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RESEARCH ARTICLE

Further insight into the sound-producing mechanism of clownfishes: what structure is involved in sound radiation?

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SUMMARY

It was recently demonstrated that clownfishes produce aggressive sounds by snapping their jaw teeth. To date, only the onset of the sound has been studied, which raises the question, what structure is involved in sound radiation? Here, a combination of different approaches has been used to determine the anatomical structure(s) responsible for the size-related variations observed in sound duration and frequency. Filling the swimbladder with physiological liquid specifically modified size-related acoustic features by inducing a significant decrease in pulse duration of approximately 3 ms and a significant increase in dominant frequency of approximately 105 Hz. However, testing the acoustics of the swimbladder by striking it with a piezoelectric impact hammer showed that this structure is a highly damped sound source prevented from prolonged vibrations. In contrast, the resonant properties of the rib cage seems to account for the size-related variations observed in acoustic features. For an equivalent strike on the rib cage, the duration and dominant frequency of induced sounds changed with fish size: sound duration and dominant frequency were positively and negatively correlated with fish size, respectively. Such relationships between sonic features and fish size are consistent with those observed in natural sounds emitted by fish. Therefore, the swimbladder itself does not act as a resonator; its wall just seems to be driven by the oscillations of the rib cage. This set of observations suggests the need for reassessment of the acoustic role of swimbladders in various fish species.

Key words: *Amphiprion clarkii*, sound production, swimbladder, rib cage, resonance.

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INTRODUCTION

Among vertebrates, teleost fishes have evolved the largest diversity of sonic organs, including stridulation of bony structures, plucking of stretched tendons and contraction of intrinsic and extrinsic sonic muscles that excite swimbladder vibration by deformation of its wall (Tavolga, 1971; Demski et al., 1973; Kratochvil, 1978; Fine et al., 1997). Damselfishes (Pomacentridae) are among the best-studied group in terms of sound production, with more than 20 species belonging to seven genera reported as sound emitters (Takemura, 1983; Myrberg et al., 1986; Lobel and Mann, 1995; Amorim, 1996; Santiago and Castro, 1997; Lobel and Kerr, 1999; Picciulin et al., 2002; Parmentier et al., 2005; Parmentier et al., 2006a; Maruska et al., 2007; Parmentier et al., 2010). Within this family, clownfishes (*Amphiprion* spp.) are well known to produce aggressive sounds during agonistic interactions (Verwey, 1930; Allen, 1972; Chen and Mok, 1988; Colleye et al., 2009).

Although sound-producing movements have been determined in the yellowtail clownfish *Amphiprion clarkii*, the mechanism of sound production remains incomplete. Indeed, only the onset of the sound has been identified; sound is initiated by teeth collisions caused by rapid jaw closure, which is attributed to a sonic ligament between the hyoid bar and the internal mandible (Parmentier et al., 2007). Thus there exists a need for further understanding of sound radiation in clownfishes, especially regarding the anatomical structure(s) involved. Some acoustic features such as dominant frequency and pulse duration are signals directly related to body size in clownfishes (Colleye et al., 2009; Parmentier et al., 2009). Such variations suggest that both acoustic features are subject to a morphological constraint.

Classically, the character of sounds changes during the development of an organism, mainly in relation to body size, and probably to the development of the sound-generating structures. This general phenomenon has been widely investigated in many groups of animals and is largely based on resonance. For example, Würdinger (Würdinger, 1970) showed that in the greylag goose, *Anser anser*, the dominant frequency of sounds is correlated negatively to the size of the tympaniform membranes; in contrast, the main frequencies are known to decrease with increasing body size in frogs (Davies and Halliday, 1978; Ryan, 1985). Similar correlations are known in several groups of fishes that produce pulsed sounds. In the grey gurnard, *Eutrigla gurnardus*, sound production differs between small juveniles and large adults during competitive feeding (Amorim and Hawkins, 2005). In the croaking gourami, *Trichopsis vittata*, sound duration increases during ontogeny whereas dominant frequency decreases (Henglmüller and Ladich, 1999). In weakfishes (Connaughton et al., 2000), pearlfishes (Parmentier et al., 2006b), damselfishes (Myrberg et al., 1993; Lobel and Mann, 1995) and clownfishes (Colleye et al., 2009; Parmentier et al., 2009), pulse duration increases and dominant frequency decreases in larger fishes. Grey gurnards and weakfishes produce sounds by contracting sonic muscles attached to the swimbladder. As such, variations in dominant frequencies and pulse durations may result from muscle-scaling effects (Wainwright and Barton, 1995), as larger fishes with larger sound-producing muscles would take

longer to complete a muscle twitch, resulting in longer pulse durations and lower dominant frequencies (Connaughton et al., 2000). In pearlfishes, the sonic muscles pull the anterior bladder and thereby stretch a thin fenestra. Sound is generated when the tension trips a release system that causes the fenestra to snap back to its resting position. Parmentier et al. (Parmentier et al., 2006b) hypothesized that the sound frequency is determined by the snapping fenestra interacting with an overlying bony swimbladder plate whose size is related to fish size. In contrast, croaking gouramis generate sounds by two enhanced pad-like tendons of the fourth and fifth pectoral fin ray (Kratochvil, 1978). Henglmüller and Ladich (Henglmüller and Ladich, 1999) suggested that the frequency changes during the ontogeny of croaking gouramis are related to the growth of the suprabranchial chamber (i.e. an airbreathing cavity dorsally of the gills in all labyrinth fishes); this structure is thought to be the main resonating structure in this species. The swimbladder has also been thought to function as a resonator that amplifies and changes the quality of sounds produced by stridulation in grunts and triggerfishes (Burkenroad, 1930; Salmon et al., 1968), but this hypothesis has never been experimentally verified. Thereby, such examples raise questions regarding the resonating structure involved in the sound-producing mechanism of clownfishes. However, it was recently shown in the oyster toadfish (Fine et al., 2009) and in the red-bellied piranha (Millot et al., 2011) that the swimbladder was a highly damped structure, prevented from sustained resonant vibrations.

The present study aims to determine the anatomical structure(s) responsible for the size-related variations observed in sound duration and frequency. More precisely, we conducted a series of experiments to determine the major acoustic radiator in the sound-producing mechanism of the yellowtail clownfish *A. clarkii*.

MATERIALS AND METHODS

Capture and holding of fish

Eighteen *Amphiprion clarkii* Bennett 1830 [standard length (SL) 55–108 mm] were collected by SCUBA diving on the fringing reef around Nakijin village (26°40′23″N–127°59′48″E, Okinawa, Japan) during May and June 2009. Specimens were caught using a fish net while they swam among the tentacles of their host sea anemone. All fish were then brought back to Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus, where they were transferred to a community tank (3.5×2.0×1.2 m) filled with running seawater at ambient temperature (28 to 30.5°C). This community tank was compartmentalised for separating fish into pairs. All fish were kept under a natural photoperiod and were fed once daily with food pellets *ad libitum*.

Recording method

Recordings were made in a smaller glass tank (1.2×0.5×0.6 m) filled with running seawater maintained at 28°C by means of a GEX cooler system (type GXC-201x, Osaka, Japan) for recording under standardised conditions. Following a published protocol (Colleye et al., 2009), one mating pair and its host were placed in the centre of the tank for an acclimation period of ~2 days. The resident pair was then challenged by introduction of conspecific intruders into the tank. Sounds were produced when the intruder approached the territory (the sea anemone) defended by the resident pair. The coloration of the caudal fin (Hattori and Yanagisawa, 1991) was used to identify males and females, which enabled the sound emitter to be identified. Recordings lasted approximately 15 min, after which the intruder was removed and placed back in the community tank until the following session.

Sound recording experiments: effects of anaesthesia and swimbladder filling

The effects of anaesthesia (negative control) and swimbladder filling on the acoustic features of aggressive sounds were examined. All experimental and animal care protocols followed all relevant international guidelines and were approved by the ethics commission of the University of Liège.

