# Biotic and abiotic sounds affect calling activity but not plasma testosterone levels in male frogs (Batrachyla taeniata) in the field and in captivity

**Article** *in* Hormones and Behavior · February 2020

DOI: 10.1016/j.yhbeh.2019.104605



**4 authors:**

Matías I. Muñoz
Vrije Universiteit Amsterdam
**9** PUBLICATIONS **22** CITATIONS
SEE PROFILE

Maricel Quispe
University of Chile
**5** PUBLICATIONS **21** CITATIONS
SEE PROFILE

Manuel Maliqueo
University of Chile
**124** PUBLICATIONS **2,624** CITATIONS
SEE PROFILE

Mario Penna
University of Chile
**73** PUBLICATIONS **987** CITATIONS
SEE PROFILE

Hormones and Behavior 118 (2020) 104605

Contents lists available at ScienceDirect

Hormones and Behavior

journal homepage: www.elsevier.com/locate/yhbeh

# Biotic and abiotic sounds affect calling activity but not plasma testosterone levels in male frogs (*Batrachyla taeniata*) in the field and in captivity

Matías I. Muñoz[a,1], Maricel Quispe[a,*], Manuel Maliqueo[b], Mario Penna[a]

[a] Programa de Fisiología y Biofísica, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Independencia 838000, Santiago, Chile
[b] Endocrinology and Metabolism Laboratory, School of Medicine, San Juan de Dios Hospital, University of Chile, Santiago, Chile

ARTICLE INFO

*Keywords:*
*Batrachyla taeniata*
Chorus sound
Rain sound
Testosterone levels
Playback experiments
Evoked vocal responses

ABSTRACT

In animals, the expression of diverse reproductive behaviors is hormonally regulated. In particular, vocalizing during courtship has been related to circulating androgen levels, and reciprocally, conspecific vocalizations are known to modulate androgen secretion in vertebrates. The effect of natural sounds of abiotic origin on hormonal status has virtually not received attention. Therefore, we evaluated the vocal responses of male *Batrachyla taeniata* frogs to conspecific chorus and rainfall sounds in natural and controlled laboratory settings, measuring the testosterone levels of exposed individuals. In field and laboratory conditions, testosterone levels of frogs exposed to 31.5 min of chorus and rain sounds and non-exposed individuals were similar. In the field, frogs increased their call rate in response to playbacks of chorus and rain sound, but the evoked calling activity was unrelated to plasma testosterone. In contrast to the field, frogs showed limited responsiveness to 31.5-min acoustic exposures in the laboratory. Similarly to the field, for vocally active males tested in the laboratory there was no association between call rate and testosterone levels. Additionally, in this group, testosterone levels were higher in vocally active males relative to non-calling individuals. Overall, these results indicate that in *B. taeniata* testosterone levels are not altered following a short-term exposure to conspecific biotic and to abiotic sounds. Our results are suggestive of a threshold influence of testosterone on the vocal activity of the species studied. Further explorations of the influence of abiotic sounds on endocrine activation are required to understand how animals respond to variable acoustic environmental conditions.

## 1. Introduction

Steroid hormones such as androgens and estrogens, are major regulators for the development of sexually dimorphic structures, and for the expression of diverse behaviors in adult individuals, including aggression, courtship and mating. In several species the circulating levels of sexual steroid hormones peak during the breeding season, often in synchrony with an increase in the expression of reproductive displays (Adkins-Regan, 2005).

The interplay between animal behavior and hormones is known to have bidirectional components. The expression of social communication by means of acoustic signals is generally related to androgen levels in vertebrates, as it has been shown in fishes (e.g., Genova et al., 2012), birds (e.g., Foerster et al., 2002; Meitzen et al., 2009; Madison et al., 2015), and mammals (e.g., Fedurek et al., 2016). Also, besides the 'activational' effects of hormones on communicative displays mentioned above, social cues may modulate the circulating hormone levels of individuals (Adkins-Regan, 2005). In a reproductive communication context, male androgen levels increase after hearing the vocalizations of conspecific males. For example, playback experiments have reported that the acoustic simulation of a territorial intrusion results in the elevation of androgen levels in male spotted antbirds (Wikelski et al., 1999), song sparrows (Moser-Purdy et al., 2017), and toadfishes (Remage-Healey and Bass, 2005). In contrast with those results, testosterone levels do not increase following an acoustic challenge in some bird species (e.g., Wingfield and Wada, 1989; Deviche et al., 2012; Rosvall et al., 2012), indicating that endocrine responses to acoustic cues are species-specific and probably associated to differences in life-history traits, such as types of mating systems or degree of parental care (Goymann, 2009).

In most anuran species (frogs and toads), during the breeding season receptive conspecific females are attracted by male advertisement vocalizations and males engage in antiphonal calling contests (Gerhardt and Huber, 2002; Wells, 2007). Because of the strong dependence of

* Corresponding author.
*E-mail address:* maricelquispe@ug.uchile.cl (M. Quispe).
[1] Current address: Department of Ecological Science, Vrije Universiteit Amsterdam, De Boelelaan 1105, 1081 HV Amsterdam, Netherlands.

https://doi.org/10.1016/j.yhbeh.2019.104605
Received 15 November 2018; Received in revised form 23 September 2019; Accepted 25 September 2019

anurans on acoustic communication, the association between the emission of male advertisement calls and circulating androgen levels has been studied in a number of taxa, revealing contrasting species-specific effects. An early laboratory study on male leopard frogs (*Rana* (=*Lithobates*) *pipiens*) found that intracranial testosterone implants located at the anterior portion of the preoptic area are effective in eliciting advertisement calls in castrated frogs, while systemic testosterone injections do not induce calling (Wada and Gorbman, 1977). In contrast, subcutaneous androgen implants are effective in stimulating the vocal activity of castrated *Xenopus laevis* males (Wetzel and Kelley, 1983). In addition, the spontaneous calling activity of male *Hyla cinerea* is positively correlated with androgen levels in testosterone implanted subjects (Burmeister and Wilczynski, 2001), and testosterone supplemented male *Hyla arborea* emit longer calling bouts as compared to control males (Desprat et al., 2015). Laboratory studies have also shown that acoustic stimulation has an effect on frog hormone levels, for example in male green treefrogs *Hyla cinerea*, long-term exposure to conspecific chorus sound elevates androgen levels (Burmeister and Wilczynski, 2000) relative to frogs hearing a control stimulus.

Similarly to laboratory experiments, studies conducted in the field have reported mixed results on the interplay between hormones and the emission of male advertisement vocalizations. Androgen levels are higher in silent relative to calling male frogs *Lithobates catesbeianus* (*Rana catesbeiana*) (Mendonça et al., 1985) and *Duttaphrynus* (*Bufo*) *melanostictus* (Gramapurohit and Radder, 2013), while in two toad species, *Anaxyrus* (*Bufo*) *woodhousii* and *A. cognatus*, vocally active and non-calling satellite males have similar plasmatic androgen levels (Leary et al., 2004; Leary et al., 2006). In other species androgen levels are higher in calling relative to silent individuals (Townsend and Moger, 1987; Marler and Ryan, 1996; Leary and Harris, 2013; Joshi et al., 2017) As pointed out by Leary (2009), the differences in the hormonal profiles of silent and calling males could be related to the various behaviors measured in the non-calling individuals tested, namely brooding (Townsend and Moger, 1987), foraging (Marler and Ryan, 1996), amplectic (Gramapurohit and Radder, 2013) and satellite status (Leary and Harris, 2013).

Natural environments comprise not only the sounds produced by animals, as additional sources of environmental noise are the sounds generated by natural geophysical and atmospheric processes, such as local weather conditions (i.e., rain, water courses, wind, sea surf and thunder). Although these are ubiquitous sources of acoustic interference, their potential effects on hormonal levels of animals remain unknown. The lack of studies on this subject is a significant gap given the growing body of evidence showing that these environmental sounds modify the vocal behavior of animals. Birds increase the amplitude of their chipping bouts in response to increasing levels of creek sound (Pytte et al., 2003) and emit songs having larger syllable lengths in the presence of sea shore sound (Gough et al., 2014). Similarly, exposure to wind sound induces vocal modifications in whales (Dunlop, 2016) and sparrows (Lenske and La, 2014). Natural abiotic sounds can also influence behavior beyond signal production, as for example females of a stream-breeding frog prefer male calls embedded in stream noise (Zhao et al., 2017), and tropical bats delay their emergence time from roosting sites in the presence of rain sound (Geipel et al., 2019). Elucidating if these responses to natural abiotic sound are accompanied by changes in the endocrine status of organisms is likely to provide valuable insights on the role of hormones in adjusting behavior to dynamic environmental conditions.

The banded wood frog, *Batrachyla taeniata* is an anuran from the temperate austral forest in South America. In this species, male calling activity is remarkably activated when exposed to natural abiotic sounds, in particular rainfall sound (Penna and Zúñiga, 2014), and a field playback study showed that the evoked vocal responses of males to synthetic advertisement calls are directly related to plasma testosterone levels (Solís and Penna, 1997).

In the present study we evaluate whether acute exposure to biotic (e.g., conspecific chorus) and abiotic (e.g., rain sound) sounds induce changes in the testosterone levels of male *B. taeniata* frogs. By comparing the endocrine and behavioral responses to short-term stimulation with biotic and abiotic acoustic cues we expect to shed light on the regulation of vocal signaling behavior under diverse acoustic conditions. We combine laboratory and field playback experiments to identify significant relationships masked by the variability and complexity of natural environmental conditions. The integration of field and laboratory experiments can contribute significantly to identify inherent complexities between behavioral and physiological processes (Calisi and Bentley, 2009), and has been recently highlighted as a valuable approach to study the effects of sound exposure in animals (Slabbekoorn, 2016).

We hypothesize that short-term stimulation with conspecific chorus and rain sound promotes male breeding activity in *B. taeniata* by increasing the call rate. Similarly to other vertebrates, testosterone levels are expected to be higher in individuals exposed to conspecific chorus as compared to individuals treated with rain sound or control silence. Based in a former field study with *B. taeniata* we predict a positive relationship between testosterone levels and evoked vocal responses.

