

Underwater acoustic communication in the macrophagic carnivorous larvae of *Ceratophrys ornata* (Anura: Ceratophryidae)

Guillermo S. Natale,¹ Leandro Alcalde,² Raul Herrera,³ Rodrigo Cajade,⁴ Eduardo F. Schaefer,⁴ Federico Marangoni⁴ and Vance L. Trudeau⁵

¹CIMA, Departamento de Química, Facultad de Ciencias Exactas, Universidad Nacional La Plata, 47 y 115 (1900), La Plata, Buenos Aires, Argentina; ²ILPLA, Sección Herpetología, Avda. Calchaqui Km 23.5, (1888), Florencio Varela, Argentina; ³Fundación Óga, Guardias Nacionales N°19 (2900), San Nicolas, Buenos Aires, Argentina; ⁴Centro de Ecología Aplicada del Litoral, Ruta 5, Km 2.5, (3400), Corrientes, Argentina; ⁵Department of Biology, Centre for Advanced Research in Environmental Genomics, University of Ottawa, Ottawa, ON K1N 6N5, Canada

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Introduction

Sound production by larval anurans has not been documented previously. In marked contrast, vocalization in adult anurans is well described for sexually active males calling and attracting females during the reproductive season. The neurobiology, hormonal dependence and evolutionary significance of frog mating calls are well understood (Hoke *et al.* 2004; Moore *et al.* 2005; Potter *et al.* 2005; Boul *et al.* 2007). Males also produce territorial calls that are distinct from mating calls (Littlejohn 1977; Wells 1977; Duellman and Trueb 1994). Adult female frog calls are rare and when they exist, are distinct from males and are related to reproduction (Tobias *et al.*

Abstract

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We provide the first evidence for sound production by anuran larvae. In this study, we describe the sounds, their context-specific emission and the structures related to sound production of the carnivorous larvae of *Ceratophrys ornata* (Amphibia, Anura, Ceratophryidae). Tadpoles emit a brief, clear and very audible metallic-like sound that consists of a short train of notes that occur at all stages of larval development. Tadpoles make sound only when a conspecific tadpole is preying upon it or when touched by an object. *Ceratophrys ornata* larvae possess the basic required anatomical structures for sound production via expulsion of atmospheric air from the lungs through the open soft-tissue glottis. The glottis is opened and closed via the larval laryngeal muscles (constrictor laryngis and dilatator laryngis). The arytenoid cartilages appear at stage 40 and the cricoid cartilage does at stage 43. Adult laryngeal muscles differentiate from the larval ones at stage 46 together with the vocal sac formation from the adult interhyoideus muscle. We demonstrate ($n = 2160$ conspecific predator-prey interactions) that larval sounds occur always under predatory attack, probably serving to diminish the chances of cannibalism. These data raise the possibility that other macrophagic carnivorous anuran larvae may produce sound.

Guillermo S. Natale, CIMA, Departamento de Química, Facultad de Ciencias Exactas, Universidad Nacional La Plata, 47 y 115 (1900), La Plata, Buenos Aires, Argentina. E-mail: gnatale@quimica.unlp.edu.ar

1998). Both sexes also produce distress calls in response to disturbance as early as the post-metamorphic stage (Duellman and Trueb 1994).

The species studied herein, *Ceratophrys ornata* (Bell 1843), and other species of the genus are explosive breeders and their macrophagic carnivorous larvae (Fig. 2A) develop rapidly in highly ephemeral ponds where the genus normally breeds and exhibits extremely rapid morphological development (Wild 1997). The eight extant species of *Ceratophrys* have terrestrial adults that inhabit both forested and non-forested areas of South America. *Ceratophrys ornata* is a typical species from the Pampas region of Argentina, Uruguay and Brazil.

In the course of fieldwork to record and catalogue mating calls of native frog populations in Buenos Aires Province, Argentina, we discovered that *C. ornata* tadpoles make sounds. The aim of this work are to describe the underwater sounds produced by the larvae of *C. ornata*, the first report of sound production by an anuran larva. We also describe the morphological structures involved in larval sound production and the specific context in which they are emitted to propose putative functions.

