particular function and that is co-opted for a new use that enhances fitness. In the case of *Haemulon*, we hypothesize that the sounds made during food processing are secondarily selected to support acoustic communication. This hypothesis of exaptation between feeding mechanism and sound production has already been formulated by Parmentier et al. (Parmentier et al., 2007) for the slam jaw mechanism in the clownfish *Amphiprion clarkii* and in the piranha *Pygocentrus nattereri* in which some sounds are produced when a fish snaps its jaws to bite a conspecific (Millot et al., 2011).

The structure and role of teeth also deserve further investigation. Scratching of teeth can cause damage to the enamel. A higher level of fluorine at the tip than at the base and in the middle of the pharyngeal jaw teeth is usually found in vertebrates (Kerebel and Le Cabellec, 1980; Miake et al., 1991; ten Cate and Featherstone, 1991). We cannot argue that *H. flavolineatum* have developed specific responses to scratching based on the chemical components of their teeth. However, enamel erosion reveals that ceratobranchial 4 teeth are subject to constraints, i.e. repeated scratching, and their higher levels of fluorine could strengthen their enamel. This might result from specialisation in the use of pharyngeal arches in sound production. Interestingly, ridges on the dorsal process of the pectoral spine of the channel catfish (*Ictalurus punctatus*), i.e. the structures involved in stridulatory sound production, were also observed to become worn over time (Fine et al.,





**Fig. 6. Classical secondary electron imaging of the jaw in *H. flavolineatum*.** (A) Left upper pharyngeal jaw. (B) Lower pharyngeal jaw (dorsal view). (C) Left ceratobranchial 4 (dorsal view).

1996; Fine et al., 1999). In addition, the teeth of pharyngeal jaws are attached to bones by means of ligaments (data not shown). This attachment, which is also observed in other fishes (e.g. Herold, 1975), may provide flexibility to resist tooth fracture (Huysseune and Sire, 1998) and allow the teeth to maintain some mobility in order to vibrate by friction (Koussoulakou et al., 2009).

In conclusion, the sound-producing mechanism of *H. flavolineatum* seems to correspond to movements made during food processing. As a result, sound production is probably an exaptation of the food-processing mechanism in this species. These sounds appear to be able to carry information about the size of the emitters but the biological role of these signals remains to be fully identified.

## MATERIALS AND METHODS

### Fish

*Haemulon flavolineatum* were purchased from a local supplier (Aqua Garden Center, Neupré, Belgium) and maintained in groups of 3–4

individuals in two holding tanks (40×110×40 cm). Each holding tank was equipped with an external filter (Pico Filter, Hydor USA, Sacramento, CA, USA) an aeration device, sand substratum and artificial rocks and algae as shelters. The temperature was maintained at 25°C, range 2°C, by an internal heater (Sera, Heinsberg, Germany) on a 12 h:12 h light:dark cycle. Fish were fed three times a week with a mixture of chopped mussels and shrimps. Individuals were identified by their standard length (SL, from the tip of the head to the basis of caudal peduncle), total length (TL, from the tip of the head to the tip of caudal fin) and different marks on their body. The gender of individuals was unknown.

### Sound analysis

Recordings were performed in an acoustically insulated room in order to minimize background noise. The experimental apparatus consisted of an aquarium (41×99×40 cm) containing a filter, an aeration device, an internal heater and a sand substratum. The apparatus was positioned on a shelf covered with a 40-mm-thick Styrofoam panel, a 200-mm-thick layer of rock wool and placed on top of a metal grid to reduce vibrations transmitted from the floor. All electrical devices were switched off during the recordings.