## 2. Methods

### 2.1. General overview

Measurements of testosterone levels and vocal activity of male *B. taeniata* frogs in response to different acoustic conditions were carried out in the field and laboratory, exposing the subjects to the same audio files in both settings. Three experimental groups of animals were used in both conditions, as subjects were either exposed to playbacks of natural conspecific chorus or rain sound (see Experimental stimuli construction), and a third group was maintained in absence of broadcast sounds as a control. The animals in the laboratory were also subjected to an acoustic maintenance period of 7–8 days previous to the experimental exposure to chorus, rain sound or control silence. During this period, individuals were exposed to a low-amplitude playback of conspecific choruses (see Maintenance stimuli construction). Half an hour after the offset of the acoustic exposures, the subjects from field and laboratory experiments were sacrificed to obtain blood samples for testosterone measurements.

### 2.2. Stimuli construction

#### 2.2.1. Experimental stimuli construction

The *B. taeniata* chorus sounds used for experimental playbacks were built from natural field recordings conducted in the locality of Tinquilco (39°07′ S, 71°46′ W) in February 1993, obtained with a cassette recorder (Sony TC D5M) and a directional microphone (AKG CK8) placed at about 3 m from the closest caller to avoid predominance of individuals in the recorded sounds. Air and substrate temperatures during these recordings ranged between 11.5 – 14.7 and 11.3–14.9 °C, respectively. With the software Audacity 2.0.3, nine 10-s duration segments of different choruses were selected and each segment was pasted successively to create nine 3-min duration audio files containing different chorusing ensembles. Care was taken to avoid discontinuities of the waveform at the points where the segments were added. The 10-s segments of recordings of different choruses are shown in Fig. 1. The 3-min duration choruses had linear fade-in and fade-out times of 2 s.

The rain sounds used for experimental playbacks were recorded in the same geographical region where the present study was conducted, using the microphone of a sound level meter (Brüel & Kjær 2230), the output of which was connected to a digital tape recorder (Sony D10 PROII). For these recordings the microphone was placed underneath a 50 × 50 cm square of a 5-cm thick sponge suspended in a metal stand to avoid the direct impact of raindrops on the microphone. 10-s segments of 9 different recordings free of other interfering sounds were chosen

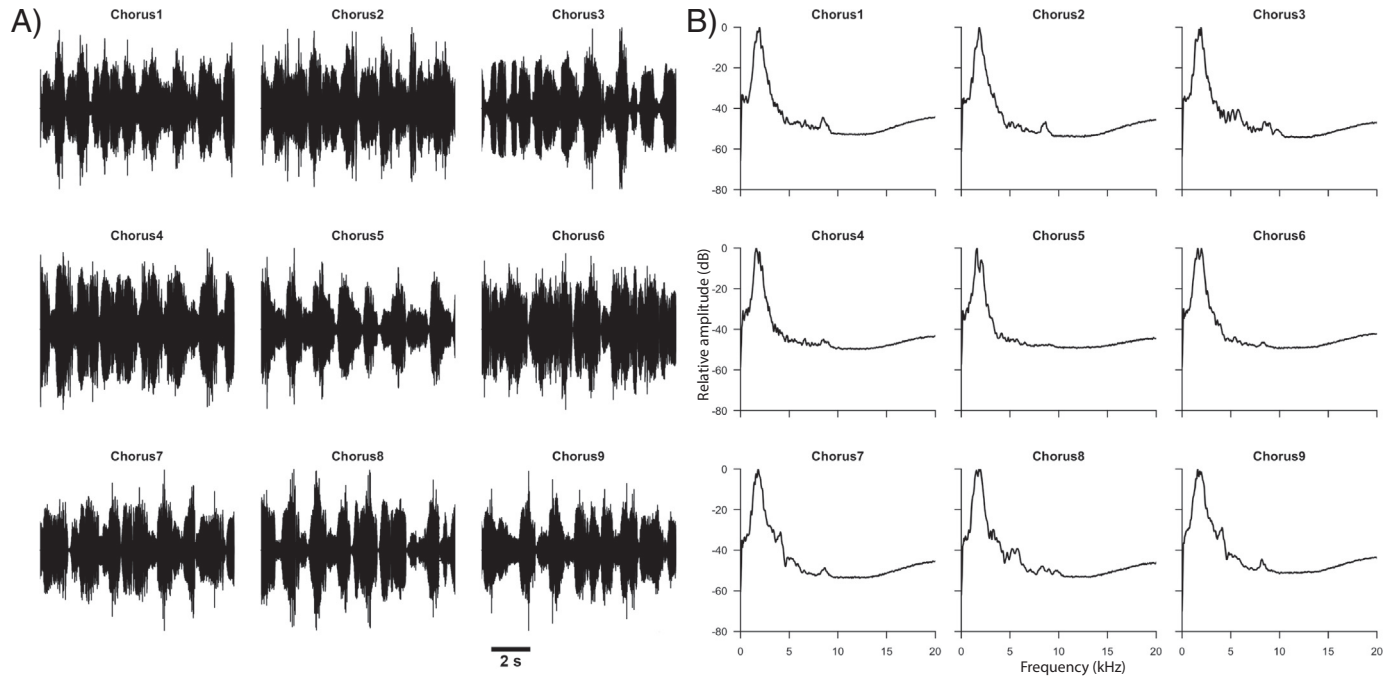


**Fig. 1.** A) Oscillograms and B) average power spectra of the nine 10-second duration *B. taeniata* chorus sounds used for stimuli edition. Power spectra were computed with a Hanning window of 1024 points and 0% overlap.

(Fig. 2) and 3-min rain sounds were created using the same procedure as for the chorus sounds.

The temporal and spectral characteristics of the *B. taeniata* choruses and rain sounds used for playbacks were judged to be representative of these signals and were devoid of other biotic or abiotic noises. A similar procedure has been previously used to create files of abiotic sounds of different kinds to study the vocal behavior of native frogs (Penna et al., 2005; Penna and Zúñiga, 2014).

### 2.2.2. Maintenance stimuli construction

Because the long-term previous acoustic experience may modulate the androgen levels of frogs (Burmeister and Wilczynski, 2000; Chu and Wilczynski, 2001), all the individuals tested in the laboratory underwent an acoustic maintenance period. For 7 or 8 consecutive nights all the frogs were exposed to conspecific chorus, to homogenize the auditory experience of the individuals before conducting the sound exposure experiments. The same nine 10-s duration *B. taeniata* chorus segments used for the construction of the experimental stimuli were

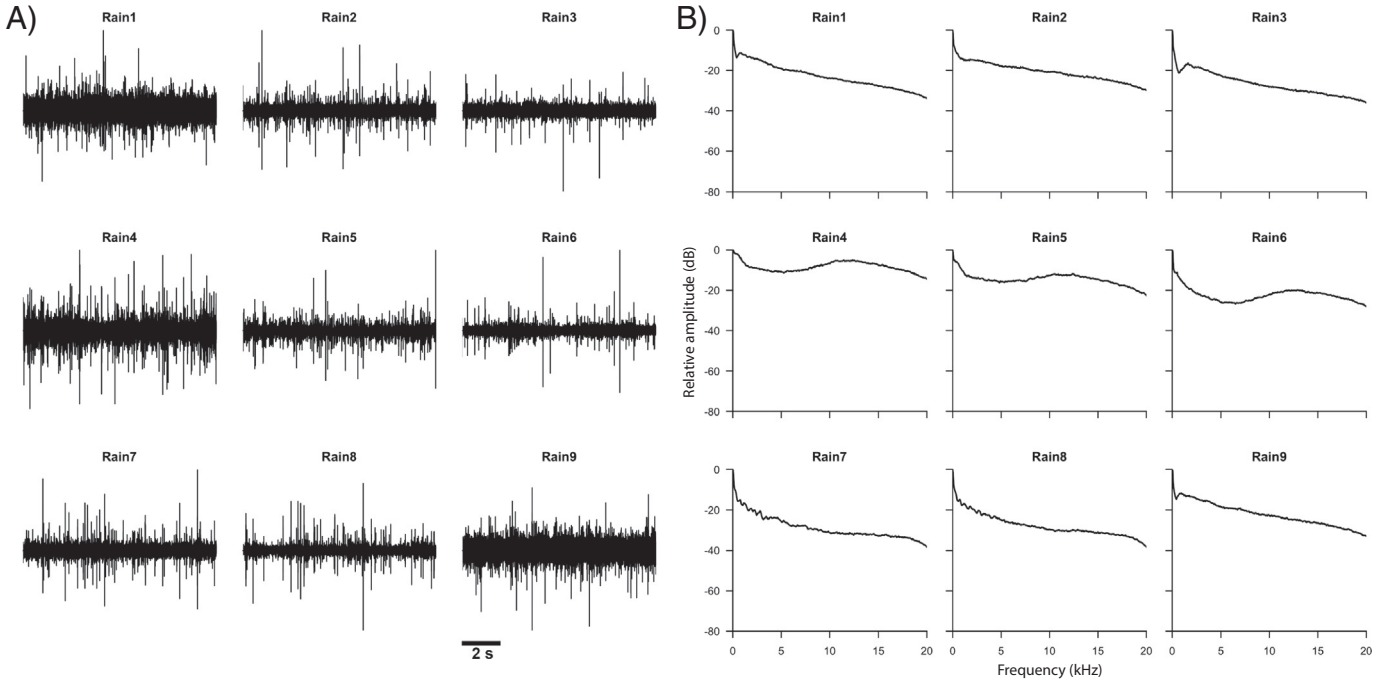


**Fig. 2.** A) Oscillograms and B) average power spectra of the nine 10-second duration rain sounds used for stimuli edition. Power spectra were computed with a Hanning window of 1024 points and 0% overlap.

pasted consecutively to create nine different 10-minute duration audio files. Each 10-min duration chorus sound had linear fade-in and fade-out of 5 s.

### 2.3. Field experiments procedure

#### 2.3.1. Study site

Field experiments were conducted at night, between 21:00 and 04:00 h, at the southwestern shore of the Tinquilco Lake (39°07′ S, 71°46′ W), near the Huerquehue National Park in southern Chile, from March 5 to 11, 2016. At the study site, male *B. taeniata* frogs called from the substrate, hidden among grasses and underneath fallen leaves and branches in a forest where *Drimys winteri* was the predominant tree.