Nine specimens (55–108 mm SL) were placed into a dedicated container with 50 mg l^{-1} of tricaine methanesulphonate (MS-222) in seawater. Fish were considered deeply anaesthetised when they turned upside down. After anaesthesia, fish were placed back into the recording tank, and after a 3 h recovery period, fish were again recorded by introducing conspecific intruders into the tank.

After a period of 5 days, the role of the swimbladder in sound modulation was tested by rapidly filling it with physiological liquid (NaCl 9‰) under general anaesthesia in 50 mg l^{-1} MS-222. Once deeply anaesthetised, fish were placed on wet cotton in a dissecting tray. Two hypodermic needles (27 gauge; 0.4×20 mm) were inserted subcutaneously into the swimbladder. The first needle was coupled to a 5 ml syringe used to fill the swimbladder and the second was used to drain the overflow, indicating when the swimbladder was completely filled. Fish were then placed back into the recording tank for a 3 h recovery period. Aggressive sounds were again recorded by challenging fish with conspecific intruders.

After carrying out both experiments, all fish were euthanised with an overdose of MS-222 (500 mg l^{-1}). Specimens were fixed in 7% buffered seawater formalin for approximately 2 weeks before being transferred to 70% ethanol for storage.

Sound analysis

Sound recordings were made using a Brüel & Kjær 8106 hydrophone (Nærum, Denmark; sensitivity: –173 dB re. 1 V µPa^{-1}) connected *via* a Nexus^{TM} conditioning amplifier (type 2690, Brüel & Kjær) to a Tascam HD-P2 stereo audio recorder (Wiesbaden, Germany, recording bandwidth: 20 Hz to 20 kHz ± 1.0 dB). This system has a flat frequency response over a wide range between 7 Hz and 80 kHz. The hydrophone was placed just above the sea anemone (±5 cm).

One hundred pulse units were analysed per individual, and for each of the experimental conditions (i.e. before anaesthesia, after anaesthesia and after swimbladder filling). Each pulse unit was digitized at 44.1 kHz (16 bit resolution) and analysed with AviSoft-SASLab Pro 4.33 software [Avisoft Bioacoustics, Berlin, Germany; 1024 point Hanning windowed fast Fourier transform (FFT)]. Recording in small tanks induces potential hazards because of reflections and tank resonance (Akamatsu et al., 2002). The resonant frequency of the recording tank was determined as 2.05 kHz using a relevant equation from Akamatsu et al. (Akamatsu et al., 2002), and a low-pass filter of 2.05 kHz was applied to all sound recordings. Temporal features were measured from the oscillograms whereas peak frequency was obtained from power spectra (filter bandwidth 300 Hz, FFT size point 256, time overlap 96.87% and a flat top window). Aggressive sounds consist of a single pulse unit that can be emitted alone or in series. Hence, the following sonic features were measured: number of pulses in a series, pulse duration in ms (the time interval between the onset of one pulse and its end), pulse period in ms (the average peak to peak interval between consecutive pulse units in a series) and dominant frequency in Hz (frequency component with the most energy).

In addition to these sonic features, the number of oscillation cycles and the quotient of the stimulation frequency that produced the maximal amplitude response divided by the bandwidth, or frequency

range, across which the amplitude of sound produced by the resonator was within 3 dB of the maximal amplitude (Q_{3dB}) (see Bradbury and Vehrenkamp, 1998) were calculated to determine the effects of the swimbladder filling experiment on these two sonic characteristics. Note that aggressive sounds produced simultaneously by several fish were excluded from acoustic analyses.

Swimbladder volume estimation

Swimbladder volume was determined using a cast method (adapted from Barnett and Bellwood, 2005). Ten specimens (55–108 mm SL) were euthanised with MS-222 (500 mg l^{-1}) and fixed in 7% buffered seawater formalin for approximately 2 weeks before being transferred to 70% ethanol for storage. They were then transversally sectioned at the level of the base of the anal fin in order to expose the posterior part of the swimbladder. After punching the swimbladder wall, an internal cast was formed by injecting specific silicone rubber (Vandamme®, Liège, Belgium) using a 5 ml syringe. Care was taken to inject silicone with constant pressure until the entire swimbladder was full. The silicone was left to partially set for 1 h with the fish maintained on ice before being transferred to 70% ethanol. After the silicone had set for one night, the swimbladder cast was removed. The volume of the silicone cast was determined by water displacement in a 500 ml volumetric flask filled with water to the graduation mark. The displaced water volume was then measured using a 1 ml volumetric pipette (accuracy: 0.01 ml). Triplicate measurements were taken for each silicone cast on separate days and the mean was calculated. The coefficient of variation between triplicates was less than 2%. The calculated volume was then used to obtain the equivalent radius of a sphere. This radius was used to calculate the resonant frequency (F) of the swimbladder according to the formula for an underwater bubble (Minnaert, 1933; Weston, 1967):

$$F = \left(\frac{1}{2\pi R}\right)\sqrt{\frac{3\gamma P}{\rho}}, \quad (1)$$

where R is the radius (cm), γ is the ratio of specific heats (=1.402), P is the pressure (atmospheric pressure + hydrostatic pressure) and ρ is the water density. To compare with natural sounds emitted by fish, the resonant frequency was calculated for a depth of 30 cm because fish were mostly situated at this depth, swimming among the tentacles of the sea anemone, just above its oral disc.

Morphological study

Specimens whose swimbladder was cast were dissected and examined with a Leica M10 stereoscopic microscope (Leica, Wetzlar, Germany) coupled to a camera lucida. Five specimens were cleared and stained with Alizarin Red S according to Taylor and Van Dyke's method (Taylor and Van Dyke, 1985) in order to visualise osseous structures. The location of the swimbladder in the body cavity and its position in relation to surrounding structures (vertebrae, ribs and parapohyses) were described. The osteology follows that of Patterson and Johnson (Patterson and Johnson, 1995).

In addition, three smaller specimens (35–41 mm SL) were purchased from a pet store and euthanised with an overdose of MS-222 (500 mg l^{-1}). Serial histological cross-sections were made to observe the detailed anatomy of the swimbladder. Samples were fixed in Bouin's fixative solution, dehydrated with ethanol, decalcified, embedded in paraffin wax (Paraplast®, VWR, Leuven, Belgium) and serially cut with a Reichert microtome (Leica, Wetzlar, Germany). Cross-sections (15 µm) were stained with haematoxylin & eosin (Gabe, 1976). The different sections were observed with a Leica DM1000 binocular microscope coupled with a digital camera (Canon Powershot S50, Diegem, Belgium).

Fig. 1. Right lateral view of the skull and the anterior part of the vertebral column of a specimen of *Amphiprion clarkii* [standard length (SL)=85 mm]. The specimen was skeletonised with boiling water to remove flesh tissue and oven dried at 60°C. The blue dot indicates the site struck with the impact hammer. Scale bar, 1 cm.

Sound resonance experiments

Two experiments were performed to determine the structure acting as the acoustic radiator. In the first experiment, three specimens (76–97 mm SL) provided by the Aquarium Oceanopolis (Brest, France) were euthanised with an overdose of MS-222 (500 mg l^{-1}). They were dissected under a Leica M10 stereoscopic microscope to expose the anterior part of the vertebral column and the skull (Fig. 1). Specimens were carefully skeletonised with boiling water to remove all remnants of flesh tissue and oven dried at 60°C (see Fine et al., 1997). To assess the role of the buccal jaws in sound radiation, specimens were placed in a dissecting tray and maintained in a lateral position using modeling clay. The lower jaws were struck (see Fig. 1) with a miniature modal analysis impact hammer (PCB model 086E80, Depew, NY, USA; ×100 gain; with a steel tip, transducer sensitivity 22.62 mV N^{-1}). Note that the force of a hammer strike was calculated using the hammer sensitivity. Sound produced by striking the buccal jaws was recorded with a Sennheiser ME62 microphone (frequency range 20 Hz–20 kHz ± 2.5 dB, sensitivity 32 mV Pa^{-1} ± 2.5 dB; Wedemark, Germany) coupled with a Sennheiser K6 modulatory system onto a Tascam recorder. The microphone was positioned 3 cm above the specimens. All recordings were made within a soundproof booth.