Fish were first net-captured and then handheld vertically, at a distance of ~10 cm from a hydrophone (HTI-96-Min; sensitivity:  $-164.4$  dB re.  $1 \text{ V } \mu\text{Pa}^{-1}$ ; flat frequency response range between 2 Hz and 30 kHz) (High Tech, Long Beach, CA, USA) positioned in the middle of the tank (15 cm above the bottom) and connected to a TASCAM DR-07 portable digital recorder (TEAC, Wiesbaden, Germany). Fish usually uttered sounds in this position; if not, they were gently shaken in order to provoke sound production. Sounds were digitized at 44.1 kHz (16-bit resolution) and analyzed with Avisoft-SASLab Pro version 5.2.07 software (Avisoft Bioacoustics, Glienicke, Germany). The resonant frequency of the tank was calculated as 3.1 kHz, using an equation in Akamatsu et al. (Akamatsu et al., 2002). Consequently, a band-pass filter between 50 Hz and 3.1 kHz was applied to all recordings.

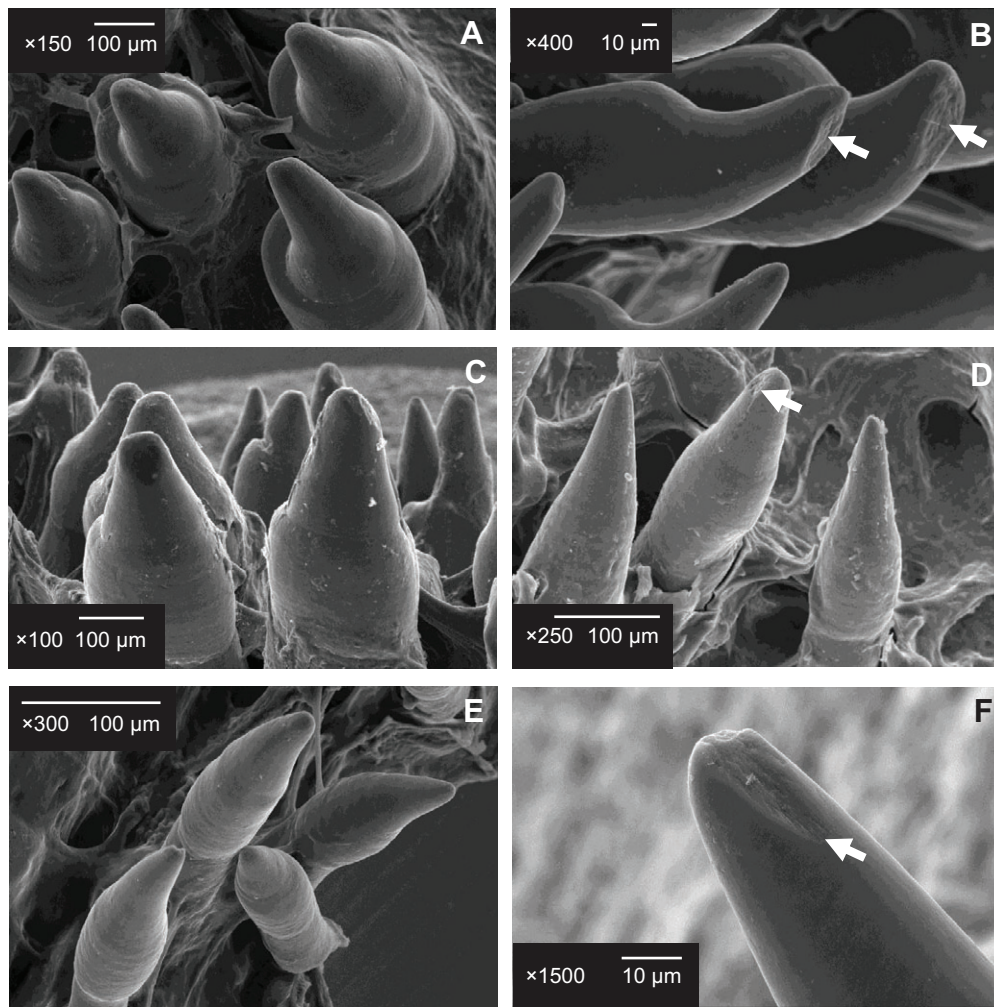
Sounds consisted of series of grunts and the following acoustic features were measured: the grunt series duration (from the beginning of the first grunt to the end of the last grunt), the number of grunts in a series, the grunt duration, the intergrunt interval (from the end of a grunt to the start of the subsequent one), the grunt period, the grunt peak frequency, the number of pulses detected within a grunt and the pulse period (average peak-to-peak interval between two consecutive pulses in a grunt). Temporal features were measured from oscillograms whereas peak frequencies were obtained from logarithmic power spectra (Hamming window, Fast Fourier Transform FFT). Sound intensity (dB re.  $1 \text{ V } \mu\text{Pa}^{-1}$ ) was measured by means of a Brüel and Kjaer 2610 measuring amplifier (Brüel and Kjaer, Naerum, Denmark; fast averaging time weighting, 22.4 Hz high-pass frequency filter) and a Brüel and Kjaer 8101 hydrophone. In order to collect a sufficient number of sounds for analysis, the experiment was divided into three series of 5 min recordings separated by 10 min of rest and was replicated twice for each individual.