Materials and Methods

Larvae of *C. ornata* from La Plata, Buenos Aires Province, Argentina were obtained following breeding of one pair in the laboratory, and by a second pair captured in amplexus in the field and transporting to the laboratory to obtain the eggs after spawning in captivity. Larvae were maintained in the laboratory (dechlorinated tap water pH 7.6–8.3; hardness 250 mg CO₃Ca/L; continuous aeration, 25 ± 1 °C; 16 : 8 h light/dark cycle) throughout metamorphosis. Tadpoles were staged using the Gosner's table of normal development (Gosner 1960). The larvae of *C. ornata* were supplied with tadpoles of species that share the ponds in nature as food. The tadpole species used as food were *Hypsiboas pulchellus* and *Scinax squalirostris* mainly (both hylids), but diet were occasionally complemented with larvae of other species such as *Odonophrynus americanus* (Cycloramphidae), *Scinax granulatus* (Hylidae), *Leptodactylus ocellatus* (Leptodactylidae), *Rhinella arenarum* and *Rhinella fernandezae* (Bufonidae).

Audio and video recordings were taken in parallel to record the various contexts in which *C. ornata* larvae produce sounds during all stages of larval development from Gosner stage 25 to end of metamorphosis. Recordings were made three times per larval stage (stages 29, 36 and 41) for approximately 1 h each session. Additionally, we recorded a series of 16 distress call emitted by a single post-metamorphic *C. ornata* to compare its call with those produced by larvae. Images of larvae were captured using a Handycam Sony DCR-HC28. Underwater sounds were recorded using an Audio-Technica ATR55 directional microphone covered with a latex sheet to protect it from water. In preliminary studies, we determined that the latex covering did not affect the sound recordings. The sounds out of water were recorded with a transformerless capacitor studio microphone Audio-Technica AT4033. Both microphones were connected to a Behringer Eurorack

MX1602 console, and computer equipped with a Creative Sound Blaster Audigy.

Audio recordings were analysed using Adobe Audition 1.0 at a sampling rate of 44 100 Hz and 16-bit resolution. Frequency information was obtained through fast Fourier transformation (FFT, with 2048 algorithms) at Blackmann–Harris window function. The FFT is an efficient algorithm to compute the discrete Fourier transformation (DFT). The Blackmann–Harris window function is a weighting method that is applied in the time domain to reduce leakage within a Fourier transformation analysis.

We analysed three underwater and three out of water sounds per stage. The arithmetic mean of the following variables were considered for sound analysis: call duration (Cd) expressed in seconds (s), number of pulse (Np), Mean interpulse interval (Mii) (s), and dominant frequency (Df) expressed in Hertz (Hz). Call variables used to describe larval sounds follow Schneider and Sinsch (2006). The variables of the distress call of the post-metamorphic froglets were the same as those considered for the larvae. Each larva was weighed using an electronic balance to the nearest 0.001 g to calculate the relationships between body mass (Bm) and each sound variable.

Scanning photographs were obtained following Alcalde and Blotto (2006). Larvae for skeletal study were prepared according to the technique of Taylor and Van Dyke (1985) but stopped before clearing the muscles. For morphological study, we used specimens of several larval stages (Gosner 31, 33, 34, 37, 41, 42, 43, 46) and one adult male. The material is housed at the Herpetological collection of the Museo de La Plata (MLP A.4849), La Plata City, Argentina.

Results

Sound analysis

The earliest age that sounds were produced by *C. ornata* was at 72 h post-hatch (Gosner stage 25), four stages later than the stage at which the mouth forms. Such sounds are produced through the larval period including metamorphic climax (Gosner stages 43–46) and also post-metamorphic froglets. The brief metallic-like sounds of *C. ornata* larvae consist of short trains of pulses during all stages of development (Table 1). The main sound features of 10 stage-36 larvae (mean ± SD) are: call duration (0.044 ± 0.014 ms),

Table 1 Arithmetic mean (±SD) for the variables of larval sounds at three developmental stages and in recently metamorphosed froglets

| | Call duration (seconds) | Number of pulses | Mean interpulse interval (seconds) | Dominant frequency (Hz) |
|----------|-------------------------|----------------------|------------------------------------|-------------------------|
| Stage 29 | 0.038 ± 0.013 (n = 10) | 6.2 ± 1.39 (n = 10) | 0.003 ± 0.003 (n = 53) | 2943 ± 500 (n = 10) |
| Stage 36 | 0.044 ± 0.014 (n = 10) | 7.4 ± 2.2 (n = 10) | 0.002 ± 0.001 (n = 64) | 2099 ± 290 (n = 10) |
| Stage 41 | 0.068 ± 0.012 (n = 10) | 9.4 ± 1.9 (n = 10) | 0.003 ± 0.001 (n = 84) | 2118 ± 625 (n = 10) |
| Froglet | 1.51 ± 0.89 (n = 10) | 60.2 ± 19.2 (n = 10) | 0.002 ± 0.002 (n = 72) | 857 ± 131 (n = 10) |