In the second experiment, three specimens (69–96 mm SL) provided by the Aquarium Oceanopolis were euthanised with an overdose of MS-222 (500 mg l^{-1}). They were dissected under a Leica M10 stereoscopic microscope. The swimbladder and surrounding structures (ribs and vertebrae) were completely exposed on one side by incision of the body musculature (Fig. 2). Then, specimens were placed in a dissecting tray and two series of hits were performed with the miniature impact hammer (×100 gain; with a vinyl tip cover, transducer sensitivity 20.80 mV N^{-1}). The force of a hammer strike was also calculated using the hammer sensitivity. Firstly, fish were struck at the level of the fourth rib; vibrations were measured with a Polytec laser Doppler vibrometer including a Vibrometer Controller (OFV 5000, Polytec BVBA, Antwerp, Belgium) and a Vibrometer Sensor Head (OFV 505; sensitivity 5 mm s^{-1} V^{-1}) with the laser beam focussing on retro-reflective laser discs placed on the sixth rib (Fig. 2A). Secondly, fish were placed, belly up in a support, in order to stimulate directly the swimbladder on the anterior part. A retro-reflective laser disc was placed on the posterior part to measure vibration of the swimbladder wall (Fig. 2B). Sounds resulting from

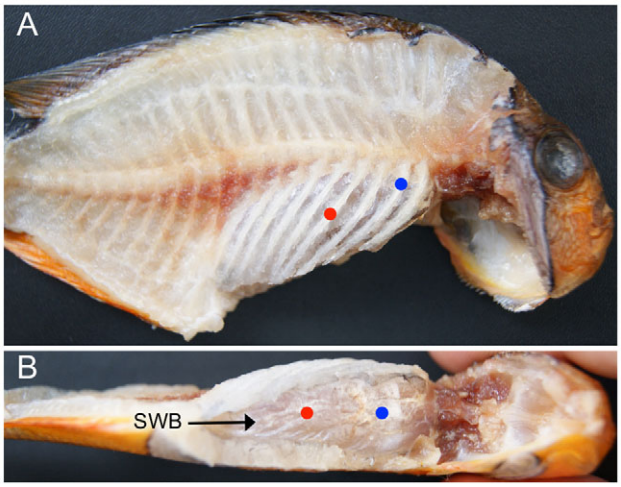

Fig. 2. (A) Right lateral view of a freshly dead specimen of *Amphiprion clarkii* (SL=96 mm) whose body musculature has been dissected. The blue dot indicates the site struck with the impact hammer at the level of the fourth rib, and the red dot is the laser target disc located on the sixth rib where vibration were measured. (B) Ventral view of the same specimen illustrating the site where the swimbladder (SWB) was directly struck with the impact hammer (blue dot), and the laser target disc where vibration was measured (red dot).

the different series of hits were recorded using the same microphone as for buccal jaws experiment. All recordings were made within a soundproof booth. All data were captured using a data acquisition box (Midas, DA Module, BNC Breakout Box, Cambridge, MA, USA). Velocity and duration were measured for the vibration trace, as well as duration for hammer force trace for each fish using LabVIEW SignalExpress 3.0 (National Instruments, Zaventem, Belgium). Vibration trace data from variable force hammer strikes were transformed with a FFT (256 point sample, Hanning window) to measure the peak frequency with the highest energy.

In both experiments, the following sonic variables were analysed: sound duration (ms) was measured from oscillograms whereas dominant frequency (Hz), relative intensity (dB rel.) and Q_{3dB} were obtained from power spectra (filter bandwidth 300 Hz, FFT size point 256, time overlap 96.87% and a flat top window).

Statistical analyses

A Shapiro–Wilk test was used to test the normal distribution of acoustic data. The non-parametric Friedman's test with subsequent Dunn's test for pairwise comparisons was used to compare acoustic features for each of the experimental conditions (i.e. before anaesthesia, after anaesthesia and after swimbladder filling). Additionally, a non-parametric Wilcoxon matched-pairs signed rank test was used to compare data on Q_{3dB} and the number of oscillation cycles before and after swimbladder filling.

An analysis of covariance (ANCOVA) was run to compare linear regressions of peak frequencies of natural sounds produced by fish and the resonant frequencies calculated on the basis of a bubble-like swimbladder of equivalent volume against SL. Note that these values were first ln-transformed because they were exponentially related to fish size.

ANCOVA was also run to compare linear regressions illustrating the effects of variable force hammer hits on the measured parameters in fish of different size. Then, a non-parametric Kruskal–Wallis one-way ANOVA by ranks with subsequent Dunn's test for pairwise comparisons was used to compare data between fish.

All statistical analyses were carried out with STATISTICA 7.1 (StatSoft, Tulsa, OK, USA). Results are presented as means ± s.d. and were considered significant at $P<0.05$.

RESULTS

Sounds were composed of short pulses (<20 ms) emitted alone or in series, and in a narrow band of low frequencies (<1 kHz). The sounds of 55–108 mm (SL) fish ranged in pulse period from 90.0 to 128.6 ms, and in number of pulses per train from 2.9 to 6.4 pulses (Table 1). Pulse duration was positively related to SL, increasing from 10.8 to 19.7 ms whereas dominant frequency was negatively related to SL, decreasing from 700 to 340 Hz (Table 1).

Effects of anaesthesia and swimbladder filling on acoustic features

The comparison of experimental conditions (i.e. before anaesthesia, after anaesthesia and after swimbladder filling) using Friedman's test revealed that experiments induce significant differences in some acoustic features such as pulse duration (χ^2=13.56, d.f.=2, P=0.0003) and dominant frequency (χ^2=13.56, d.f.=2, P=0.0003), but not in pulse period (χ^2=4.667, d.f.=2, P=0.1066) or number of pulses per sound (χ^2=1.556, d.f.=2, P=0.5690; Table 2). Pairwise comparisons

Table 1. Summary (means ± s.d.) of the four acoustic features analysed from 18 live *Amphiprion clarkii*

Standard length (mm)	Pulse duration (ms)	Dominant frequency (Hz)	Pulse period (ms)	Number of pulses
55	10.8±1.3	700±77	114.9±10.1	3.7±1.6
72	13.0±0.7	553±63	104.6±19.3	4.4±3.3
75	13.5±0.9	537±46	125.1±23.5	3.3±1.6
76	13.7±0.8	530±61	95.4±15.2	5.5±2.0
76	13.6±0.9	531±57	110.9±14.5	4.2±2.4
81	14.8±0.6	500±54	90.0±13.3	4.3±1.6
82	14.9±0.7	502±56	101.4±12.4	6.4±2.6
83	14.8±0.9	503±59	101.6±20.4	5.0±1.9
83	14.9±0.8	497±59	103.2±16.4	3.9±2.1
85	15.1±0.6	495±65	128.0±15.5	2.9±1.1
85	14.9±0.8	493±54	118.5±15.4	4.9±2.1
87	15.2±0.8	482±69	106.9±12.9	4.8±1.7
90	15.3±0.9	433±52	128.2±20.3	4.6±1.9
90	15.5±0.9	431±43	128.6±13.5	5.5±2.6
94	15.9±0.7	426±42	115.2±20.2	4.4±2.5
95	15.8±0.7	425±35	106.9±11.9	3.4±1.8
97	15.9±0.9	415±43	124.6±14.3	4.1±2.1
108	19.7±0.9	340±29	120.5±16.3	5.0±2.1

Table 2. Effects of anaesthesia and swimbladder (SWB) filling on the acoustic features in *Amphiprion clarkii*

Acoustic variable	Before anaesthesia	After anaesthesia	After SWB filling	*P*
Pulse duration (ms)	15.04±2.3	15.01±2.3	12.11±2.3	<0.001
Dominant frequency (Hz)	484.7±99.4	483.1±100.1	589.3±100.2	<0.001
Pulse period (ms)	110.8±13.9	113.2±13.2	111.4±12.4	n.s.
Number of pulses	4.7±0.6	4.7±0.8	4.4±0.6	n.s.