Sounds were also recorded during feeding. The hydrophone (HTI-96-Min; sensitivity:  $-164.4$  dB re.  $1 \text{ V } \mu\text{Pa}^{-1}$ ; flat frequency response range between 2 Hz and 30 kHz) was placed in the storage tank 20 minutes prior to providing food close by. The hydrophone was connected to a TASCAM DR-07 recorder and the same acoustic features as above were measured.

### Hearing

Hearing was measured using the auditory-evoked potential (AEP) technique, which is an electrophysiological method for measuring hearing thresholds in fish and other vertebrates (Kenyon et al., 1998). Electrodes inserted subdermally in proximity to the brainstem, directly measure bulk neural responses generated in the VIIIth nerve and brain in response to sounds (Corwin et al., 1982). Signal averaging was used to filter the evoked potential signal from background noise.

The experimental set-up in the present study was similar to that used by Parmentier et al. (Parmentier et al., 2011). During the experiment, individuals ( $N=8$ ) were secured in a vertical cylindrical steel vessel (115 cm high, 22 cm diameter) filled with saltwater maintained at 26°C. An UW-30 underwater speaker (Lubell Labs, Columbus, OH, USA) was placed on the bottom. The apparatus was located in an acoustically insulated room and four anti-vibration floor mounts (51700 Series, Tech Products, Philadelphia,



**Fig. 7. High-magnification classical secondary electron imaging of teeth in *H. flavolineatum*.** (A) Central teeth of the upper pharyngeal jaw. (B) Outer teeth of the upper pharyngeal jaw. (C,D) Teeth in the lower pharyngeal jaw. (E,F) Teeth in ceratobranchial 4. Traces of erosion are indicated by arrows.

PA, USA) were placed under each corner of the base of the steel tube. Fish were wrapped in a fine-mesh steel screen maintained 10 cm below the surface by a clamp. Sub-dermal stainless steel needle electrodes (Rochester Electro-Medical, Lutz, FL, USA) were used for recording the AEP signal. An electrode was inserted about 1 mm into the head, over the otic region. The reference electrode was placed within the epaxial musculature of the fish and a ground electrode was placed in the water close by.

A Tucker-Davis Technologies (TDT, Alachua, FL, USA) AEP workstation was used to generate sound stimuli and record brain responses (Egner and Mann, 2005). TDT SigGen and BioSig software were used to generate sound stimuli with an RP2.1 enhanced realtime processor, a PA5 programmable attenuator to control sound level, and a power amplifier (Hafler Trans Ana P1000 110 W professional power amplifier; Tempe, AZ, USA) before being sent to the UW-30 underwater speaker (Lubell Labs, Columbus, OH, USA). Stimuli consisted of 50-ms-pulsed tones gated with a Hanning window. The phase of the tone was alternated between presentations to minimize electrical artefacts from the recordings. Acoustic