#### 2.3.2. Field playback experiments

Vocally active male *B. taeniata* were located in the field and different groups were exposed to conspecific chorus ($N = 5$), natural rain sound ($N = 5$), and not exposed to sound broadcast ($N = 5$). The snout-vent lengths and body weights of frogs were similar across acoustic treatments (Kruskal-Wallis rank sum test, $\chi^2 = 3.83$, $d.f. = 2$, $P = 0.147$ and $\chi^2 = 1.15$, $d.f. = 2$, $P = 0.563$, respectively). Acoustic stimuli were played back with an Ipod nano (Apple Inc.) connected to an attenuator (Hewlett-Packard 355-3560) and an amplifier (Alpine 3540), and delivered through a loudspeaker (Behringer, Monitor 1C) placed at 60–80 cm from the experimental subject. The amplitude of the acoustic stimuli was measured with the microphone of a sound level meter (Brüel & Kjær 2238) placed next to the focal individual and adjusted with the attenuators to 67 dB SPL RMS (linear frequency weighting, slow time weighting) during brief sound broadcasts of short segments of one chorus and one rain stimulus before proceeding with the experiment. The 67 dB SPL RMS amplitude was chosen because this value is within the range of amplitudes of calls of nearest neighbors at the position of focal subjects at the study site (M. Penna, unpublished data) and is also within the range amplitudes of rain noise measured in the geographic region where the study was conducted (Penna et al., 2005). This amplitude has proved to be effective to expose individuals of *B. taeniata* and related species to abiotic and synthetic noises (Penna and Zúñiga, 2014; Penna et al., 2017). The calibration broadcasts prior to the experiment were brief, lasting about 5 s and the subjects were left undisturbed for at least 5 min before the experiment onset. The experimental subjects were subsequently exposed to the nine 3-min experimental stimuli leaving 30 s of silence in between successive stimuli in order to prevent fatigue of vocally active individuals. The order of presentation of the nine 3-min chorus and rain experimental stimuli followed a random sequence. To reduce acoustic interferences during the experiments, neighboring frogs were either captured or silenced by gently tapping the substrate. Following 31.5-min of exposure to chorus or rain sounds, or an equivalent interval in which no sound was broadcast, all the experimental individuals were left in silence for 30 additional minutes. The vocal activity of the focal subjects was recorded during 2 min prior to sound broadcast onset, and throughout the experiment (2 min of basal activity + 31.5 min of sound exposure + 30 min of silence, total recording time: 63.5 min) with a directional microphone (Sennheiser ME66 with K6 power module) placed 20–40 cm in front of the subject and connected to the left channel of a digital recorder (Tascam DR-100). Stimuli delivered with the Ipod nano were recorded on the right channel of the same digital recorder. Fig. 3 shows a schematic diagram of the stimulation protocol used for the playback experiments.

At the end of each experimental session, the environmental noise level was measured by placing the microphone of the sound level meter (Brüel & Kjær 2238) at the position of the experimental subject. Air and substrate temperature were measured with a thermometer (Ebro TFN 520) and the relative humidity with a hygrometer (Extech Instruments RH390).

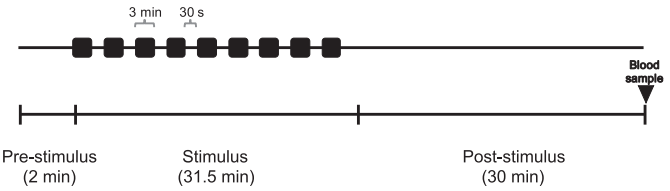


**Fig. 3.** Schematic diagram of the stimulation protocol. The experimental procedure included two initial minutes of spontaneous activity, followed by 31.5 min of acoustic stimulation and finally 30 min of post-stimulation vocal activity. After completing the stimulation protocol males were rapidly captured and a blood sample was obtained. The vocal activity of males was recorded throughout the complete duration of the experiment. The nine top black boxes during the stimulation period depict the nine 3-minute duration audio files corresponding to chorus or rain sound used for playback experiments. The vocal activity of frogs treated with silence was recorded for an equivalent time, but individuals were not exposed to acoustic stimulation.

### 2.4. Laboratory experiments procedure

#### 2.4.1. Animal housing

Laboratory experiments were conducted during February 2016 and March–April 2017. A total of 24 male *B. taeniata* ($N = 12$ during 2016 and N = 12 during 2017) were captured at the northern shore of the Tinquilco lake (39°07′ S, 71°46′ W), near Huerquehue National Park in southern Chile. All the frogs were captured during the breeding season and presented morphological traits indicating active reproductive status (i.e., nuptial pads and pigmented vocal sacs). Frogs were transported to the Faculty of Medicine of the University of Chile in Santiago, Chile, and housed individually inside acoustically transparent cylinder-shaped plastic containers enclosed with fine plastic mesh (diameter: 15 cm, height: 23 cm) and kept at 12 ± 1 °C inside a refrigerated room and an inverted 14:10 light:dark cycle. Each plastic enclosure contained mosses, leaves and twigs collected at the capture site, resembling the natural microenvironment where males of this species call from. Frogs were fed with tenebrionid larvae and crickets once per week.

#### 2.4.2. Laboratory acoustic maintenance period

For the laboratory experiments conducted during 2016 and 2017, each plastic container with an individual male *B. taeniata* was placed inside acoustically isolated wooden chambers (80 × 20 × 35 cm) at 12 ± 1 °C and the frogs were kept under an inverted 14:10 light:dark cycle. Each chamber contained a loudspeaker (Behringer, Monitor 1C) placed at 60 cm from the plastic container housing an individual frog. The chambers' internal walls were partially covered with acoustic foam to reduce internal resonances and six LEDs positioned on the ceiling of each chamber provided illumination.

During 7 or 8 consecutive nights the individual frogs were stimulated with the chorus maintenance stimuli played back with a Mac mini computer (Apple Inc.) connected to an attenuator (Tucker-Davis Technologies PA4 or Hewlett-Packard 350D) and an integrated amplifier (Topaz AM5, Cambridge Audio) and delivered through a loudspeaker inside each chamber. The experimental subjects were not exposed to the same number of days to chorus sound due to the prolonged duration of the experimental procedure (see "Laboratory playback experiments" subsection). The nine 10-min choruses were presented in random succession leaving 3 min of silence in between choruses. On each night this sequence of choruses was presented for 5 h starting 1 h after the beginning of the dark phase. The exposure level used was 50 dB SPL RMS (A frequency weighting, slow time weighting). This amplitude corresponds to the amplitude of distant *B. taeniata* chorusing aggregations at the site where frogs were captured (M. Penna, unpublished data).

Following the 7 or 8 nights of conspecific chorus exposure, stimulation was discontinued, and individuals were left in silence for one day before experimental testing. This was done in order to allow time for

immediate early gene expression following the procedures of Gall and Wilczynski (2014), as the brains of the experimental subjects were used for immunohistochemical procedures in a parallel study.

Throughout the acoustic maintenance period, and the day of silence, the vocal activity of each frog was recorded with a small omnidirectional tie-clip microphone (AKG C417) placed inside each chamber and connected to a channel of two six-channel digital recorders (Tascam DR-680). Recordings were restricted to the 5-hour period of maintenance chorus presentation. All the experiments were completed within 3 weeks of the arrival of the frogs in the laboratory.

#### 2.4.3. Laboratory playback experiments

The overall experimental protocol used for the sound exposure experiments in the laboratory was similar to the one used in the field. After the acoustic maintenance period and the day of silence, frogs were exposed to *B. taeniata* chorus sound ($N = 8$), natural rain sound ($N = 8$) or control silence ($N = 8$). The snout-vent lengths (Kruskal-Wallis rank sum test, $\chi^2 = 0.35$, $d.f. = 2$, $P = 0.840$) and body weights (Kruskal-Wallis rank sum test, $\chi^2 = 0.39$, $d.f. = 2$, $P = 0.822$) of frogs were similar across acoustic treatments. Due to a failure of the recording system during the sound exposure experiments, the vocal activity of one individual exposed to conspecific chorus was not recorded and therefore the final sample size for this group was $N = 7$. Sound exposure experiments were conducted within the same 5-hour interval of time during which the maintenance sound was played back on previous days. Because of the prolonged duration of the experimental protocol and the blood collection procedure, the number of individuals tested was restricted to five per day. The basal vocal activity of males was recorded for 2 min before the onset of the stimuli playback and the post-stimulation vocal activity was recorded for additional 30 min. The vocal activity of individuals that were not exposed to any broadcast sound was recorded for the same time as the duration of the playback experiments.

### 2.5. Body measurements and blood sampling

The same blood sampling protocol was used for frogs tested in the laboratory and the field. Once the sound exposure experiments were completed, subjects were captured by hand, weighted and their snout-vent lengths measured with a digital caliper to the nearest 0.1 mm. Individuals were then anesthetized by immersion in 0.2% MS-222 for < 3 min and blood samples were collected by puncturing the heart ventricle with a heparinized 1-ml syringe. Blood samples were centrifuged and the plasma stored at −20 °C until radioimmunoassay was conducted. Plasma samples collected in the field were initially stored at 4 °C for 1–2 weeks, and later stored at −20 °C. Plasma samples were stored in 27 μl aliquots, unless a lower volume was obtained.

### 2.6. Plasma testosterone analysis

Plasma levels of testosterone were measured using a competitive binding radioimmunoassay (RIA) kit (DIA source ImmunoAssays, DIAsource TESTO-RIA-CT kit, catalog #: KIP1790). The RIA kit used had a high specificity for testosterone, having a cross reactivity with dihydrotestosterone of 0.31%, and a detection limit of 0.05 ng/ml. In order to determine the most appropriate dilutions to be used in the study, serial dilutions of plasma samples of blood overflown inside the thoracic cavities during heart puncturing of 15 individuals used in the present study were obtained. Dilutions 1:2 and 1:4 were judged as appropriate by parallelism between the standard dilution and standard curves (for details see Fig. S1). As such, samples having volumes of 27 μl were diluted with 27 μl of the zero calibrator of the kit. Plasma samples having volumes lower than 27 μl were diluted with the zero calibrator until reaching 54 μl. Following the kit instructions, 50 μl of the diluted samples were used to perform the assay. Testosterone levels reported were corrected by the dilution factor and expressed in nanograms per milliliter of plasma (ng/ml). Individuals with undetectable testosterone levels were assigned 0.05 ng/ml values, corresponding to the detection limit of the kit. Samples were run in duplicate when enough plasma was collected. Average intra-assay variation was 8.91% and 17.04% for plasma samples collected in the field and in the laboratory, respectively. Samples collected in the field were run on a single assay, and mean inter-assay variation for laboratory samples was 6.76%.