Fish (N=9) ranged in standard length from 55 to 108 mm and all recordings were made at 28°C. Results are presented as means ± s.d. of N=100 measurements per fish and P-values refer to the results of the non-parametric Friedman's test (n.s., not significant).

showed that pulse duration (15.04 *vs* 15.01 ms, Dunn's test, $P>0.05$), dominant frequency (484.7 *vs* 483.1 Hz, Dunn's test, $P>0.05$), pulse period (110.8 *vs* 113.2 ms, Dunn's test, $P>0.05$) and number of pulses (4.7 *vs* 4.7, Dunn's test, $P>0.05$; Table 2) were not affected by anaesthesia. In contrast, the swimbladder filling induced a significant decrease in pulse duration of approximately 3 ms (2.93±0.07, N=9) from 15.0 to 12.1 ms (Dunn's test, $P<0.001$; Table 2), and a significant increase in dominant frequency of approximately 105 Hz (104.6±2.71, N=9) from 485 to 589 Hz (Dunn's test, $P<0.001$; Table 2). Overall, it clearly appears that the swimbladder filling specifically acted on size-related acoustic features.

Deeper attention to the oscillograms showed changes in the sound waveform after filling the swimbladder. Although the number of oscillation cycles did not vary (6.53 *vs* 6.55; Wilcoxon matched-pairs signed rank test, P=0.4961), all recorded pulses exhibited a waveform with a decrease in the period of oscillation cycles (Fig. 3B). This observation matched the upward shift observed in peak frequency (see white arrows in Fig. 3C,D). Such changes in acoustic features indicate that sound radiation appeared to be affected by the swimbladder filling. These results were also supported by the change in Q_{3dB}, decreasing from 4.1 to 3.7 (Wilcoxon matched-pairs signed rank test, P=0.0039) after the swimbladder filling.

Swimbladder volume and resonant frequency

Swimbladder volume was exponentially related to fish size (r=0.99, $P<0.0001$; Fig. 4A): the more fish size increased, the more swimbladder volume increased. Natural dominant frequencies produced by fish decreased by an octave from 700 Hz in a 55 mm SL individual with a 0.49 cm^3 swimbladder volume to 340 Hz in a 108 mm SL individual with a 3.85 cm^3 swimbladder volume (Fig. 4A, Table 1). Frequencies emitted by fish were then compared with the resonant frequency calculated on the basis of the radius of a bubble-like swimbladder of equivalent volume (Fig. 4B). Although values were rather close and displayed the same scatter plot (ANCOVA, test for common slopes: $F_{1,16}$=0.0059, P=0.9398), the calculated resonant frequency values were inferior to natural dominant frequencies emitted by fish. This finding strongly suggested that sound frequency is not determined by the natural resonant frequency of the swimbladder.

Morphological study

Generally speaking, the swimbladder of *A. clarkii* is a thin-walled sac located in the dorsal portion of the body cavity and surrounded by bony structures such as vertebrae and ribs. *Amphiprion clarkii* possesses 11 precaudal vertebrae (Fig. 5). The first two each possess a pair of epineural ribs, the second being the longest. From the third to the eleventh vertebrae, the vertebrae possess pairs of ribs and intermuscular bones as well. From the sixth to the eleventh vertebrae, there are pairs of ventral parapohyses on which ribs articulate (Fig. 5). These ribs are dorsally closely attached to the serosa of the abdominal cavity. The anterodorsal part of the swimbladder leans against the vertebral column. From the second to the seventh vertebrae, there is a groove at the midline that makes the swimbladder anteriorly bilobate (Fig. 6). Both lobes surround the

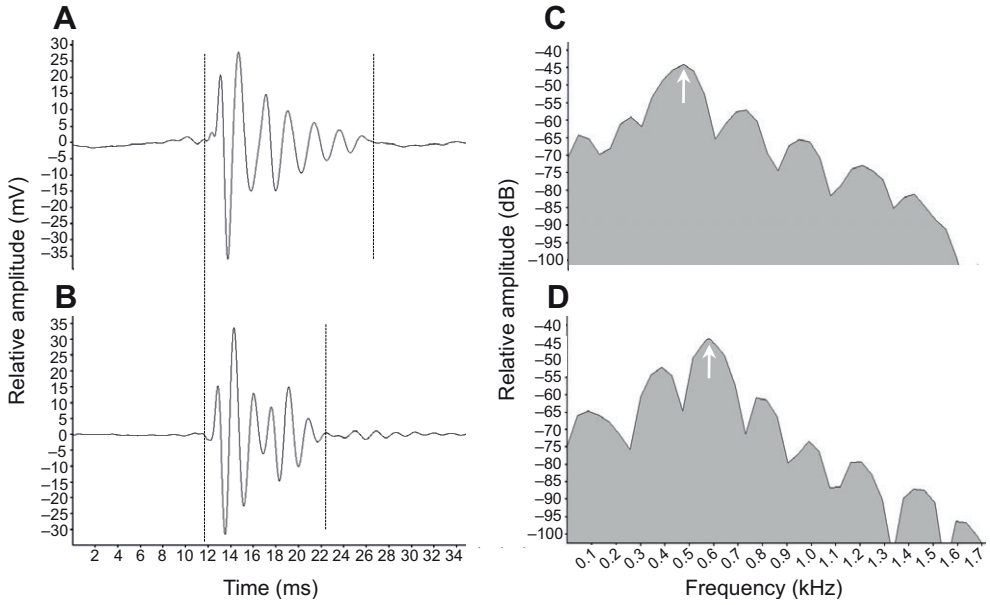

Fig. 3. Oscillogram of one pulse illustrating the difference in sound waveform between an intact *Amphiprion clarkii* (A) and a fish with the swimbladder filled (B). The vertical lines delimit the pulse duration. Power spectra of the same pulses showing the peak frequency (see white arrows) in an intact fish (C) and a fish in which the swimbladder was filled (D).

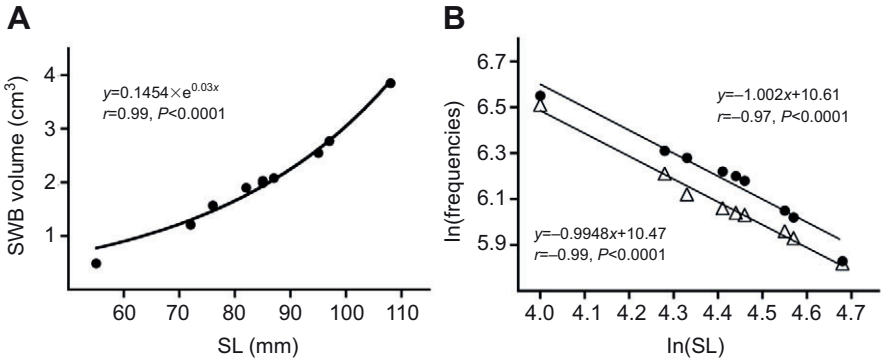

Fig. 4. (A) Relationship between swimbladder volume and fish size (SL) in *Amphiprion clarkii*. Fish (N=10) ranged in SL from 55 to 108 mm. (B) Comparison between dominant frequencies of natural sounds (filled circles) and calculated resonant frequencies (open triangles) in *Amphiprion clarkii*. Note that data were ln-transformed because they were exponentially related to SL. Resonant frequencies were calculated for a depth of 30 cm.

ventral apophysis of the first vertebra (onto which the retractor dorsalis muscle inserts). The dorsocaudal part of the swimbladder is connected to the last four precaudal vertebrae *via* the ventral parapohyses that are more and more elongated (Fig. 5). The swimbladder is laterally delimited by the different pairs of ribs onto which the wall is attached (Fig. 6). The posterior part of the swimbladder presses against the second pterygiophore of the anal fin (Fig. 5). The swimbladder wall is very thin (~20 µm) and histological cross-sections cannot clearly identify all of its layers (Fig. 7). The tunica interna of the swimbladder is a cuboidal epithelium. The tunica externa contains a thicker fibrous layer, which is more developed ventrally. At this level, the tunica externa is also doubled by the coelomic epithelium lining the abdominal cavity. Moreover, the tunica externa is also connected to a second fibrous layer lining the myosepta in relation with the ribs (Fig. 7C). As a result, the movements of the ribs have a direct influence on the whole fibrous layer.