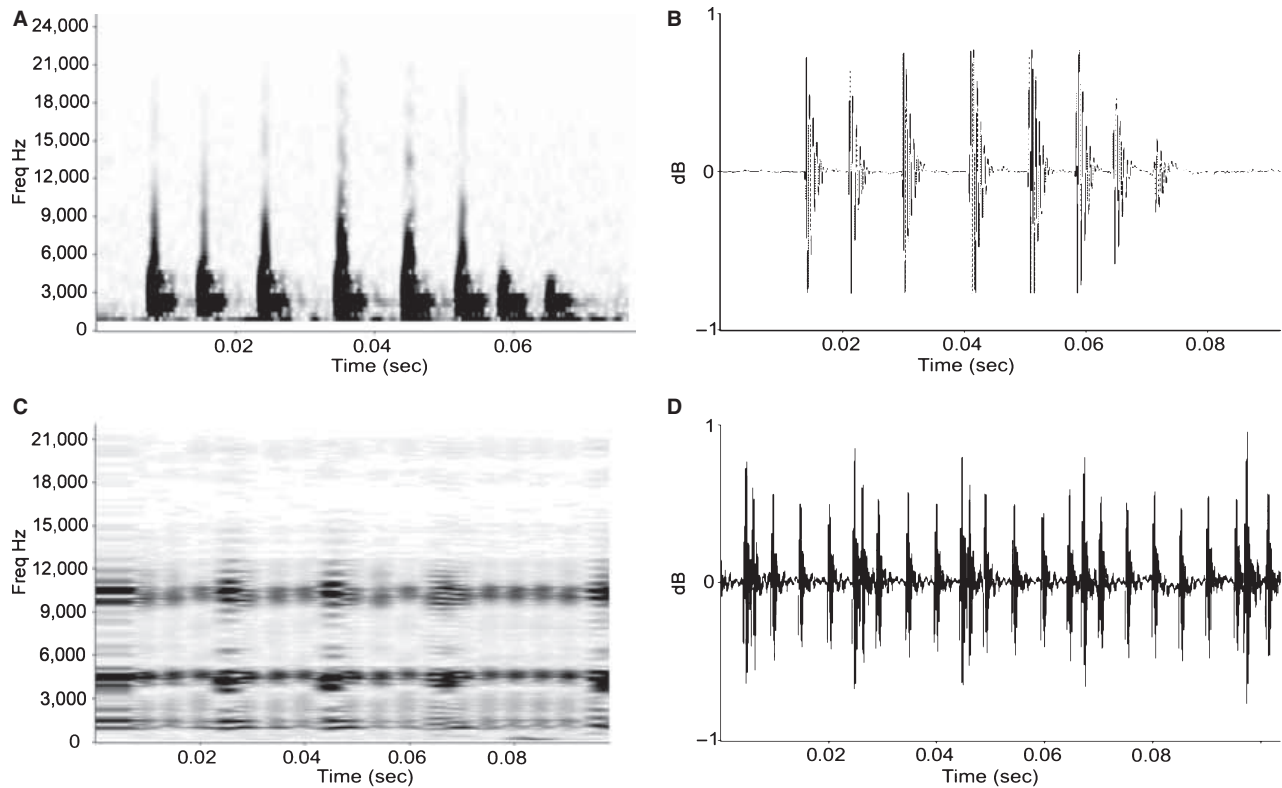


Fig. 1—Sonograms and oscillograms for the sounds produced by a *Ceratophrys ornata* larvae at Gosner stage 41. — **A.** Sonogram of an underwater sound. — **B.** Oscillogram of the same underwater sound; Sonogram of the pulsed distress call emitted by froglets of *C. ornata* in laboratory. — **C.** Sonogram of a distress call with pulsed structure. — **D.** Oscillogram of the same distress call with pulsed structure.

number of pulses (7.4 ± 2.22), interpulse interval (0.002 ± 0.001) and dominant frequency (2099 ± 290). The sound features of Gosner stage-29, and stage-41 larvae are similar to stage-36 larvae and are presented in Table 1. Such larval sounds (Fig. 1A,B) have a generally similar structure to that of froglets (Table 1; Movie S3). Both larval and froglet sounds have a long series of pulses, often referred to as trills (Duellman and Trueb 1994), with the mean interpulse frequency being stable across development.