### 2.7. Acoustic analysis of vocal responses

Calls emitted by frogs during the field and laboratory playback experiments were manually selected using RavenPro 1.4 (Cornell Laboratory of Ornithology, Ithaca, NY, USA). For each individual, we counted the number of calls emitted before (2 min), during (31.5 min) and after (30 min) the acoustic stimulation. The call rate of individuals was computed for each recording period.

Calls emitted by male *B. taeniata* frogs during the laboratory acoustic maintenance period were automatically counted with a custom-written R script (version 3.3.3, R Core Team, 2017) using the library 'seewave' (version 2.0.5, Sueur et al., 2008). Vocalizations that were undetected by the script (about 10–20% of the calls) were manually selected with RavenPro 1.4. Call rate was calculated from the number of calls emitted by the individuals over 5 h of daily exposure to conspecific chorus at low level (50 dB SPL RMS). Call rate during the day of silence was calculated for the corresponding 5 h during which the subjects were exposed to chorus sound on previous days. The vocal activity of six individuals was not recorded during the day 6 of the acoustic conditioning phase due to a failure of the recording devices, and thus these values were included as missing data in the analyses.

### 2.8. Statistical analysis

All the statistical analyses were performed using R (version 3.3.3, R Core Team, 2017), and the figures were created the libraries "ggplot2" (version 2.2.1, Wickham, 2009), "seewave" (version 2.0.5, Sueur et al., 2008), and "tuneR" (version 1.3.2, Ligges et al., 2016).

#### 2.8.1. Field experiments

The vocal responsiveness of frogs to the acoustic playback was evaluated with linear mixed-effects models (LMM) fitted through maximum-likelihood with the package "lme4" (version 1.1-12, Bates et al., 2015). The model included the square-root transformed call rate as the dependent variable. The recording period (categorical variable with 3 levels: pre, during and post-exposure), the acoustic treatment (categorical variable with 3 levels: chorus, rain and silence), and their interaction were included as fixed-effects. As temperature may influence the vocal activity of frogs (e.g., Ziegler et al., 2016), substrate temperature was included in the models as a continuous covariate. Individuals were included as a random-effect to account for data dependencies derived from recording the same subjects at three consecutive time periods. The significance of fixed-effects was evaluated by means of sequential likelihood ratio tests (LRT). Differences among treatment levels were evaluated by means of Tukey's Honest-Significant-Difference (Tukey HSD) planned comparisons performed with the library "lsmeans" (version 2.26-3, Lenth, 2016).

Analysis of covariance (ANCOVA) was used to investigate differences in plasma testosterone level of frogs exposed to the three different acoustic treatments (chorus, rain or silence). Because a former study showed that the evoked vocal activity and testosterone levels were correlated in male *B. taeniata* (Solís and Penna, 1997), the call rate of individuals during the acoustic exposure period was included as a continuous covariate. Additionally, we tested the effect of the acoustic treatment on plasma testosterone levels after excluding the call rate as a covariate from the ANCOVA model (i.e., a one-way ANOVA).

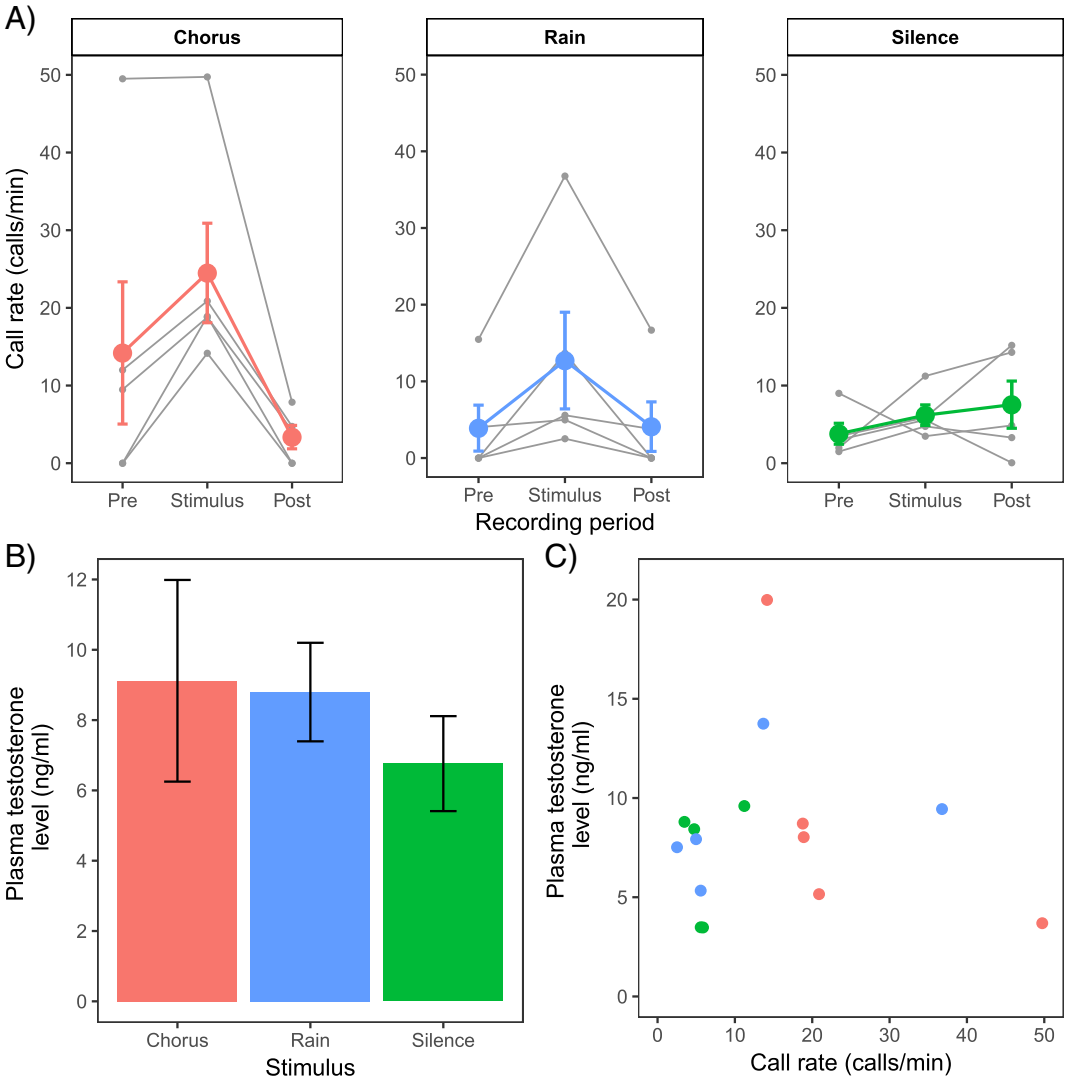


**Fig. 4.** A) Call rates of male *B. taeniata* exposed to conspecific chorus ($N = 5$), rain sound ($N = 5$) and silence ($N = 5$) in the field. Grey lines and dots indicate responses of individual males. Colored dots and error bars correspond to means and s.e.m., respectively. B) Plasma testosterone levels of male *B. taeniata* exposed to chorus, rain sound, and silence in the field. Bars and error bars correspond to means and s.e.m., respectively. Data shown in Fig. 4B correspond to the mean testosterone levels not corrected by the call rate. C) Scatterplot of testosterone levels and calling rate of frogs exposed to the three acoustic treatments in the field. Red, blue and green colors depict the behavior and hormone levels of individuals exposed to chorus, rain and silence, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

*2.8.2. Laboratory experiments*

The vocal responsiveness of frogs to the different acoustic treatments in the laboratory was investigated by fitting a generalized linear mixed-effects model (GLMM). A large number of individuals tested in the laboratory did not call during the playback experiments (see Results section), and therefore to analyze their vocal responses we fitted a zero-inflated negative binomial GLMM using the library 'glmmTMB' (version 0.2.3, Brooks et al., 2017). We used the number of calls as the dependent variable, and included the log-10 transformed duration of each recording period as an offset to account for the different duration of the recording intervals. The offset was transformed because of model convergence issues. Similar to field experiments, we included the recording period, the acoustic treatment, and their interaction as fixed-effects in the model. Because experiments were restricted to a maximum of 5 individuals per day, we included the individuals nested within the day of the test to incorporate this experimental blocking in the model, and to account for data dependencies derived from repeatedly recording the same individuals. Temperature was stable in the laboratory settings where frogs were housed and tested and therefore it was not included as a covariate. The significance of fixed-effects was evaluated by means of type III Wald tests using the library 'car' (version 3.0-2, Fox and Weisberg, 2011).

We used ANCOVA to compare testosterone levels of frogs exposed to the different acoustic treatments (chorus, rain or silence). Testosterone levels were $\log_{10}$-transformed to improve the normality of the residuals, and three call-related continuous covariates were included in the model to assess their possible influence on individual testosterone levels: (1) the evoked call rate during the 31.5 min of sound exposure; (2) the average call rate during the 7 or 8 days of acoustic maintenance period, and (3) the call rate during the day of silence prior to testing. The ANCOVA analysis showed a significant association between testosterone levels and the call rate of frogs during the playback period (see Results section). To further explore this relationship we pooled together the data from the three acoustic treatments and fitted a linear regression between the testosterone levels ($\log_{10}$-transformed) and the call rate of frogs. We also fitted the linear regression model after excluding silent individuals from the data set. In the field only vocally active individuals were tested, and therefore excluding the silent individuals

from laboratory analysis allows to compare the hormone-behavior association between the two experimental settings. Finally, we also evaluated the effect of the acoustic treatment on plasma testosterone levels after excluding the three covariates related to the vocal activity of frogs from the ANCOVA model (i.e., a one-way ANOVA).

For laboratory experiments, we used the Wilcoxon rank sum test with continuity correction to compare the testosterone levels of individuals that emitted calls and frogs that remained silent during the acoustic stimulation.

To test the effects of captivity on the testosterone levels of *B. taeniata*, we used the Wilcoxon rank sum test to compare the testosterone of individuals tested in the field and in laboratory.