Sound resonance experiments

Buccal jaws

Striking the buccal jaws using the impact hammer generated a waveform with an asymmetrical half-cycle with a shorter rise than fall time (6.3 and 8.5 ms, respectively, N=45; Fig. 8). The rise time represents the period during which the hammer was transferring energy to the jaws. It started with a relatively low force as pressure on the jaws increased and then continued with a steeper slope. The fall time represents the time when the hammer bounced back from the surface of the jaws. The slope of the fall time decreased just before return to the baseline.

The sound waveform induced by the hammer strike on buccal jaws displayed differences with that of natural sounds emitted by fish. It suddenly disappeared into the background noise without displaying decay (see Fig. 8). Comparison of hammer and sound traces indicated that the onset of the hammer trace and the sound waveform were closely aligned (Fig. 8). Generally speaking, hammer duration (rise and fall time) closely corresponded to sound duration.

Changes in hammer force (i.e. harder hits) caused significant changes in sound parameters (Fig. 9). Sound duration increased from 8.9 to 13.5 ms (r=0.87, P<0.0001, N=45), and sound amplitude increased from –64.6 to –58.1 dB rel. (r=0.87, P<0.0001, N=45) with harder hits. Inversely, peak frequency dropped from 1650 to 1100 Hz (r=–0.84, P<0.0001, N=45) and Q_{3dB} decreased from 11.2 to 5.2 (r=–0.87, P<0.0001, N=45) with harder hits.

Comparisons of values calculated from regressions of variable force hammer strikes in three specimens of different size showed that hammer strikes had the same effects on sound duration (ANCOVA, test for common slopes: $F_{2,39}$=0.1842, P=0.8325), sound amplitude (ANCOVA, test for common slopes: $F_{2,39}$=0.3754, P=0.6894), sound frequency (ANCOVA, test for common slopes: $F_{2,39}$=0.1678, P=0.8461) and Q_{3dB} (ANCOVA, test for common slopes: $F_{2,39}$=2.9250, P=0.0652), regardless of fish size. In addition, there were no significant differences between individuals of different

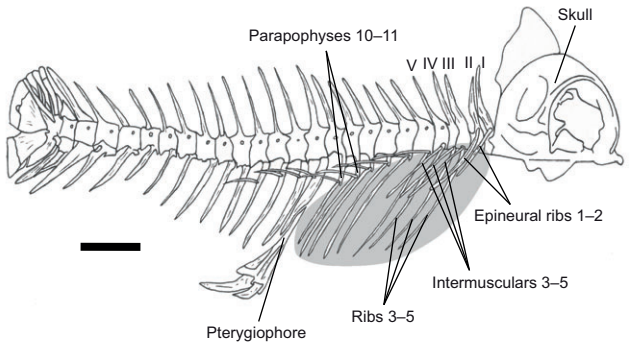

Fig. 5. Lateral view of the skull, axial skeleton ad hypural structure in *Amphiprion clarkii* showing the location of the swimbladder (in grey) in relation to the rib cage. Roman numerals refer to the first five vertebrae. Scale bar, 5 mm.

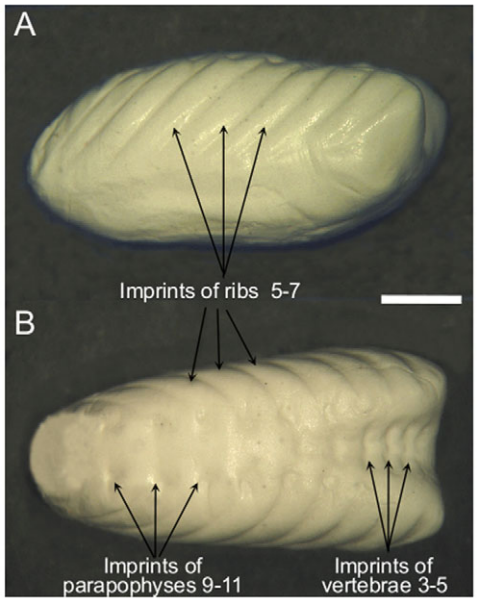

Fig. 6. Internal silicone cast of the swimbladder of *Amphiprion clarkii*. (A) Lateral view showing the imprints of the ribs. (B) Dorsal view with the imprints of the parapophyses and the first vertebrae. A midline groove is formed in the anterodorsal part because the swimbladder leans agaisnt the vertebral column. Scale bar, 5 mm.

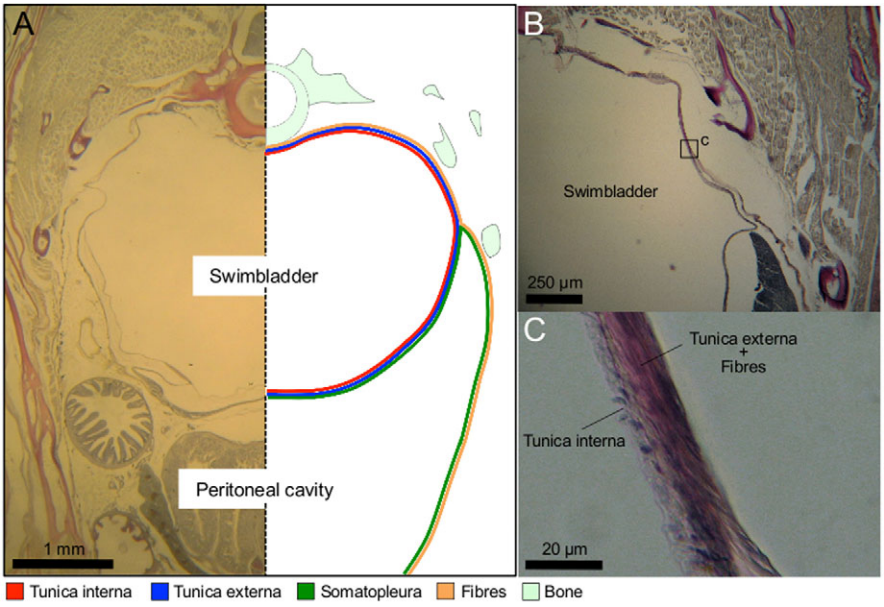

Fig. 7. Cross-section of *Amphiprion clarkii* at the level of the abdominal cavity. The schematic drawing in A helps to distinguish the different swimbladder tissues. The tunica externa is connected to a fibrous layer lining the myosepta tissues, as shown in B and C. (C) Enlargement of the area in B marked by 'c'.

size in sound duration (H=1.155, d.f.=2, P=0.5613), sound amplitude (H=1.140, d.f.=2, P=0.5656), sound frequency (H=1.754, d.f.=2, P=0.4161) or Q_{3dB} (H=0.932, d.f.=2, P=0.6275), which suggests that buccal jaws may maintain similar acoustic properties as they increase in size.

Ribs and swimbladder

A typical hammer strike (10 mV, 0.48 N), the velocity of the rib vibration and the induced sound waveform are represented in Fig. 10 for a 96 mm SL specimen of *A. clarkii*. The hammer waveform was an asymmetrical half-cycle with a shorter rise than fall time (6.1 and 8.7 ms, respectively, N=45).

The rib vibration displayed a sinusoidal pattern that began with a negative deflection (N1), indicating that the ribs were pushed in (i.e. away from the laser sensor; Fig. 10). This was followed by a longer and greater positive peak (P1). Generally speaking, the signal was composed of three oscillation cycles that became progressively longer and greater, whereupon there was vibration decay, indicating that the ribs lost velocity. The N1 vibration was approximately two-thirds as long as the P1 vibration (6.7 and 8.6 ms, respectively, N=45; Fig. 10). Likewise, the N1 velocity was approximately half as large as that of P1 (11.2 and 24.8 mm s^{-1}, respectively, N=45). Moreover, the frequency spectrum obtained from the vibration of the ribs highlighted a very low peak frequency (24.84±7.82 Hz, N=45; Fig. 11).