The post-metamorphic froglets of *C. ornata* emit a 72 pulses-sound following opening of the mouth and expelling air (see Fig. 1C,D and Movie S3). This sound has two bands of dominant frequency, one ranged from 3832 to 4565 Hz and another ranged from 9560 to 10 550 Hz.

Context of sound production

The larvae of *C. ornata* reared by us spend most of their time quietly on the bottom of the aquaria without movement. We observed that when a *C. ornata* larva is laying in such manner and a second larva of a different species (see food supply in Materials and Methods) approaches *C. ornata*, this second larva is attacked. The attack consists of a bite using the large keratinized jaw sheets followed by the ingestion of the attacked larva. Larval prey smaller than the oral disc gap of

C. ornata larvae were ingested whole whereas the larger prey were bitten into pieces before ingestion. The *C. ornata* larva attacks a larger prey item by biting systematically at the same point using the jaw sheets. Once chopped, the body parts are engulfed. It is important to note that *C. ornata* larvae do not produce sounds during the whole sequence of attack (bite - ingestion or bite - cut of - ingestion). This predatory behaviour in which *C. ornata* larvae lay on the bottom waiting for prey that swim near the larval mouth resembles the 'sit and wait' behaviour described by Duellman and Trueb (1986) for the adults of the same species.

On the other hand, when a *C. ornata* larva is touched or bitten by a conspecific larvae, or when it is touched by an object, such as metal spatula or glass pipette, it produces a brief metallic-like sound (see Movies S1 and S4). Sound production is generally accompanied by a contraction at the body-tail junction (see Movies S4-S6). This contraction consists of a fast tail movement towards the body. When this movement reaches a maximum, with the tail nearly touching the lateral wall of the body, there are two associated behaviours: (i) a rapid escape of the attacked larva and (ii) the exhalation of the air contained within lungs and the production of the described sound. These behaviours were observed in 2160 interactions between pairs of *C. ornata* larvae and there was the complete absence of cannibalism observed in all cases. Moreover, larvae

experimentally removed from water emit a sound similar to that recorded underwater (see Movie S2), but the sounds produced out of water are repeated frequently, perhaps because of easy access to the air used for sound production (see Mechanism of sound production).

Mechanism of sound production

The scanning electron micrographs of the buccal cavity and lungs of larvae of *C. ornata* reveal that this species possesses medium-sized inflated lungs and an opened soft-tissue glottis since early stages of development (~Gosner 30–31) (Fig. 2B,C). The double staining and clearing techniques reveal that arytenoid cartilages appear at stage 40 whereas the cricoid cartilage appears latter at stage 43 (Fig. 2F,G). In addition, the muscular dissections at stage 31 before clearing show the presence of both larval laryngeal muscles (muscles dilatator laryngis and constrictor laryngis) since early in development (Fig. 2F). The presence of the open glottis, laryngeal muscles and lungs provides the basic structural requirements for sound production in early post-hatching stages of development. The sound production by *C. ornata* larva seems not to need laryngeal cartilages. The glottis receives the attachment of the muscle dilatator laryngis at each side. Contraction of this paired muscle opens the glottis. In addition, there is an annulus of muscular fibres surrounding the soft-tissue glottis (larval muscle constrictor laryngis). Contraction of this unpaired muscle closes the glottis.