For ANOVAs and ANCOVAs we computed the partial eta-squared ($\eta_p^2$) as a measure of the effect sizes of main factors using the library 'sjstats' (Lüdeke, 2019, version 0.17.4). For mixed-models, standardized effect sizes were not computed because it is currently unclear which of the variability estimates of these models to employ for effect size calculations (Rights and Sterba, 2019).

For field and laboratory experiments, the assumptions of the parametric tests employed were evaluated by visually inspecting the model residuals, and if necessary, dependent variables were transformed to attain the normality assumption. For the GLMM, residuals were visually inspected using the library 'DHARMa' (version 0.2.4, Hartig, 2019) in R.

## 3. Results

### 3.1. Field experiments

#### 3.1.1. Field vocal activity during exposure to experimental stimuli

In the field, the chorus and rain sounds evoked vocal responses of male *B. taeniata* effectively. The LMM analyses revealed a significant effect of the interaction between the recording period and the acoustic treatment on call rate (Fig. 4A, $\chi^2 = 14.30$, *d.f.* = 4, $P = 0.006$). For frogs exposed to chorus and rain sounds, planned comparisons showed that the call rate was higher during sound exposure as compared to the pre- and post-exposure periods, and no differences were found between the pre- and post-recording periods (Table 1). In contrast to the vocal responses of frogs under playback treatment, the call rate of frogs during silence treatment was similar across recording periods (Table 1).

#### 3.1.2. Field testosterone levels

The ANCOVA showed that testosterone levels did not differ between the frogs exposed to conspecific chorus, rain sound and silence (Fig. 4B, $F = 0.40$, *d.f.* = (2, 11), $P = 0.682$, $\eta_p^2 = 0.067$), and there was no effect of the call rate on plasma testosterone levels (Fig. 4B, $F = 0.68$, *d.f.* = (1,11), $P = 0.426$, $\eta_p^2 = 0.059$). The one-way ANOVA showed that the effect of acoustic treatment on testosterone levels was not significant after removing the call rate from the model ($F = 0.41$, *d.f.* = (2, 12), $P = 0.674$, $\eta_p^2 = 0.064$).

**Table 1**
Post hoc analyses of the vocal responses of male *B. taeniata* to different acoustic treatments in the field (see text for description of the experimental conditions).

| Stimulus | Comparison | t-Ratio (*d.f.*) | *P* |
|---|---|---|---|
| Chorus | Pre - stimulus | −3.29 (37.5) | **0.006** |
| | Stimulus - post | 5.34 (37.5) | < **0.001** |
| | Pre - post | 2.05 (37.5) | 0.113 |
| Rain | Pre - stimulus | −3.14 (37.5) | **0.009** |
| | Stimulus - post | 3.11 (37.5) | **0.010** |
| | Pre - post | −0.03 (37.5) | 1.000 |
| Silence | Pre - stimulus | −0.93 (37.5) | 0.628 |
| | Stimulus - post | 0.08 (37.5) | 0.674 |
| | Pre - post | −0.85 (37.5) | 0.997 |

Bold characters indicate significant P values (< 0.05)

**Table 2**
Call rates of males of *B. taeniata* during the maintenance period prior to experimental exposure to different acoustic treatments in the laboratory. Values correspond to mean ± s.e.m. On day 6 six individuals were not recorded due to a technical failure. See text for description of experimental condition.

| Day | *N* of individuals | Call rate (calls/min) |
|---|---|---|
| 1 | 24 | 0.98 ± 0.53 |
| 2 | 24 | 1.99 ± 0.88 |
| 3 | 24 | 3.78 ± 1.33 |
| 4 | 24 | 4.47 ± 1.47 |
| 5 | 24 | 3.99 ± 1.34 |
| 6 | 18 | 1.71 ± 0.87 |
| 7 | 24 | 4.63 ± 1.45 |
| 8 | 13 | 5.48 ± 2.28 |
| Last exposure day (7 or 8) | 24 | 4.90 ± 1.47 |
| Silence day | 24 | 1.27 ± 0.52 |

### 3.2. Laboratory experiments

#### 3.2.1. Acoustic maintenance period

The vocal activity of males during the 7–8 days of acoustic maintenance period was highly variable. While the conspecific chorus playback elicited strong vocal responses in some individuals, other experimental subjects called only occasionally or remained silent throughout the maintenance period. Table 2 shows the call rates of frogs during the acoustic maintenance period. A within-individual comparison showed that the call rate during the day of silence was significantly lower as compared to the call rate during the last day of chorus exposure (Wilcoxon signed rank test with continuity correction, $V = 75$, $P = 0.005$).

#### 3.2.2. Laboratory vocal activity during exposure to experimental stimuli

Unlike field experiments, individuals tested in the laboratory showed limited responsiveness to sound exposures (Fig. 5A). Two out of seven and two out of eight experimental subjects emitted advertisement calls in response to conspecific chorus and rain sounds, respectively. Four out of eight frogs not exposed to any broadcast sound were vocally active. The GLMM analysis showed that neither the recording period ($\chi^2 = 0$, *d.f.* = 2, $P = 1$), the acoustic treatment ($\chi^2 = 0$, *d.f.* = 2, $P = 1$), or their interaction ($\chi^2 = 2.61$, *d.f.* = 4, $P = 0.625$) had a significant effect on the number of calls emitted by males tested in the laboratory.

#### 3.2.3. Laboratory testosterone levels

The ANCOVA showed that testosterone levels did not differ between the frogs exposed to conspecific chorus, rain sound and silence (Fig. 5B, Table 3, $F = 0.31$, *d.f.* = (2,17) $P = 0.735$, $\eta_p^2 = 0.036$). In addition, plasma testosterone was not related to the average call rate during the maintenance period, or the call rate during the day of silence (Table 3). However, the call rate of frogs during the 31.5 min sound exposure was significantly related to testosterone plasma levels (Table 3, $F = 8.97$, *d.f.* = (1, 17), $P = 0.008$, $\eta_p^2 = 0.345$). After pooling together the data from frogs exposed to the three acoustic treatments, including individuals that did not call, linear regression analysis showed a significant positive association between plasma testosterone and call rate ($F = 10.60$, *d.f.* = (1, 21), $P = 0.004$, $R^2 = 0.335$). However, this significant association is driven by the large number of silent individuals with relatively low testosterone levels. Removing these individuals from the linear regression analysis yields a non-significant association between call rate and testosterone for vocally active males ($F = 0.10$, *d.f.* = (1, 6), $P = 0.762$, $R^2 = 0.02$). Fig. 5B shows a scatterplot of the testosterone levels and call rates of frogs exposed to the three acoustic treatments in the laboratory. The one-way ANOVA analysis showed that the effect of acoustic treatment on testosterone levels was not significant after removing the three covariates related to the calling activity of males from the model ($F = 0.27$, *d.f.* = (2, 21), $P = 0.763$,

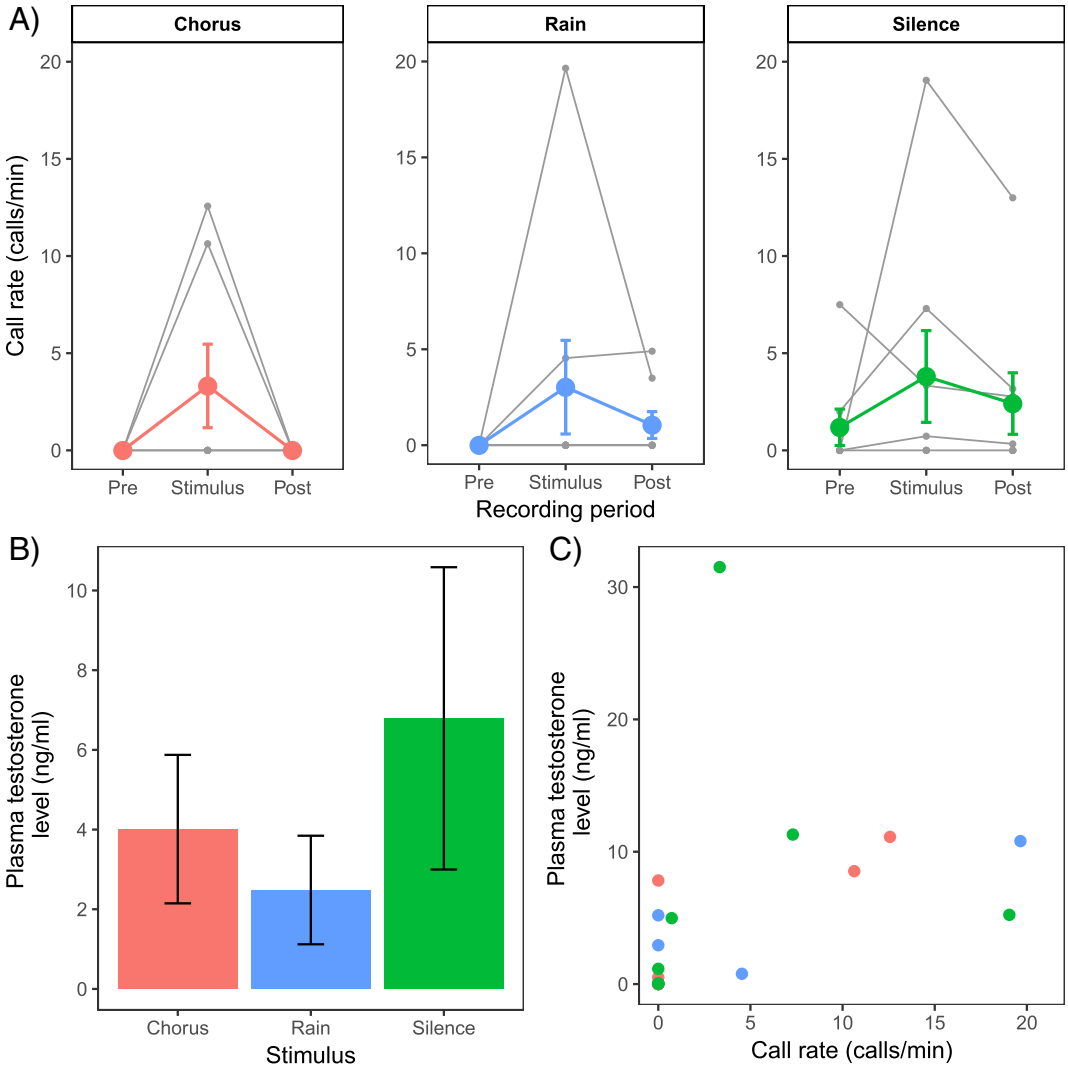


**Fig. 5.** A) Call rates of male *B. taeniata* exposed to conspecific chorus ($N = 7$), rain sound ($N = 8$) and silence ($N = 8$) in the laboratory. B) Plasma testosterone levels of male *B. taeniata* exposed to chorus, rain sound, and silence in the laboratory. Data shown in Fig. 5B correspond to the mean testosterone levels not corrected by the call rate. C) Scatterplot of testosterone levels and calling rate of frogs exposed to the three acoustic treatments in the laboratory. Symbols and colors as in Fig. 4. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

$\eta_p^2 = 0.025$).