Interestingly, striking the ribs with the impact hammer produced a sound waveform that exhibited a progressive damping effect (Fig. 10), as observed for natural sounds emitted by fish.

Comparison of hammer, velocity and sound indicated that the onset of the traces corresponded closely (Fig. 10). The peaks of the hammer trace and N1 vibration were closely aligned, and the end of the rebound of the hammer trace almost coincided with P1, suggesting that expanding ribs were pushing the hammer back. The highest peaks of the sound waveform occurred within the rise time of the hammer hit as the ribs were compressed. As the force of the hammer declined and the ribs expanded, the positive acoustic pressure of the signal declined. The remaining sound waveform occurred between N2 and P3, with the sound decaying to background level at P3. The vibration decay of the ribs (after P3) did not result in audible sound. Therefore, the ribs could still vibrating weakly without producing sounds, or at least without producing detectable sounds.

Changes in hammer force (i.e. harder hits) caused significant changes in rib vibration (duration and velocity) and sound parameters (Fig. 12). The total duration of N1+P1 for the first cycle of vibration decreased from 26 to 7 ms (r=–0.86, P<0.0001, N=45), whereas N1+P1 velocity increased from 11.5 to 73.1 mm s^{-1} (r=0.94, P<0.0001, N=45) with increasing hammer force, indicating that harder hits transferred higher frequency energy to the ribs.

Sound duration increased from 26.1 to 39.3 ms (r=0.37, P=0.0114, N=45), and sound amplitude increased from –83.7 to –75.1 dB rel. (r=0.86, P<0.0001, N=45) with harder hits, which transferred more energy to the ribs. Inversely, peak frequency dropped from 900 to 300 Hz (r=–0.49, P=0.0006, N=45) and Q_{3dB} decreased from 22.5 to 7.5 (r=–0.51, P=0.0004, N=45) with harder hits.

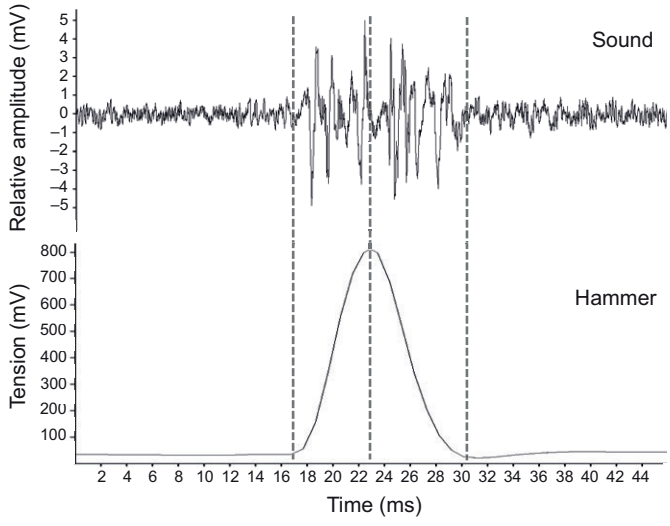

Fig. 8. Waveform of a hammer strike (8 mV, 0.35 N) on buccal jaws and induced sound for a 85 mm SL specimen of *Amphiprion clarkii*. Vertical dashed lines mark the beginning, peak and end of the hammer strike.

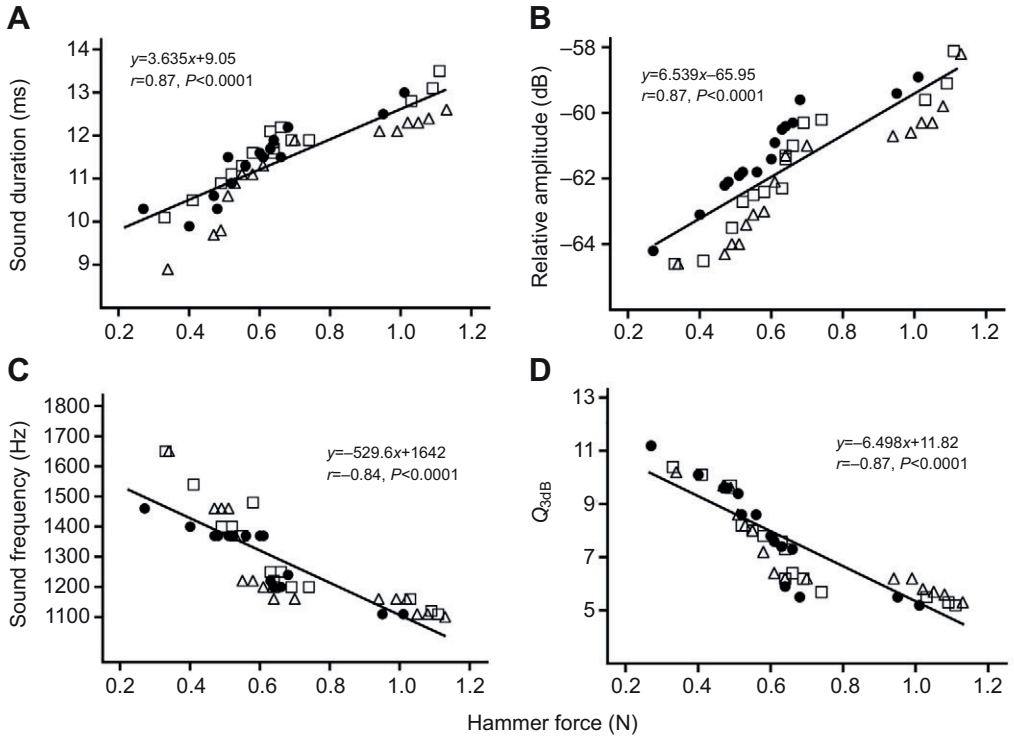

Fig. 9. Comparisons of (A) sound duration, (B) relative amplitude, (C) sound frequency and (D) Q_{3dB} for sounds induced by variable force hammer hits on buccal jaws in *Amphiprion clarkii* (*N*=3) of different size (filled circles, 97 mm SL; open squares, 85 mm SL; open triangles, 76 mm SL).

Comparisons of values calculated from regressions of variable force hammer strikes in three specimens of different size showed that hammer strikes had the same effects on N1+P1 duration (ANCOVA, test for common slopes: $F_{2,39}$=0.8813, P=0.4223) and N1+P1 velocity (ANCOVA, test for common slopes: $F_{2,39}$=1.4883, P=0.2383), regardless of fish size.

The effects of variable force hammer hits on sound duration (ANCOVA, test for common slopes: $F_{2,39}$=0.8002, P=0.4565), sound amplitude (ANCOVA, test for common slopes: $F_{2,39}$=1.7003, P=0.1959), sound frequency (ANCOVA, test for common slopes: $F_{2,39}$=2.3959, P=0.1044) and Q_{3dB} (ANCOVA, test for common slopes: $F_{2,39}$=3.0142, P=0.0606) were similar regardless of fish size.

In addition, there were no significant differences between individuals of different size in N1+P1 duration (H=1.994, d.f.=2, P=0.3690), N1+P1 velocity (H=0.281, d.f.=2, P=0.8690) or sound amplitude (H=4.011, d.f.=2, P=0.1346). By contrast, sound duration (H=34.35, d.f.=2, P<0.001), sound frequency (H=32.69, d.f.=2, P<0.001) and Q_{3dB} (H=32.03, d.f.=2, P<0.001) showed significant changes in relation to fish size, regardless of hammer force (Fig. 12). This result suggests that the acoustic properties of the ribs vary with increasing size.

Sound duration and dominant frequency were closely related to fish size, and varied in the same way as natural sounds produced by fish. The more fish size increased, the more sound duration increased and dominant frequency decreased (Fig. 12). These results were also supported by the frequency spectra for the sound of the small, medium and large fish with SL ranging from 69 to 96 mm (Fig. 13); the smallest fish actually exhibited the highest frequency energy, whereas the largest had the lowest frequency energy. In addition, the Q_{3dB} was also negatively related to fish size, being lower in larger individuals. This observation strongly suggested that longer ribs could vibrate with a longer period of oscillation.