stimuli were calibrated with a Brüel and Kjær 8101 hydrophone (sensitivity  $-184$  dB re.  $1 \text{ V } \mu\text{Pa}^{-1}$ ; bandwidth 0.1 Hz to 200 kHz; Brüel and Kjær, Nærum, Denmark) connected to the RP2.1. During calibration, the hydrophone was positioned near the fish position in the experimental setup, and the sound levels were measured with BioSig, without phase alternation. During each trial, nine different frequencies were presented: 150, 300, 450, 600, 750, 900, 1050 and 1200 Hz. Sound levels at each frequency were presented at up to 164 dB re.  $1 \text{ } \mu\text{Pa}$  and were decreased in 6 dB steps until a threshold level was determined. Evoked potentials recorded by the electrode were fed through a TDT HS4-DB4 amplifier ( $\times 10,000$  gain) connected to an RP2.1, routed into the computer and averaged by BioSig software. Hearing thresholds were determined using power spectra calculated using a 4096-point FFT (fast Fourier transform) for all AEP waveforms and were analyzed for the presence of peaks at twice the frequency of the stimulus that were at least 3 dB above background levels. AEP thresholds were defined as the lowest sound level at which significant FFT peaks for the dominant frequency were apparent.

**Table 1. Relative atomic percentage of the four main elements in pharyngeal tooth enamel of *Haemulon flavolineatum***

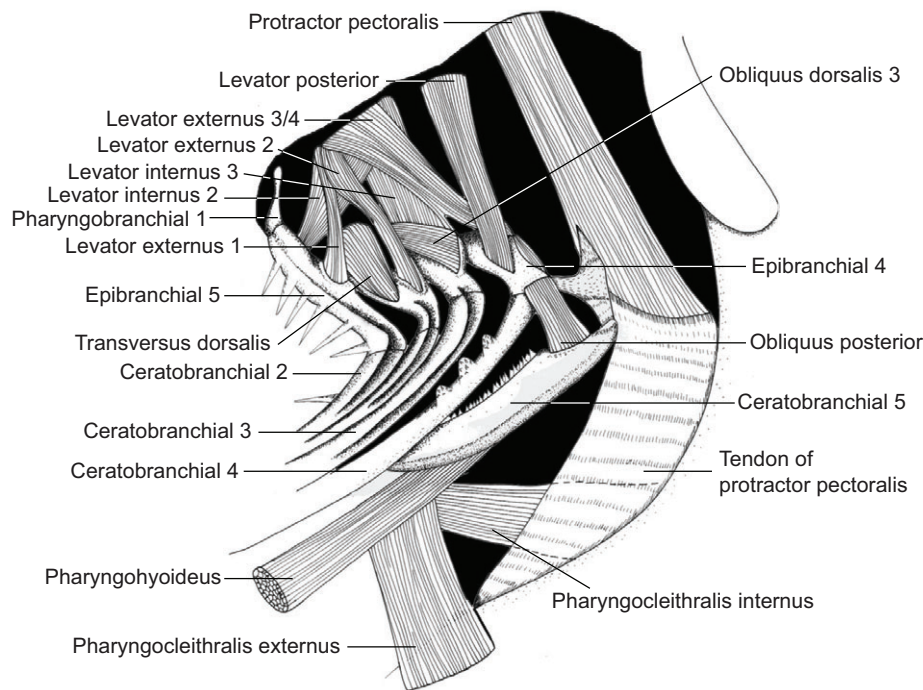
	UPJ	LPJ	CB4
Calcium	21 $\pm$ 5	18 $\pm$ 3	17 $\pm$ 3
Phosphorus	12 $\pm$ 3	11 $\pm$ 2	10 $\pm$ 2
Fluorine	1.0 $\pm$ 0.6	1.4 $\pm$ 0.7	1.8 $\pm$ 0.6
Magnesium	0.3 $\pm$ 0.1	0.3 $\pm$ 0.1	0.2 $\pm$ 0.1

Pooled values (means  $\pm$  s.d.) obtained on bulk samples and polished slices. UPJ, upper pharyngeal jaw; LPJ, lower pharyngeal jaw; CB4: ceratobranchial 4.