In addition, testosterone levels were higher in calling individuals (mean ± s.e.m.: 10.54 ± 3.27 ng/ml) as compared to non-calling males (mean ± s.e.m.: 1.21 ± 0.60 ng/ml) (Wilcoxon rank sum test, $W = 113$, $P < 0.001$).

### 3.3. Comparison of testosterone levels between field and laboratory subjects

Because the three groups of experimental subjects stimulated with chorus sound, rain sound or maintained in silence in the field, had similar testosterone levels, as occurred with animals in captivity, subjects of each group were pooled together to compare their plasma hormone concentration. Testosterone levels of frogs tested in the field (mean ± s.e.m.: 8.23 ± 1.11 ng/ml) were significantly higher than the levels of individuals tested in laboratory settings (mean ± s.e.m.: 4.46 ± 1.49 ng/ml) (Wilcoxon rank sum test with continuity correction, W = 265, $P = 0.006$).

## 4. Discussion

In the present study, male *B. taeniata* frogs were exposed to different acoustic stimuli to evaluate how these sounds influence the circulating testosterone levels and the vocal activity of this species. Field and laboratory experiments revealed that short-term exposure to conspecific

**Table 3**
Results of the ANCOVA analysis showing the effects of stimulus type and call rates of frogs on the plasma testosterone levels of males of *B. taeniata* in the laboratory.

| Dependent variable | Factor | *F* (*d.f.*) | *P* | $\eta_p^2$ |
|---|---|---|---|---|
| $\log_{10}$(testosterone) | Stimulus type | 0.31 (2, 17) | 0.735 | 0.036 |
| | Call rate evoked by experimental stimuli | 8.97 (1, 17) | **0.008** | 0.345 |
| | Average call rate during the acoustic maintenance period (7 or 8 days) | 0.74 (1, 17) | 0.403 | 0.042 |
| | Call rate during the day of silence | 0.11 (1, 17) | 0.746 | 0.006 |

Bold characters indicate significant P values (< 0.05)

chorus sounds and rainfall sounds do not elevate testosterone above the levels of frogs that were not exposed to any broadcast sound. The short duration of the acoustic exposure used in the present study may account for the similar testosterone levels between experimental and control individuals, as long-term exposure to conspecific acoustic cues has been demonstrated to regulate hormone levels in frogs. Male leopard frogs, *Rana sphenocephala* and green treefrogs *Hyla cinerea*, present higher androgen levels after hearing conspecific advertisement vocalizations during successive days as compared to frogs hearing control sounds (Burmeister and Wilczynski, 2000, 2005; Chu and Wilczynski, 2001), and in males of *Rana temporaria* exposed for ten days to conspecific advertisement calls, testicular size and interstitial cell size does not decrease as in non-exposed animals (Brzoska and Obert, 1980). In a study using short exposures Assis et al. (2012) showed that males of *Hypsiboas faber* receiving 10-min stimulation with conspecific calls had lower testosterone plasmatic levels relative to non-stimulated individuals. Furthermore, a former field study in *B. taeniata* reported similar testosterone levels in frogs exposed to about 11 min of acoustic stimulation relative to individuals not subjected to any acoustic treatment, suggesting a lack of effect of short acoustic stimulation on plasmatic testosterone levels in this species (Solís and Penna, 1997).

In studies in other vertebrates using short exposures to sounds of communicational significance, dissimilar results have been found. In spotted antbirds *Hylophylax n. naevioides*, testosterone levels increase after 2 h of song playback but not at earlier times (Wikelski et al., 1999). In contrast with this study, acoustic exposures to conspecific calls lasting 30 min or less cause testosterone increases in toadfishes (Remage-Healey and Bass, 2005) and song sparrows (Moser-Purdy et al., 2017).

In the current study we chose to expose the experimental subjects to short sound treatments, although most of the evidence indicates that long-term exposures are needed to obtain plasmatic testosterone increases, because we were interested in comparing results from field and laboratory settings, and long exposures cannot be managed accurately amid varying conditions inherent to field work. Our choice is justified to some extent by the studies that have shown short-term sound exposures to be effective in increasing levels of this hormone in other vertebrates (Remage-Healey and Bass, 2005; Moser-Purdy et al., 2017).

Rainfall sound effectively evokes vocal responses in male *B. taeniata* (Penna and Zúñiga, 2014, this study), however, testosterone levels do not increase in frogs exposed to this sound relative to individuals kept in silence. Although the influence of natural abiotic sounds on the endocrine status of animals has not been sufficiently explored, recent studies have investigated the physiological stress response associated to anthropogenic noise exposure, showing that human-generated sounds increase circulating glucocorticoid levels in a number of taxa (e.g., Wysocki et al., 2006; Blickley et al., 2012; Rolland et al., 2012; Westlund et al., 2012), including anurans (Tennessen et al., 2014; Kaiser et al., 2015; Troïanowski et al., 2017). Because of the similarity of some general features between anthropogenic noises and natural abiotic sounds, as both types of sound have an important proportion of the energy concentrated in the low-frequency range, glucocorticoid levels may be affected following abiotic sound exposure. Indeed, a study reported that after heavy rains male cane toads (*Rhinella marina*) exhibit increased clasping behavior and elevated corticosterone levels, while testosterone levels remain unchanged (Orchinik et al., 1988). Yet, it is unclear if this elevation of corticosterone in cane toads is induced by the natural sound of rainfall, or other environmental factors related to rainy conditions. Elucidating how abiotic acoustic cues influence the activity of the endocrine system will contribute valuable information on the regulation of behavior under variable environmental conditions; however, this remains an important gap in our current knowledge.

In the field, males of *B. taeniata* increase their call rate in response to chorus and rain sounds, while the vocal activity of control individuals not exposed to these sounds remains constant, and testosterone levels were unrelated to the vocal output of frogs. This result agrees with other studies that have failed to find correlations between androgens and vocal effort in a number of frog species (Burmeister and Wilczynski, 2000; Leary et al., 2008; Leary et al., 2015; Joshi et al., 2017). Also, in free-ranging dark-eyed juncos (*Junco hyemalis carolinensis*) song playback drastically increases the song rate of experimental subjects without any apparent increase in circulating plasma testosterone (Rosvall et al., 2012). It is worth mentioning that the results of the present study are in partial contrast with a previous field study on the same species, in which the call rate elicited by synthetic *B. taeniata* calls was positively correlated with testosterone levels (Solís and Penna, 1997). One major difference between both studies is that in the present investigation we used natural choruses broadcast at 67 dB SPL RMS for stimulation, while in Solís and Penna, 1997 synthetic calls of a single individual were broadcast at amplitudes of 67–79 dB SPL RMS. Such synthetic stimuli are likely to be perceived as a more threatening challenge as compared to the sound of chorusing aggregations and may account for the contrasting results between both studies.

In contrast to the field results, frogs in laboratory settings exhibited limited responsiveness to acoustic stimulation. Individuals that were vocally responsive during the test had higher testosterone levels than individuals that remained silent, but call rate and testosterone levels of calling males did not covary, a lack of association that has been reported to occur in other anurans and related to target-tissue receptors, enzymes acting on hormone pathways and hormone-binding proteins (Leary, 2009). These results are consistent with models proposing that hormone action is exerted through a threshold mechanism, above which no graded relationship occurs between hormone plasma concentration and behavioral effects (Adkins-Regan, 2005; Ball and Balthazart, 2008). A formal evaluation this hypothesis would require hormonal measurements of silent males in the field, which we did not perform in the present study, and thus we consider our results to be suggestive of such an association, but further research is required to better evaluate the nature of hormone-behavior associations in frogs.

Laboratory conditions allowed to homogenize the acoustic experience prior to quantifying the vocal activity of frogs and facilitated a precise control of the acoustic environment in which playbacks were performed. Experiments conducted in this context have the potential of revealing androgen-behavior relationships that may remain obscured amid the complexity of the social and environmental conditions of natural field settings, yet a potential drawback of this approach are the side-effects of captivity on the endocrine system of experimental subjects.

The physiological stress associated to captivity may explain in part the low activity of males in the laboratory, as glucocorticoid levels have been found to increase in birds (Adams et al., 2011; Love et al., 2017) and frogs (Assis et al., 2015; Titon et al., 2017) kept in laboratory settings. Moreover, increased corticosterone levels inhibit the vocal activity of male anurans (reviewed in Leary, 2009), suggesting that different hormones interact in complex ways to influence the vocal behavior of frogs. However, in the laboratory experiments of the current study it is apparent that the low levels of vocal activity displayed by the experimental subjects do not result from general aspects of housing and maintenance, but by the particular experimental schedule chosen, as the vocal activity dropped significantly after the day of acoustic deprivation preceding the experimental acoustic exposures.

The “Energetics-Hormone Vocalization” model (EHV, Emerson, 2001) proposes feedback interactions between corticosterone and androgens, and relates the levels of these hormones with the vocal behavior of frogs. Some studies have shown that higher corticosterone levels are associated with lower testosterone in frogs (e.g., Marler and Ryan, 1996; Leary and Harris, 2013), supporting the inhibitory influence of glucocorticoids on androgens proposed by the EHV model. In the present study, testosterone levels were lower in individuals tested in the laboratory relative to frogs tested in the field, and in captivity silent individuals showed almost undetectable testosterone, results that could be related to the secretion of corticosterone in response to housing, as

discussed above. However, a negative association between corticosterone and testosterone has not been found in studies other than the ones quoted above (e.g., Leary et al., 2004; Joshi et al., 2017), suggesting that other hormonal factors may also play a role in modulating frogs' vocal communication. For example, the neuropeptide arginine-vasotocin (AVT) exerts strong effects on the vocal activity of males, and is known to interact with other hormones, such as androgens, corticosterone and melatonin (reviewed in Wilczynski et al., 2017). Additional potential mediators of acoustic environmental effects on vocal behavior are steroidal hormones produced de novo locally in brain areas reported originally in mammals, namely neurosteroids (Robel and Baulieu, 1994). Such steroids have been reported to occur in the anuran brain (reviewed in Do Rego et al., 2009) and the presence of enzymes implicated in their production has also been documented in these vertebrates (Mensah-Nyagan et al., 1994, 1996, 1999). Acute fluctuations of neurosteroids in response to acoustic social interactions occur in telencephalic nuclei controlling song in birds (Remage-Healey et al., 2008). Neurosteroid regulation has not been demonstrated in frogs' brains, but the strong dependence of communication on acoustic environments in these vertebrates offers opportune avenues for exploring effects of such molecules in vocal activation in breeding contexts.