Note that the experimental trial in which the swimbladder was stimulated directly with the impact hammer did not generate usable results. Even a soft hit (~0.10 N) was tricky because it nearly popped the swimbladder. This latter was pushed in by the hammer but never recovered its initial form, resulting in a deformed swimbladder wall. As a result, the vibration trace at the measurement site was distorted and leveled off. Such results are likely caused by the thinness of the wall.

DISCUSSION

Acoustical properties of the swimbladder

It has been hypothesised that the swimbladder may act as a resonator and thus change the quality of sounds produced by stridulation in some fish species. For example, Burkenroad (Burkenroad, 1930) noted that if the swimbladder of the white grunt *Haemulon plumieri* was deflated, the character of the sound became ‘dry’ and lost its ‘gruntlike’ quality. Salmon et al. (Salmon et al., 1968) revealed that sounds were produced by the rubbing of the pectoral fins against the body sides where skin thinly covered evaginations of the swimbladder in the triggerfish *Rhinecanthus rectangulus*. Additionally, swimbladders have long been considered to function as underwater bubbles that are excited to pulsate at their resonant frequency (Harris, 1964; Van Bergeijk, 1964). Because of the compressibility of gas in the bladder compared with the surrounding water, an acoustic pressure wave is believed to excite the bladder into vibration.

In clownfishes, the sound-producing mechanism of aggressive sounds is initiated by teeth collisions caused by rapid mouth closure attributed to a sonic ligament between the hyoid bar and the internal mandible (Parmentier et al., 2007). Sound duration and frequency are known to be morphologically determined signals strongly related to fish size (Colleye et al., 2009; Parmentier et al., 2009; Colleye et al., 2011), which suggests that both acoustic features are subject to a morphological constraint. Considering the positive relationship existing between fish size and swimbladder volume (Fig. 4A), it appears that the swimbladder could be the structure responsible for the size-related variations in acoustic features. The energy of the vibrating jaws resulting from the snapping could be

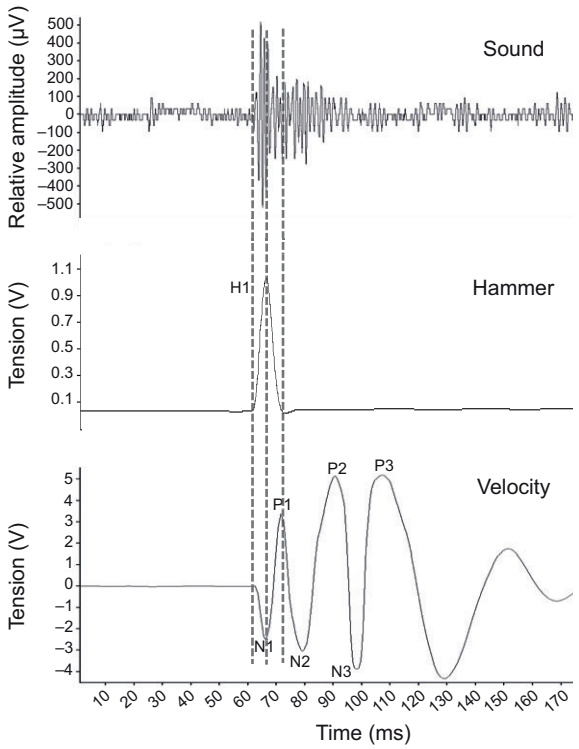

Fig. 10. Waveform of a hammer strike (10 mV, 0.48 N), the velocity of the ribs vibration and the sound waveform induced for a 96 mm SL specimen of *Amphiprion clarkii*. Vertical dashed lines mark the beginning, peak and end of the hammer strike (H1). Note that the highest peaks of the sound waveform are complete by the peak of the hit, which occurs at N1 of vibration when the ribs are compressed, and that the positive acoustic pressure of the signal declines as the force of the hammer declines and the ribs expand. N1–N3, negative deflections 1–3; P1–P3, positive peaks 1–3.

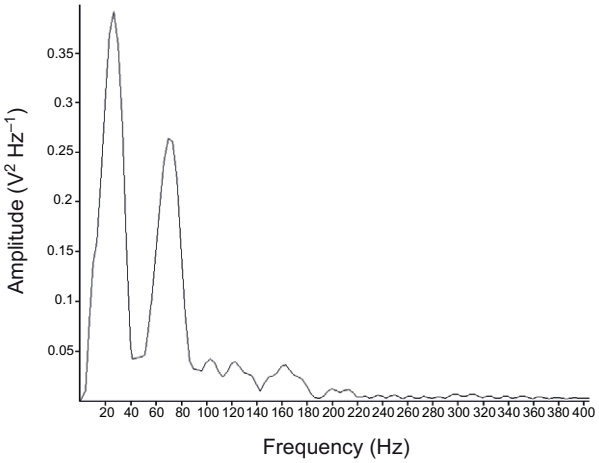

Fig. 11. Frequency spectrum for a 96 mm SL specimen of *Amphiprion clarkii* showing the peak frequency (~20 Hz) obtained from the vibration of the ribs induced by a hammer hit of 0.48 N.

transferred through bones to the swimbladder, which may excite it to vibrate. This forced vibration could be possible because the swimbladder wall is rigidly attached to the vertebral column and ribs (Figs 6, 7). Filling the swimbladder with physiological liquid also supports this possibility because this experiment specifically modified size-related acoustic features (Table 2). For example, in the channel catfish, *Ictalurus punctatus*, removing the gas in the swimbladder did not induce changes in sonic features of sounds produced by pectoral spine stridulation, suggesting that this organ does not play an active role in sound production (Fine et al., 1997). Conversely, deflating the swimbladder with a needle significantly modified some acoustic features in the Nile tilapia, *Oreochromis niloticus* (Longrie et al., 2009). In clownfishes, the swimbladder filling induced pulsed sounds with slight but significant differences in pulse duration and dominant frequency, which implies that this structure plays a role in sound radiation.

Calculations of the resonant frequency of the swimbladder in clownfishes using Minnaert's formula (Minnaert, 1933) indicated that swimbladder resonance itself does not explain the recorded frequencies (Fig. 5B). The dominant frequencies of natural sounds were higher than the calculated swimbladder resonant frequencies. This could be due to the nonspherical shape of the swimbladder because the resonance frequency of a bubble of constant volume increases with any deformation of its spherical shape (Weston, 1967; Sand and Hawkins, 1973). In contrast, the mechanical properties of the swimbladder wall and the viscosity of the surrounding fish tissue can affect the presumably resonant bubble within (Sand and Hawkins, 1973; Love, 1978). This notion was supported by Feuillade and Nero (Feuillade and Nero, 1998), who showed that the presence of an elastic shell (representing the swimbladder wall) enclosed by a viscous shell (representing the surrounding fish tissue) induces a shift in resonance to a higher frequency.

Finally, the use of an impact hammer allowed deeper investigation of the contribution of the swimbladder as an acoustic source to sound properties. Interpretation of the mechanical events during a hammer strike is complicated because of separation of the hammer strike from the site used to measure displacement (Fig. 2). Striking the ventral surface of the swimbladder forced it inward, which increased the pressure within the bladder. However, this manipulation also highlighted that the swimbladder is an inefficient resonator. As a result, the hammer strike created a deformation of the swimbladder wall, and energy in the compressed bladder did not rebound to cause vibration.

This observation seems to indicate that generalisations about fish swimbladders are incorrect when applied to clownfishes. Actually, the results of the present study support other recent findings (Fine et al., 2009; Millot et al., 2011), and indicate that swimbladders are highly damped and thus prevented from prolonged resonant vibrations. An alternative explanation is that swimbladders are inefficient low Q sources (the quality factor Q is a descriptor of the sharpness of tuning) with minimal dependence on resonance because of rapid damping by the swimbladder wall (Fine et al., 2001; Connaughton et al., 2002). Indeed, clownfish sounds display characteristics of a broadly tuned, highly damped sound source. They possess low Q values (Q_{3db}=4.1) and the acoustic waveform of pulses decays rapidly (<20 ms). In the oyster toadfish, *Opsanus tau*, and the weakfish, *Cynoscion regalis*, for example, swimbladder sounds are not sharply tuned and display such characteristics [Q_{3db}<2, pulse duration <10 ms (see Fine et al., 2001; Connaughton et al., 2002)].

