Efecto de la exposición a ruidos sobre el estado hormonal y respuesta vocal del anuro *Batrachyla taeniata*

Tesis

Entregada A La
Universidad De Chile
En Cumplimiento Parcial De Los Requisitos
Para Optar Al Grado De

Magíster en Ciencias Biológicas

Facultad De Ciencias

Por

Matías Igor Muñoz Sandoval

Enero, 2018

Director de Tesis: Dr. Mario Penna

FACULTAD DE CIENCIAS

UNIVERSIDAD DE CHILE

INFORME DE APROBACION

TESIS DE MAGÍSTER

Se informa a la Escuela de Postgrado de la Facultad de Ciencias que la Tesis de Doctorado presentada por la candidata.

Matías Igor Muñoz Sandoval

Ha sido aprobada por la comisión de Evaluación de la tesis como requisito para optar al grado de Magíster en Ciencias Biológicas, en el examen de Defensa Privada de Tesis rendido el día 11 de noviembre 2017.

Director de Tesis:

Dr. Mario Penna .....

Comisión de Evaluación de la Tesis:

Dr. Jorge Mpodozis .....

Dr. Nelson Velásquez .....

AGRADECIMIENTOS

Agradezco a mis papás por su constante apoyo.

A Maricel, José y Claudio, del laboratorio, por su amistad, y por siempre estar dispuestos a conversar.

CONICYT-PCHA/MagisterNacional/2016 - 22161325.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

RESUMEN

En animales con reproducción estacional, la expresión de diversas conductas se encuentra regulada hormonalmente. En este contexto, la emisión de señales acústicas durante el cortejo e interacciones agresivas ha sido relacionada con los niveles de andrógenos. Adicionalmente, las señales acústicas sociales, como por ejemplo las vocalizaciones emitidas por conespecíficos, modulan la secreción de andrógenos en vertebrados. En la presente investigación se evaluó la respuesta vocal de la rana *Batrachyla taeniata* al sonido de coro conespecífico y ruido de lluvia, tanto en condiciones naturales de terreno como en el laboratorio. Además, se midieron los niveles de testosterona plasmática en los individuos expuestos a estos estímulos acústicos. Para los experimentos realizados en terreno y laboratorio, los niveles de testosterona de ranas expuestas a sonido de coro y ruido de lluvia fueron similares en comparación a los niveles de individuos en silencio. En terreno, los sujetos experimentales incrementaron la tasa de canto y la duración de las vocalizaciones en respuesta al coro y al ruido de lluvia. La actividad vocal evocada por estos sonidos no se relacionó con la testosterona plasmática. En el laboratorio, los individuos mostraron una baja sensibilidad a la estimulación acústica, y a diferencia de los experimentos en terreno, no incrementaron significativamente su tasa de canto en respuesta al ruido de coro ni de lluvia. A pesar de la escasa actividad vocal, la tasa de cantos y los niveles de testosterona medidas en el laboratorio se relacionaron positivamente.

Adicionalmente, los niveles de testosterona fueron mayores en machos que emitieron vocalizaciones respecto a individuos que permanecieron en silencio. En conjunto, estos resultados indican que la exposición a ruidos de origen biótico y abiótico no modifica los niveles de testosterona plasmática en machos de *B. taeniata*. La relación positiva entre actividad vocal y niveles de testosterona en condiciones de laboratorio podría indicar que en condiciones controladas, relaciones entre andrógenos y comportamiento son más susceptibles de ser observadas que en medio de la complejidad de las condiciones ambientales y sociales de terreno.

ABSTRACT

In seasonally breeding animals the expression of diverse reproductive behaviors is hormonally regulated, and the emission of acoustic signals during courtship and aggressive interactions has been related to circulating androgen levels. Also, social acoustic cues, such as the vocalizations of conspecifics, are known to modulate the secretion of androgens in vertebrates. Through playback experiments we evaluated the vocal responses of male *Batrachyla taeniata* frogs to conspecific chorus sound and rain noise in natural field conditions and under controlled laboratory settings. Additionally, we measured the plasmatic testosterone levels of individuals exposed to these acoustic stimuli. For experiments conducted in the field and the laboratory, circulating testosterone levels of frogs exposed to chorus sound and rain noise were similar as

compared to the levels of individuals in silence. In the field, frogs increased their call rate and call duration in response to playbacks of chorus and rain noise, and the evoked calling activity was unrelated to the plasma testosterone. In the laboratory, frogs showed limited responsiveness to acoustic stimulation and, unlike field experiments, did not increase their call rate in response to noise exposure. In spite of the limited vocal activity displayed by males, the overall calling activity and plasma testosterone levels were positively correlated in frogs tested in the laboratory. Additionally, testosterone levels were higher in vocally active males as compared to non-calling individuals. Taken together, these results indicate that *B. taeniata* testosterone levels are not altered following an acute short-term exposure to biotic and abiotic noises. The positive relationship between the calling activity of frogs tested in the laboratory and testosterone levels could indicate that under controlled conditions relationships between androgens and behavior are more susceptible to be observed than amid the complexity of environmental and social conditions of natural settings.