In summary, the basic structures required for sound production appear at early larval stages and they are: (i) a soft-tissue open glottis, (ii) the lungs, and (iii) the larval muscles related to the glottis (mm. dilatator laryngis and constrictor laryngis). With the progression of larval development there is the sequential appearance of (i) arytenoids (stage 40), (ii) cricoid (stage 43), (iii) adult laryngeal muscles (stage 46), and (iv) other adult muscles related to sound production (e.g. muscles described by Walkowiak 2007; stage 46). These adult muscles are: (i) the m. dilatator laryngis and m. hyolaryngeus (both derived from the larval m. dilatator laryngis), (ii) the muscles sphincter anterior and sphincter posterior (both derived from the larval m. constrictor laryngis), (iii) mm. Petrohyoideus I–IV (derived from the respective larval mm. levatorae arcuum branchiales), (iv) the mm. interhyoideus and intermandibularis (both derived from the respective muscles of the larva, see Fig. 2D,E), (v) the lateral and medial parts of the m. geniohyoideus (homologous to the single larval m. geniohyoideus), (vi) the m. sternohyoideus (homologous to the m. recus cervicis of the larva), (vii) the m. genioglossus (formed during metamorphosis into the lingual anlagen, without larval homology), and finally, (viii) the m. hyoglossus (derived from the deepest fibres of the pars medialis of the m. geniohyoideus). The intramuscular space of the m. interhyoideus (vocal sac) used for sound amplification is absent in the larva and first appears in froglets (Fig. 2E).

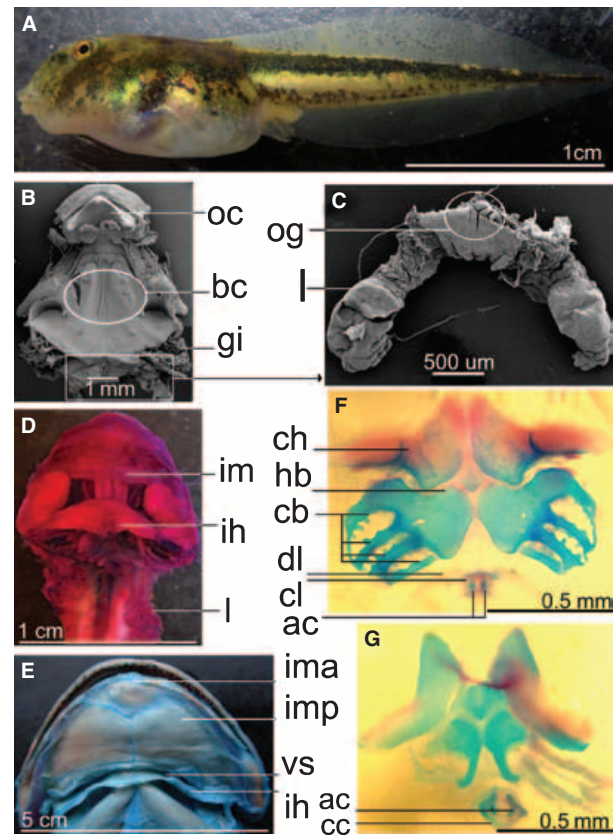


Fig. 2—Morphological study of *Ceratophrys ornata* larvae and adult showing the structures implied in the mechanism of sound production. — **A**. Lateral view of a Gosner stage-34 larva. — **B,C**. Stage-31 larva observed by scanning electron micrographs: Note the open glottis and the inflated lungs at the posterior end of the buccal cavity floor (**B**) and, the detail of both structures in (**C**). — **D**. Ventral view of the muscles associated to the mandibular arch and hyobranchial apparatus in a dissected stage-42 larva. These set of muscles are the principal ones that act in lung ventilation and sound production in the adults. — **E**. An adult dissected male showing the most superficial muscles of the throat that act in both lung ventilation and sound production. Note the vocal sac into the m. interhyoideus. — **F**. Hyobranchial apparatus in a stage-40 larva of *C. ornata* stained for cartilage (blue) and almost completely cleared. Note the presence of the arytenoid cartilages at the posterior end of the hypobranchial sinus and the muscles constrictor laryngis and dilatator laryngis (both partially cleared). — **G**. Stained and cleared stage-43 larva showing the recently chondrified cricoid cartilage, the previously formed arytenoids, and the rest of the hyobranchial apparatus in metamorphic remodeling and reabsorption processes. ac, arytenoids; bc, buccal cavity; cb, cenatobranchials; cc, cricoid; ch, cenatohyal; cl, m. constrictor laryngis; dl, m. dilatator laryngis; gi, gills; hb, hypobranchial plate; ih, m. interhyoideus; im, m. intermandibularis; ima, m. intermandibularis anterior; imp, m. intermandibularis posterior; l, luma; oc, oral cavity; og, open glottis; vs, vocal sac.