Implication of the rib cage as acoustic radiator?

Striking the buccal jaws with an impact hammer generated an acoustic waveform different from that of natural sounds produced by fish; the percussive event suddenly ended (without decaying) before the call disappeared in the background noise (Fig. 8). Moreover, sonic characteristics of sounds induced by hammer strike

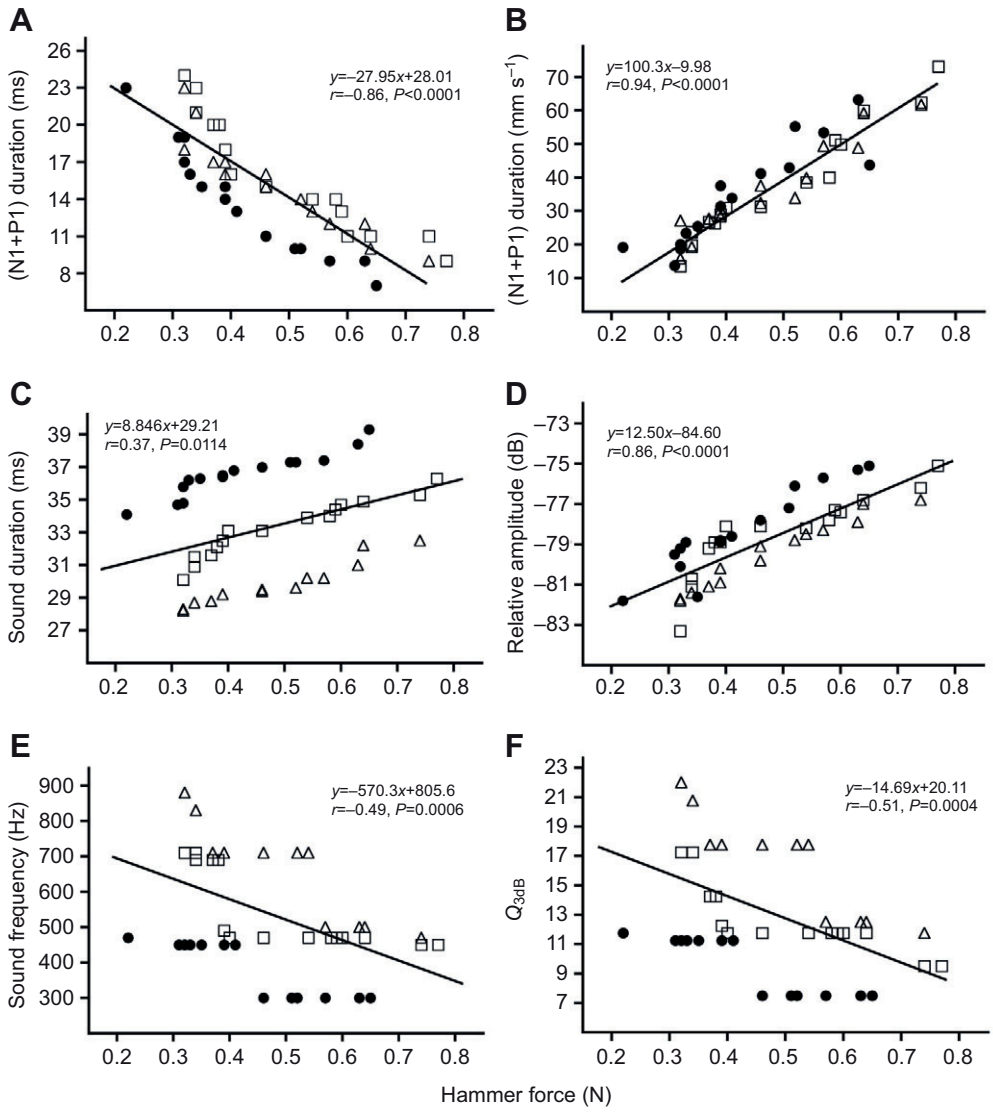

Fig. 12. Comparisons of (A) N1+P1 duration and (B) N1+P1 velocity of the first cycle of rib vibration induced by variable force hammer hits in *Amphiprion clarkii* (*N*=3) of different size (filled circles, 96 mm SL; open squares, 84 mm SL; open triangles, 69 mm SL). Comparisons of (C) sound duration, (D) relative amplitude, (E) sound frequency and (F) Q_{3dB} for sounds induced by variable hammer hits on ribs in the same fish.

did not display differences between individuals of different sizes (Fig. 9), suggesting that buccal jaws would maintain similar acoustic properties as they increased in size. Conversely, striking the rib cage with the impact hammer generated sounds with size-related variation in some acoustic features: sound duration and frequency were positively and negatively related to fish size (Figs 12, 13). Such relationships between sonic features and fish size are consistent with those observed in natural sounds, suggesting that the resonant properties of the rib cage might be responsible for the size-related variations observed in acoustic features. Additionally, manually induced sounds also exhibited an acoustic waveform similar to that of natural sounds (Fig. 10). Note that some acoustic features were a bit different from natural sounds, e.g. sound duration and the sharpness of tuning (Q_{3dB} values) were both higher. However, our findings must be considered in light of the fact that the exposed parts of the fish were in air rather than underwater, a much denser medium. Because the radiation mass was lower in a gas such as air (Kinsler et al., 2000), it is likely that the dramatic decrease in the mass loading and radiation resistance affected the vibrational properties of the ribs as a result of moving the fish from water to air. Such differences may also be explained by the elimination of the damping attributable to the body musculature that has been dissected (see Fine et al., 2001).

Despite the fact that all of these considerations indicate that the swimbladder is an inefficient resonator, this structure might be involved in clownfish sound production. Vibrating ribs should not be very efficient radiators of sound, being much the same as a vibrating tuning fork. Thereby, the swimbladder might function like a loudspeaker cone, which is a highly damped driven radiator. Being closely connected to the swimbladder, the vibrating ribs drive the

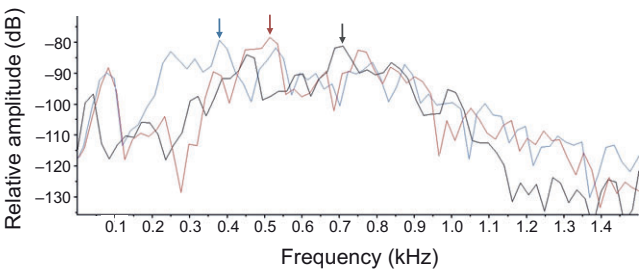

Fig. 13. Power spectra for sounds induced from a small (black line, 69 mm SL), medium (red line, 84 mm SL) and large (blue line, 96 mm SL) individual of *Amphiprion clarkii* by variable force hammer hits on ribs. Note the inverse relationship between dominant frequency and fish size, being the highest for the small fish (black arrow), the lowest for the large fish (blue arrow) and intermediate for the medium fish (red arrow).

swimbladder wall. The tight connection between the swimbladder wall and the rib cage would also explain the low peak values related to the frequency spectrum obtained from the vibration of the rib cage induced by hammer strikes (Fig. 11). Likewise, the swimbladder filling with physiological liquid increased the pressure inside, and thus changed the tension in the swimbladder wall. Therefore, modifying the physical properties of the swimbladder should affect the vibrational properties of the rib cage, which may explain the resultant differences in sound duration and frequency.

Overall, the swimbladder itself does not act as a resonator, its wall just seems to be driven by the vibrations of the rib cage. In contrast, the resonant properties of the rib seem to account for the size-related variations observed in the acoustic features of aggressive sounds in clownfishes. A vibrational wave resulting from buccal jaw snapping is likely to be transferred to the rib cage *via* different functional units of the skeleton such as the suspensorium, the neurocranium and the vertebral column. Therefore, ribs articulate with the distal ends of all parapophyses (Fig. 5) could vibrate easily because they are mobile.

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