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).

Among vertebrates, the expression of social communication by means of acoustic signals has been shown to relate closely to androgen levels. For example, in chimpanzees the rate of pant-hoot emission, a type of call thought to be involved in male-male competition, is positively correlated with urinary testosterone (Fedurek et al. 2016). Also, vocally active midshipman fish, *Porichthys notatus*, have higher plasma and testis androgen levels than silent satellite males (Genova et al. 2012). Particularly, the relationship between steroid hormones and vocal behavior has been extensively studied in passerine birds, where androgen levels and singing effort are typically positively correlated (e.g., Foerster et al. 2002, Meitzen et al. 2009, Madison et al. 2015). Androgen receptors are widespread in the brain song control system (SCS) of passerines, and testosterone acting on these nuclei modulates the acoustic complexity of the song (reviewed in Alward et al. 2017), while androgen-dependent increases in singing rate result from the action of testosterone on the medial preoptic nucleus (Alward et al. 2013, Shevchouk et al. 2017). Similarly to birds, the

presence of brain androgen receptors and the neuromodulatory effects of sex steroids on the vocal control systems have been described to considerable detail in other vertebrates (reviewed in Yamaguchi & Kelley 2003, Bass & Remage-Healey 2008). As such, the endocrine regulation of acoustic communication, as well as the specific brain circuitry underlying this association, is a common theme across vertebrates.

Furthermore, the interplay between behavior and hormones is known to have bidirectional components, as hormones have 'activational' effects on the expression of reproductive behaviors (see above), but social cues may in turn modulate circulating hormone levels (Adkins-Regan 2005). The 'Challenge Hypothesis' proposed by Wingfield et al. 1990 to explain the inter-individual variability in androgen levels of free-living birds posits that unstable social conditions, such as male-male interactions, elevate circulating testosterone above baseline levels. In a reproductive communication context, male androgen levels increase after hearing the vocalizations of conspecific males. For example, playback experiments reported that plasma testosterone increases in male spotted ant birds *Hylophylax naevioides naevioides* after being exposed to conspecific songs for two hours (Wikelski et al. 1999). Similarly, in male green treefrogs *Hyla cinerea*, long-term exposure to conspecific chorus sound elevates androgen levels (Burmeister & Wilczynski 2000) and increases the number of brain gonadotropin-releasing hormone cells (Burmeister & Wilczynski 2005) to a larger extent relative to frogs hearing a control stimulus. In contrast with those results, testosterone levels do not increase following an acoustic challenge in

some bird species (e.g., Wingfield & 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).

Natural environments comprise not only the sounds produced by animals, but also the sounds derived from human activities (i.e., anthropogenic noise). The diverse behavioral strategies used by animals to overcome the impact of anthropogenic noise are well documented (reviewed in Brumm 2013) and the associated endocrine responses have been described in some vertebrates. For instance, the low-frequency noise of ship traffic increases fecal glucocorticoid levels in whales (Rolland et al. 2012) and cortisol secretion in fishes (Wysocki et al. 2006). Similarly, exposure to anthropogenic noises, such as drilling and traffic noise, increase corticosteroid metabolites in male greater-sage grouse *Centrocercus urophasianus* (Blickley et al. 2012). Additional sources of environmental noise are the sounds generated by natural geophysical and atmospheric processes, such as local weather conditions (i.e., the natural noises of rain, wind or thunder, among others). Although these are ubiquitous sources of acoustic interference in natural environments, potential effects of these sounds 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. Blue-throated hummingbirds (*Lampornis clemenciae*) increase the amplitude of their chipping bouts in response to increasing levels of creek noise (Pytte et al. 2003) and

Pacific Wrens (*Troglodytes pacificus*) emit songs having larger syllable lengths near the coastal shoreline, where sea shore noise is louder (Gough et al. 2014). Similarly, exposure to wind noise induces vocal modifications in humback whales (*Megaptera nevaeangiae*) and white-throated sparrows (*Zonotrichia albicolis*) (Lenske & La 2014, Dunlop 2016). Elucidating if these responses to natural abiotic noises are accompanied by changes in the endocrine status of organisms will provide valuable insights on the role of hormones in adjusting behavior under dynamic environmental conditions.