Based in the morphological data we believe that the mechanism of sound production by *C. ornata* larvae is similar to that of adults. All larvae reared by us in the aquaria take

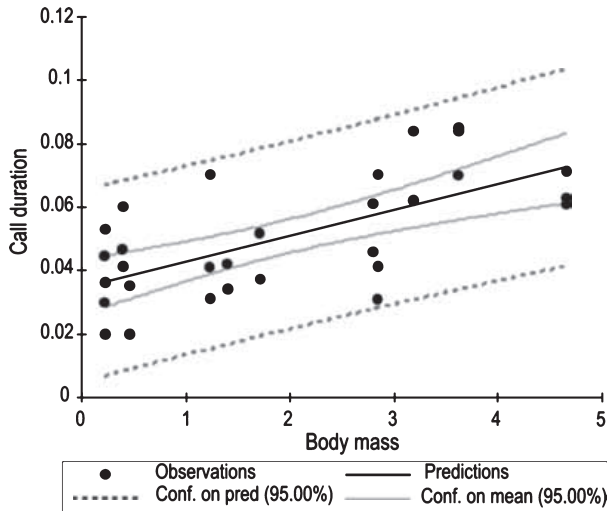


Fig. 3—Linear regression between Body mass (g) and Sound duration. Note that both variables are positively correlated increasing sound duration with larger body mass (a putative indicative of larger lung volume).

atmospheric air periodically (see Movie S5), and we think that this air is used for sound production by passing it from lungs to the glottis as occurs in adults, but without sound amplification. Air-breathing occurs when a larva swims rapidly to the surface of water, at which time the larva engulfs the air via the mouth, and then returns to the bottom. Lung exhalation in adult anurans occurs via the action of many post-cranial axial muscles as the mm. rectus abdominis, obliquus externus and transversus (Walkowiak 2007). The larvae of *C. ornata* have all these muscles but with poor development during premetamorphic larval stages. We postulate that exhalation is aided by the previously described rapid tail movement used for escape. Such movement produces a slight pressure on one side of the larva and causes the necessary force to produce lung exhalation. Some sound variables (Sound duration, Fig. 3, Table 2; Number of pulses, Fig. 4, Table 2) correlate positively with body mass, reinforcing our idea that the sound production mechanism involve air exhalation via the lungs–glottis route. The positive correlation between body mass with both sound duration and number of pulses may relate to the increase of lung volume. As the lung capacity increases, the amount of air expelled through the larynx can increase with a subsequent increase in both acoustic variables. Contrary, dominant frequency of the sound

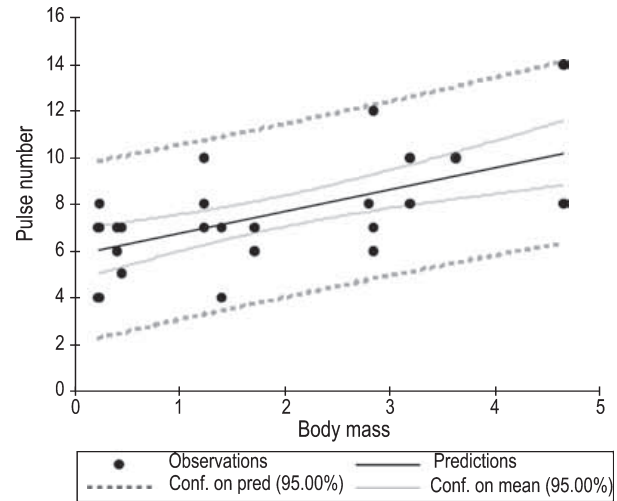


Fig. 4—Linear regression between Body mass (g) and Pulse number (number of pulses in a single train). Note that the number of pulses correlates positively with body mass.

correlated negatively with body mass (Fig. 5, Table 2), as occurs in most anurans (Ryan 1988; Gerhardt 1994).