Finally, another possibility to be considered in interpreting our current results is that sound environments produce changes in the expression of androgen receptors rather than on androgen plasma levels. To our knowledge no studies have addressed such an effect, remaining an issue open for further experimental efforts. In order to elucidate the intricate links between vocal behavior, the acoustic environment and the endocrine status of anurans, future studies should attempt to measure simultaneously different hormones and their corresponding receptors. However, the small blood samples obtained from *B. taeniata* frogs precluded measuring other hormones in the same experimental males.

## 5. Conclusion

In summary, we sought to evaluate the interplay between different acoustic cues, the vocal behavior and testosterone levels of male *B. taeniata* frogs. In the field, frogs increased their vocal output in response to chorus and rain sound playback, and their vocal activity was unrelated to testosterone levels. In the laboratory, the overall vocal activity of males tested was low, and males gave limited responses to acoustic playback, probably due to the particular experimental protocol used in the present study combined with stressful captivity conditions. Similarly to frogs tested in the field, no association between calling rate and testosterone was found in the laboratory. Also, calling males had higher testosterone levels than non-calling males in the laboratory. Our results are suggestive of a threshold relation of testosterone and the calling activity of the species studied, where testosterone levels above the threshold level permit calling behavior without any apparent graded-dose association with the call rate. Undoubtedly further work is required in order to elucidate how environmental acoustic cues other than the largely studied conspecific social signals influence the behavior and hormone secretion in animals, and the complexities of hormone-behavior associations.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2019.104605.

## Data availability

Data are available upon request from the corresponding author.

## Acknowledgments

We thank José Serrano and Cristián Sánchez for their assistance during field and laboratory work. We thank Jorge Mpodozis and Nelson Velásquez for contributing valuable comments on an early version of the manuscript.

## Funding

Supported by FONDECYT grant 1140014 to MP and MQ, Guillermo Puelma Foundation award and CONICYT-PCHA/MagisterNacional/2016-22161325 scholarship to MIM.

## Ethics

Capture of animals was authorized by Permit 7626/2015 of the Poultry and Livestock Service, Chile. Experimental procedures were authorized by Protocol CBA# 0652 of the Committee of Bioethics for Animal Research, Faculty of Medicine, University of Chile.

## Author's contributions

MQ and MP conceived and designed the experiments. MIM, MQ and MP conducted the experiments. MIM, MQ and MM performed the hormone analyses. MIM performed the statistical analyses. All the authors reviewed, edited and approved the manuscript.

## Declaration of competing interest

The authors report no competing interests.

## References

Adams, N.J., Farnworth, M.J., Rickett, J., Parker, K.A., Cockrem, J.F., 2011. Behavioural and corticosterone responses to capture and confinement of wild blackbirds (*Turdus merula*). Appl. Anim. Behav. Sci. 131, 246–255.

Adkins-Regan, E., 2005. Hormones and Animal Social Behavior. Princeton University Press, Princeton, New Jersey.

Assis, V.R., Navas, C.A., Mendonça, M.T., Gomes, F.R., 2012. Vocal and territorial behavior in the Smith frog (*Hypsiboas faber*): relationships with plasma levels of corticosterone and testosterone. Comp. Biochem. Phys. A. 163, 265–271.

Assis, V.R., Titon, S.C.M., Barstotti, A.M.G., Titon Jr., B., Gomes, F.R., 2015. Effects of acute restraint stress, prolonged captivity stress and transdermal corticosterone application on immunocompetence and plasma levels of corticosterone on the cururu toad (*Rhinella icteica*). PLoS One 10, e0121005.

Ball, G.F., Balthazart, J., 2008. Individual variation and the endocrine regulation of behavior and physiology in birds: a cellular/molecular perspective. Phyl. Trans. R. Soc. B. 363, 1699–1710.

Bates, D., Maechler, M., Bolker, B., Walker, S., 2015. Fitting linear mixed-effects models using lme4. J. Stat. Soft. 67, 1–48.

Blickley, J.L., Word, K.R., Krakauer, A.H., Phillips, J.L., Sells, S.N., Taff, C.C., Wingfield, J.C., Patricelli, G.L., 2012. Experimental chronic noise is related to elevated fecal corticosteroid metabolites in lekking male greater sage-grouse (*Centrocercus urophasianus*). PLoS One 7, e50462.

Brooks, M.E., Kristensen, K., van Benthem, K.J., Magnusson, A., Berg, C.W., Nielsen, A., Skaug, H.J., Maechler, M., Bolker, B.M., 2017. glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. The R Journal 9, 378–400.

Brzoska, J., Obert, H.J., 1980. Acoustic signals influencing the hormone production of the testes in the grass frog. J. Comp. Physiol. A. 140, 25–29.

Burmeister, S.S., Wilczynski, W., 2000. Social signals influence hormones independently of calling behavior in the treefrog (*Hyla cinerea*). Horm. Behav. 38, 201–209.

Burmeister, S.S., Wilczynski, W., 2001. Social context influences androgenic effects on calling in the green treefrog (*Hyla cinerea*). Horm. Behav. 40, 550–558.

Burmeister, S.S., Wilczynski, W., 2005. Social signals regulate gonadotropin-releasing hormone neurons in the green treefrog. Brain Behav. Evol. 65, 26–32.

Calisi, R.M., Bentley, G.E., 2009. Lab and field experiments: are they the same animal? Horm. Behav. 56, 1–10.

Chu, J., Wilczynski, W., 2001. Social influences on androgen levels in the southern leopard frog, *Rana sphenocephala*. Horm. Behav. 121, 66–73.

Desprat, J.L., Lengagne, T., Dumet, A., Desouhant, E., Mondy, N., 2015. Immunocompetence handicap hypothesis in tree frog: trade-off between sexual signals and immunity? Behav. Ecol. 26, 1138–1146.

Deviche, P., Sharp, P.J., Dawson, A., Sabo, J., Fokidis, B., Davies, S., Hurley, L., 2012. Up to the challenge? Hormonal and behavioral responses of free-ranging male Cassin's sparrows, *Peucaea cassinii*, to conspecific song playback. Horm. Behav. 61, 741–749.

Do Rego, J.-L., Seong, J.Y., Van Luu-The, D.B., Larhammar, D., Tsutsui, K., Pelletier, G., Tonon, M.C., Vaudry, H., 2009. Steroid biosynthesis within the frog brain: a model of neuroendocrine regulation. Trends in comparative endocrinology and neurobiology. Ann. N. Y. Acad. Sci. 1163, 83–92.

Dunlop, R.A., 2016. The effect of vessel noise on humpback whale, *Megaptera novaeangliae*, communication behavior. Anim. Behav. 111, 13:21.