#### High-speed X-ray video recordings during sound production

To visualize the movements of the pharyngeal jaw apparatus during sound production, barium sulphate powder was applied onto the upper and lower pharyngeal jaws using a small brush. This radio-opaque powder allowed an increase in the contrast of those bony structures on videos. Fish were filmed at 500 frames  $\text{s}^{-1}$  with a Redlake MotionPro high-resolution digital camera (1280 $\times$ 1024 pixels; Redlake, San Diego, CA, USA) attached to the image intensifier of a Philips Optimus M200 X-ray system (Royal Philips Electronics, Eindhoven, The Netherlands). X-rays were generated at 50 kV and the fish were filmed during sound production in air. Two recordings were produced on two individuals, filmed laterally. The Midas player





**Fig. 8.** Left lateral line drawing of the muscles of the branchial basket of *H. flavolineatum*.

software (Xcitex, Cambridge, MA, USA) was used for data acquisition and to follow the movements of the pharyngeal jaws during sound production.

#### Observation of the pharyngeal jaw apparatus

After recording, seven individuals were euthanized with an overdose of tricaine methanesulfonate (MS-222). Fish were then dissected under a binocular microscope (Leica Wild M10, Leica, Solms, Germany) to observe the branchial basket morphology. Pharyngeal jaws and ceratobranchial 4 of five individuals were placed in 99% ethanol and split into three sets. The first set (from two individuals) was prepared for classical SEM surface examination. The samples were thus critical-point dried with CO<sub>2</sub>, mounted on aluminium stubs and platinum sputter-coated (20 nm) in a Balzers SCD-030 unit (Oerlikon Balzers Coating, Balzers, Liechtenstein). A second set of samples (from two individuals) was prepared for low-vacuum SEM observation and elemental X-ray microanalysis of enamel at the teeth surface. These samples were critical-point dried and directly mounted on glass slides with carbon tape. The third set of samples (from one individual) was used to prepare polished thin slices. These samples were embedded in AGAR low viscosity resin (AGAR, R1078), sectioned with metal saw, mounted on glass slides, polished with SiC-sand papers (ESCIL) of decreasing grain size (down to PSA 4000), then mirror polished with non-aqueous 1 µm diamond suspension (ESCIL IPS-1MIC) and carbon coated in a Balzers MED-010 evaporator (Oerlikon Balzers Coating, Balzers, Liechtenstein). The samples were examined either in a SEM Jeol JSM-840A (JEOL, Tokyo, Japan) for classical SE imaging or in a FEI ESEM-FEG XL30 (FEI Europe, Eindhoven, The Netherlands) for GSE/BSE (gaseous secondary electron/back scatter electron) imaging of bulk samples under low-vacuum conditions and BSE imaging of polished slides under high-vacuum conditions.

The elemental composition of the enamel was measured at the tip of 10 randomly selected teeth from the UPJ, LPJ and ceratobranchial 4 by means of an energy-dispersive X-ray microanalyzer Bruker 125eV (Bruker Nano, Germany). Atomic percentages (atm %) were calculated using the ESPRIT software (Bruker Nano, Germany).

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#### Competing interests

The authors declare no competing financial interests.

#### Author contributions

F.B., L.R. and E.P. conceived and designed the experiments; F.B. and L.R. performed audio recordings, data were analyzed by L.R.; L.R. performed AEP experiments and analyzed the data; F.B., L.R.; S.V.W. and E.P. performed X-ray video recordings; F.B. analyzed X-ray movies; F.B., L.R. and E.P. performed dissections; L.R. and P.C. performed electromicroscopy and ultrastructural analysis of teeth; E.P. described the musculature of the branchial basket; F.B., L.R. and E.P. wrote the paper.

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