Discussion

Until now, sound production by anuran larvae was totally unknown. Larval sound production by other animal forms is restricted to airborne warning sounds, such ‘clicking’ in caterpillars (i.e. acoustic aposematism) (Yack *et al.* 2001; Brown *et al.* 2007). In marked contrast with the complete lack of knowledge about larval sounds in anurans, the neurobiology and evolutionary significance of mating and territorial calls of adult frogs are well understood (Littlejohn 1977; Duellman and Trueb 1994; Hoke *et al.* 2004; Moore *et al.* 2005). The advertisement call of adults of *C. ornata* is well described (Barrio 1963; Salas *et al.* 1998) and confirmed here. The adult advertisement call, however, is different from the sounds we recorded for the larvae of the same species. Importantly, there is a clear similarity in structure among the adult distress call described by Barrio (1963) for adults of *C. ornata* and the sounds described herein for post-metamorphic froglets and larvae of the same species. This similarity raises the following question: Is the larval sound of *C. ornata* a distress call? We

Table 2 Values for the regression analysis between body mass and selected call variables. y , linear regression equation ($y = a + b \cdot x$); n , number of observations; r , correlation coefficient; r^2 , determination coefficient; F (critical value for significance test)

| | y | n | r | r^2 | $F(1.28)$ | P -value |
|-----------------------------------|---------------------|-----|------|-------|-----------|------------|
| Call duration–body mass (g) | $0.0349 + 0.0081x$ | 30 | 0.66 | 0.43 | 21.24 | 0.0001 |
| Number of pulses–body mass | $5.835 + 0.937x$ | 30 | 0.63 | 0.4 | 18.26 | 0.0002 |
| Dominant frequency (Hz)–body mass | $271.120 + 160.33x$ | 30 | 0.54 | 0.29 | 11.22 | 0.002 |

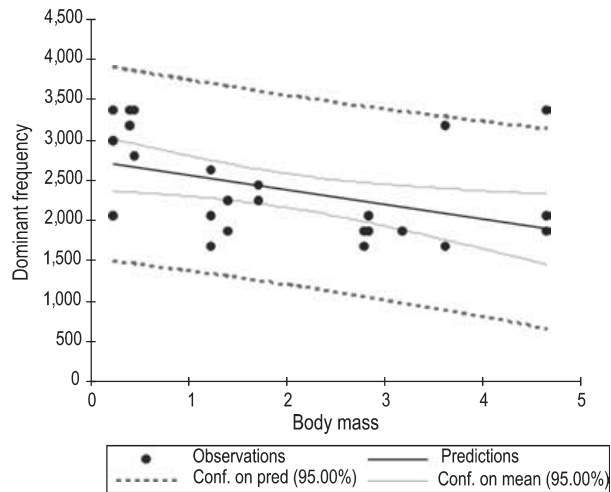


Fig. 5—Linear regression between body mass (g) and dominant frequency (Hz). Note that dominant frequency of the sound correlated negatively with body mass.

demonstrate that larvae of *C. ornata* produce underwater sounds only in the case of physical interactions with conspecific larvae or when being touched experimentally by an object. A tadpole never produced sounds in other test situations (e.g. when it was the predator and not the prey). Such a context of sound production in which larvae emit sounds only when they are ‘attacked’ fills the definition of ‘distress calls’.

Ceratophrys ornata larvae, like the adults, are cannibalistic as first noted by Fernandez and Fernandez (1921), who mention the impossibility of rearing more than one *C. ornata* larva in the same aquarium because they eat each other. In disagreement with these authors, our experience indicates absence of cannibalism in larvae reared at low densities (5 larvae/L) and supplemented with abundant food. When cannibalism occurs, it is more common that larger animals prey upon smaller larvae that are somewhat delayed in their development. We recorded at least 2160 predator–prey interactions between conspecific larvae during the course of this work and no cannibalism was observed. We observed that sound production is coupled with the fast movement and escape by an attacked larva. Although the exact significance of the larval sound to the receiving conspecific larvae remains obscure, we hypothesize that both sound production and fast tail movement are linked to the almost complete absence of cannibalism among the *C. ornata* larvae reared by us. This idea is reinforced by the high incidence of predation upon the larvae of other frog species provided as food. None of these other larvae survived within the aquaria containing *C. ornata* larvae.