Emerson, S.B., 2001. Male advertisement calls: behavioral variation and physiological processes. In: Ryan, M.J. (Ed.), Anuran Communication. Smithsonian Institution Press, Washington, pp. 36–44.
Fedurek, P., Slocombe, K.E., Enigk, D.K., Thompson, M.E., Wrangham, R.W., Muller, M.N., 2016. The relationship between testosterone and long-distance calling in wild male chimpanzees. Behav. Ecol. Sociobiol. 70, 659–672.
Foerster, K., Poesel, A., Kunc, H., Kempenaers, B., 2002. The natural plasma testosterone profile of male blue tits during the breeding season and its relation to song output. J. Avian Biol. 33, 269–275.
Fox, J., Weisberg, S., 2011. An R Companion to Applied Regression, 2nd ed. Sage, Thousand Oaks, CA.
Gall, M.D., Wilczynski, W., 2014. Prior experience with conspecific signals enhances midbrain responsiveness to conspecific vocalizations. J. Exp. Biol. 217, 1977–1982.
Geipel, I., Smeekes, M.J., Halfwerk, W., Page, R., 2019. Noise as an informational cue for decision-making: the sound of rain delays bat emergence. J. Exp. Biol. 222, jeb192005.
Genova, R.M., Marchaterra, M.A., Knapp, R., Fergus, D., Bass, A.H., 2012. Glucocorticoid and androgen signaling pathways diverge between advertisement calling and non-calling fish. Horm. Behav. 62, 426–432.
Gerhardt, H.C., Huber, F., 2002. Acoustic Communication in Insects and Anurans. University of Chicago Press, Chicago.
Gough, D.C., Mennill, D.J., Nol, E., 2014. Singing seaside: Pacific wrens (*Troglodytes pacificus*) change their songs in the presence of natural and anthropogenic noise. Wilson J. Ornithol. 126, 269–278.
Goymann, W., 2009. Social modulation of androgens in male birds. Gen. Comp. Endocr. 163, 149–157.
Gramapurohit, N.P., Radder, R.S., 2013. Changes in plasma sex steroids in relation to reproductive behavior of the toad, *Bufo melanostictus*. Copeia 2, 332–337.
Hartig, F., 2019. DHARMa: Residual Diagnostics for Hierarchical (Multi-level/Mixed) Regression Models. (R package version 0.2.4).
Joshi, A.M., Narayan, E.J., Gramapurohit, N.P., 2017. Interrelationship among steroid hormones, energetics and vocalization in the Bombay night frog (*Nyctibatrachus humayuni*). Gen. Comp. Endocr. 246, 142–149.
Kaiser, K., Devito, J., Jones, C.G., Marentes, A., Perez, R., Umeh, L., Weickum, R.M., McGovern, K.E., Wilson, E.H., Saltzman, W., 2015. Effects of anthropogenic noise on endocrine and reproductive function in White's treefrog, *Litoria caerulea*. Conserv. Physiol. 3, cou061.
Leary, C.J., 2009. Hormones and acoustic communication in anuran amphibians. Integr. Comp. Biol. 49, 452–470.
Leary, C.J., Harris, S., 2013. Steroid hormone levels in calling males and males practicing alternative non-calling mating tactics in the green treefrog, *Hyla cinerea*. Horm. Behav. 63, 20–24.
Leary, C.J., Jessop, T.S., Garcia, A.M., Knapp, R., 2004. Steroid hormone profiles and relative body condition of calling and satellite toads: implications for proximate regulation of behavior in anurans. Behav. Ecol. 15, 313–320.
Leary, C.J., Garcia, A.M., Knapp, R., 2006. Elevated corticosterone levels elicit non-calling mating tactics in male toads independently of changes in circulating androgens. Horm. Behav. 49, 425–432.
Leary, C.J., Garcia, A.M., Knapp, R., Hawkins, D.L., 2008. Relationships among steroid hormone levels, vocal effort and body condition in an explosive-breeding toad. Anim. Behav. 76, 175–185.
Leary, C.J., Lippincott, J., Harris, S., Hawkins, D.L., 2015. A test of the energetics-hormone vocalization model in the green treefrog. Gen. Comp. Endocr. 213, 32–39.
Lenske, A.K., La, V.T., 2014. White-throated sparrows alter songs differentially in response to chorusing anurans and other background noise. Behav. Process. 105, 28–35.
Lenth, R.V., 2016. Least-squares means: the R packages lsmeans. J. Stat. Soft. 69, 1–33.
Ligges, U., Krey, S., Mersmann, O., Schnackenberg, S., 2016. tuneR: analysis of music. URL. http://r-forge.r-project.org/projects/tuner/.
Love, A.C., Lovern, M.B., DuRant, S.E., 2017. Captivity influences immune responses, stress endocrinology, and organ size in house sparrows (*Passer domesticus*). Gen. Comp. Endocr. 252, 18–26.
Lüdeke, D., 2019. Sjstats: Statistical Functions for Regression Models. version 0.17.4. https://doi.org/10.5281/zenodo.1284472.
Madison, F.N., Rouse Jr., M.L., Balthazart, J., Ball, G.F., 2015. Reversing song behavior phenotype: testosterone driven induction of singing and measures of song quality in adult male and female canaries (*Serinus canaria*). Gen. Comp. Endocr. 215, 61–75.
Marler, C.A., Ryan, M.J., 1996. Energetic constraints and steroid hormone correlates of male calling behavior in the túngara frog. J. Zool. Lond. 204, 397–409.
Meitzen, J., Thompson, C.K., Choi, H., Perkel, D.J., Brenowitz, E.A., 2009. Time course of changes in Gambel's white-crowned sparrow song behavior following transitions in breeding condition. Horm. Behav. 55, 217–227.
Mendonça, M.T., Licht, P., Ryan, M.J., Barnes, R., 1985. Changes in hormone levels in relation to breeding behavior in male bullfrogs (*Rana catesbeiana*) at the individual and population levels. Gen. Comp. Endocr. 58, 270–279.
Mensah-Nyagan, A.G., Feuilloley, M., Dupont, E., Do-Rego, J.L., Leboulenger, F., Pelletier, G., Vaudry, H., 1994. Immunocytochemical localization and biological activity of 3β-hydroxysteroid dehydrogenase in the central nervous system of the frog. J. Neurosci. 14, 7306–7318.
Mensah-Nyagan, A.G., Feuilloley, M., Do-Rego, J.-L., Marcual, A., Lange, C., Tonon, M.C., Pelletier, G., Vaudry, H., 1996. Localization of 17β-hydroxysteroid dehydrogenase and characterization of testosterone in the brain of the male frog. Proc. Natl. Acad. Sci. U. S. A. 93, 1423–1428.
Mensah-Nyagan, A.G., Do-Rego, J.-L., Beaujean, D., Luu-The, V., Pelletier, G., Vaudry, H., 1999. Neurosteroids: expression of steroidogenic enzymes and regulation of steroid biosynthesis in the central nervous system. Pharmacol. Rev. 51, 63–81.
Moser-Purdy, C., MacDougall-Schackleton, S.A., Bonier, F., Graham, B.A., Boyer, A.C., Menill, D.J., 2017. Male song sparrows have elevated testosterone in response to neighbors versus strangers. Horm. Behav. 93, 47–52.
Orchinik, M., Licht, P., Crews, D., 1988. Plasma steroid concentrations change in response to sexual behavior in *Bufo marinus*. Horm. Behav. 22, 338–350.
Penna, M., Zúñiga, D., 2014. Strong responsiveness to noise interference in an anuran from the southern temperate forest. Behav. Ecol. Sociobiol. 68, 85–97.
Penna, M., Pottstock, H., Velásquez, N., 2005. Effect of natural and synthetic noise on evoked vocal responses in a frog of the temperate austral forest. Anim. Behav. 70, 639–651.
Penna, M., Moreno-Gómez, F.N., Muñoz, M.I., Cisternas, J., 2017. Vocal responses of austral forest frogs to amplitude and degradation patterns of advertisement calls. Behav. Proc. 140, 190–201.
Pytte, C.L., Rusch, K.M., Ficken, M.S., 2003. Regulation of vocal amplitude by the blue-throated hummingbird, *Lampornis clemenciae*. Anim. Behav. 66, 703–710.
R Core Team, 2017. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria URL. https://www.R-project.org/.
Remage-Healey, L., Bass, A.H., 2005. Rapid elevation in both steroid hormones and vocal signaling during playback challenge: a field experiment in gulf toadfish. Horm. Behav. 25, 297–305.
Remage-Healey, L., Maidment, N., Schlinger, B.A., 2008. Forebrain steroid levels fluctuate rapidly during social interactions. Nat. Neurosc. 11, 1327–1334.
Rights, J.D., Sterba, S.K., 2019. Quantifying explained variance in multilevel models: an integrative framework for defining R-squared measures. Physchol. Methods. 24, 309–338.
Robel, P., Baulieu, E.-E., 1994. Neurosteroids biosynthesis and function. Trends Endoc. Metabol. 5, 1–8.
Rolland, R.M., Parks, S.E., Hunt, K.E., Castellote, M., Corkeron, P.J., Nowacek, D.P., Wasser, S.K., Kraus, S.D., 2012. Evidence that ship noise increases stress in right whales. P. Roy. Soc. B-Biol. Sci. 279, 2363–2368.
Rosvall, K.A., Reichard, D.G., Ferguson, S.M., Whittaker, D.J., Ketterson, E.D., 2012. Robust behavioral effects of song playback in the absence of testosterone or corticosterone release. Horm. Behav. 62, 418–425.
Slabbekoorn, H., 2016. Aiming for progress in understanding underwater noise impact on fish: complementary need for indoor and outdoor studies. In: Popper, A.N., Hawkins, A. (Eds.), The Effects of Noise on Aquatic Life II. Springer, New York, pp. 1057–1065.
Solís, R., Penna, M., 1997. Testosterone levels and evoked vocal responses in a natural population of the frog *Batrachyla taeniata*. Horm. Behav. 31, 101–109.
Sueur, J., Aubin, T., Simonis, C., 2008. Seewave: a free modular tool for sound analysis and synthesis. Bioacoustics 18, 213–226.
Tennessen, J.B., Parks, S.E., Langkilde, T., 2014. Traffic noise causes physiological stress and impairs breeding migration behavior in frogs. Conserv. Physiol. 2, cou032.
Titon, S.C.M., Assis, V.R., Titon Jr., B., Cassettari, B.O., Fernandes, P.A.C.M., Gomes, F.R., 2017. Captivity effects on immune response and steroid plasma levels of a Brazilian toad (*Rhinella schneideri*). J. Exp. Zool. 1–12. https://doi.org/10.1002/jez.2078.
Townsend, D.S., Moger, W.H., 1987. Plasma androgen levels during male parental care in a tropical frog (*Eleutherodactylus*). Horm. Behav. 21, 93–99.
Troïanowski, M., Mondy, N., Dumet, A., Arcanjo, C., Lengagne, T., 2017. Effects of traffic noise on tree frog stress levels, immunity, and color signaling. Conserv. Biol. https://doi.org/10.1111/cobi.12893.
Wada, M., Gorbman, A., 1977. Relation of mode of administration of testosterone to evocation of male sex behavior in frogs. Horm. Behav. 8, 310–319.
Wells, K.D., 2007. The Ecology and Behavior of Amphibians. The University of Chicago Press, Chicago.
Westlund, K., Fernström, A.-L., Wergard, E.-M., Fredlund, H., Hau, J., Spangberg, M., 2012. Physiological and behavioural stress responses in cynomolgus macaques (*Macaca fascicularis*) to noise associated with construction work. Lab. Anim. 46, 51–58.
Wetzel, D.M., Kelley, D.B., 1983. Androgen and gonadotropin effects on male mate calls in South African clawed frogs, *Xenopus laevis*. Horm. Behav. 17, 388–404.
Wickham, H., 2009. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag, New York.
Wikelski, M., Hau, M., Wingfield, J.C., 1999. Social instability increases plasma testosterone in a year-round territorial neotropical bird. P. Roy. Soc. B-Biol. Sci. 266, 551–556.
Wilczynski, W., Quispe, M., Muñoz, M.I., Penna, M., 2017. Arginine vasotocin, the social neuropeptide of amphibians and reptiles. Front. Endocrinol. 8, 186.
Wingfield, J.C., Wada, M., 1989. Changes in plasma-levels of testosterone during male-male interactions in the song sparrow, *Melospiza melodia* – time course and specificity of response. J. Comp. Physiol. A. 166, 189–194.
Wysocki, L.E., Dittami, J.P., Ladich, F., 2006. Ship noise and cortisol secretion in European freshwater fishes. Biol. Conserv. 128, 501–508.
Zhao, L., Zhu, B., Wang, J., Brauth, S.E., Tang, Y., Cui, J., 2017. Sometimes noise is beneficial: stream noise informs vocal communication in the little torrent frog *Amolops torrentis*. J. Ethol. 35, 259–267.
Ziegler, L., Arim, M., Bozinovic, F., 2016. Intraspecific scaling in frog calls: the interplay of temperature, body size and metabolic condition. Oecologia 181, 673–681.