Such is the situation in laboratory conditions, but what about in nature? The full diet of *C. ornata* larvae remains undescribed but, we suspect that cannibalism rarely occurs in nature for several reasons. Our field estimations indicate a frequency of two *C. ornata* tadpoles per 1000 tadpoles of other species. Therefore, there is a low tadpole density and

conspecific larval interactions are predicted to be infrequent. Adults of this species are explosive breeders that reproduce in large ponds only during intense rains. There is usually only one cohort per pond and larvae reach the metamorphosis rapidly (20 days). Additionally, *C. ornata* reproduces synchronously with *R. fernandezae* and *R. arenarum*, two species with small larvae (30 mm) and high reproductive potential (e.g. a single female of *R. arenarum* produces about 30 000 eggs). The abundance of bufonid larvae provides food for both larvae and froglets of *C. ornata* because these species usually reach metamorphosis synchronously. Although many species of *Rhinella* larvae are considered unpalatable prey at least at early stages of development (Jara and Perotti 2009) our laboratory reared *C. ornata* larvae ate the *R. fernandezae* and *R. arenarum* larvae without problem when they were supplied as food. For natural populations, low densities of *C. ornata* in proximity with the very abundant small larvae of *Rhinella* spp., suggests that larval conspecific predator–prey interactions would be relatively infrequent. However, in the case that conspecific predator–prey interactions take place, sound production and the associated fast tail movement used to escape may prevent cannibalism.

Early stage larvae of *C. ornata* have lungs, a soft-tissue open glottis and a pair of laryngeal muscles (mm. constrictor and dilatator laryngis). These equipment incorporates the cartilaginous laryngeal support at larval stages 40 (arytenoids) and 43 (cricoid). The mechanism of sound production is well documented for numerous adult anurans and involves the elastic lungs, the contractile vocal sac formed by the interhyoideus muscle, and the larynx formed by the arytenoid and cricoid cartilages containing the vocal cords (Wistcht 1949; de Jongh and Gans 1969). The vocal sac acts to modify and amplify the sounds produced by the passage of air through the larynx, but it is not the element, which produces the sound. The observation that anuran larvae of many genera have laryngeal cartilages (at least the arytenoids), laryngeal muscles (not as complex as in adults), lungs, and open glottis (Wiens 1989; Maglia and Pugener 1998; Haas 1999; Alcalde 2005) raises the possibility that larval sound production is more widespread. This possibility is most likely for the carnivorous macrophagic larvae of *Ceratophrys cranwelli* and *Ceratophrys aurita*, which also have lungs and open glottis since early larval stages (Wassersug and Heyer 1988; Vera Candiotti 2005). However, laryngeal cartilages were not reported for other species of Ceratophryinae (including *C. ornata*) (see Wild 1997), but such omissions do not imply absence since laryngeal cartilages are usually omitted in chondrocranial morphological descriptions of anuran larvae.

Our data suggest that vocalizations related to distress can play an important role in early life-history stages of *C. ornata*. We hypothesize that sound production reduces the chances of larval cannibalism in aggressive, rapidly developing predators. In the case of the carnivorous larvae studied herein, vocalizations in the human audible range are produced by laryngeal structures within 3 days of life. While anurans differ

considerably in the timing of the formation of a functional adult-like auditory system, it is clear that this begins early in development (Quick and Serrano 2005; Horowitz *et al.* 2007). The developmental neurobiology of the genus *Ceratophrys* is completely unexplored but it is likely that sound detection via lungs and bronchial columella is a possibility as reported for other anuran larvae (see Mason 2007). For adult Anura and other vertebrates, auditory communication is fundamental to speciation, population dynamics, reproduction and survival (Littlejohn 1977; Wells 1977; Schneider and Sinsch 2006; Boul *et al.* 2007). The importance of larval auditory communication for other members of the highly apomorphic *Ceratophryine* clade, and in the larger context of anuran ecology should now be investigated.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Movie S1 A stage-29 larva of *Ceratophrys ornata* is experimentally touched using a spoon. Note the sound produced by

the larva in response to the touch a resting tadpole and the later produces a sound.

Movie S2 A stage-41 larva emits sounds when it is netted out of water.

Movie S3 A post-metamorphic individual emits the distress call when touched by a pipette.

Movie S4 A stage-29 resting tadpole is first approached by one tadpole and then it is bitten by a third tadpole. Note the sound produced in such situation.

Movie S5 A stage-36 tadpole bites another tadpole which produces the sound and then swims away.

Movie S6 A stage-41 tadpole attacks another tadpole, they bite each other, produce sounds and finally swim in opposite directions. Note an additional tadpole taking atmospheric air once finished the attacked interaction.

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