In most anuran species (frogs and toads) courtship involves the emission of advertisement calls by males. During the breeding season, receptive conspecific females are attracted by male advertisement vocalizations and males engage in antiphonal calling contests (Gerhardt & Hubert 2002, Wells 2007). Because of the strong dependence of 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 & Gorbman 1977). In contrast, androgen implants are effective in stimulating the vocal activity of castrated *Xenopus laevis* males (Wetzel & Kelley 1983). In addition the spontaneous calling activity of male *Hyla cinerea* is positively correlated with androgen levels in testosterone

implanted subjects (Burmeister & Wilczynski 2001) and testosterone supplemented male *Hyla arborea* emit longer calling bouts as compared to control males (Desprat et al. 2015).

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 bullfrogs (Mendonça et al. 1985), while in two toad species, *Bufo woodhousii* and *Bufo cognatus*, vocally active and non-calling satellite males have similar plasmatic androgen levels (Leary et al. 2004, Leary et al. 2006). Moreover, other studies in different species have found that androgen levels are higher in calling individuals relative to silent conspecifics (Townsend & Morger 1987, Marler & Ryan 1996, Leary & Harris 2013, Joshi et al. 2017) and the evoked vocal activity of male *Batrachyla taeniata* frogs is positively correlated with testosterone levels (Solís & Penna 1997). As pointed out by Leary (2009), the different behaviors expressed by the non-calling males could explain the discrepancies, as studies have investigated in addition to calling males, brooding (Townsend & Morger 1987), foraging (Marler et al. 1996) and satellite (Leary & Harris 2013) silent individuals.

In correspondence with studies in other vertebrates, recent research has also revealed that exposure to anthropogenic noise increases corticosterone levels in male (Kaiser et al. 2015, Troïanowski et al. 2017) and female (Tennessen et al. 2014) frogs, highlighting the relevance of the acoustic

environmental components other than conspecific social signals on the hormonal status of anurans.

The banded wood frog, *Batrachyla taeniata*, is an anuran from the temperate austral forest in South America that has an extended breeding period lasting about three months from late summer to early autumn. The advertisement call consists of a series of brief pulses produced in a fast sequence having a call duration of about 0.5 s (Penna and Veloso 1990). More recent studies have shown that the evoked vocal responses of males are selective for temporal features of the calls (Penna 1997, Penna and Velásquez 2011) and its calling activity is remarkably activated when exposed to natural abiotic noises, in particular rain noise (Penna and Zúñiga 2014). A field playback study showed that the responsiveness of males of this species to synthetic advertisement calls is directly related to plasma testosterone levels (Solís and Penna 1997).

Because acoustic social stimuli elicit fast (within minutes) endocrine and behavioral responses in vertebrates (e.g., Remage-Healey & Bass 2005, Moser-Purdy et al. 2017), in the present study we sought to investigate whether acute exposure to biotic (e.g., conspecific chorus) and abiotic (e.g., rain noise) sounds induce changes in the testosterone levels of male *B. taeniata* frogs. Previous research has shown that conspecific calls and rain noise induce strong vocal responses in male *B. taeniata* frogs (Penna & Zúñiga 2014), and thus represent salient environmental cues for this species. By comparing the endocrine and behavioral responses to short-term stimulation with biotic and abiotic acoustic

cues I expect to shed light on the regulation of vocal signaling behavior under diverse acoustic conditions.

GOALS

General goal

To evaluate the effect of acute exposure to conspecific chorus and rain noise on the vocal behavior and testosterone levels of male *B. taeniata* frogs under laboratory and field settings. By combining laboratory and field playback experiments we sought to identify significant relationships that may undergo masked amid the variability and complexity of natural environmental conditions.

Specific goals

1.- To measure the effect of acute exposure to conspecific choruses and rain noise on the vocal responsiveness and on testosterone levels in natural populations of *B. taeniata* frogs.

2.- To measure the effect of acute exposure to conspecific choruses and rain noise on the vocal responsiveness and on the testosterone levels of *B. taeniata* frogs in controlled laboratory conditions.

3.- To evaluate potential relationships between the emission of advertisement calls and testosterone levels in *B. taeniata* frogs tested in the field and in laboratory conditions.

HYPOTHESES

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

MATERIALS AND METHODS

Stimuli synthesis

Test stimuli synthesis

The hormonal (i.e., testosterone levels) and behavioral responses (i.e., vocal activity) of male *B. taeniata* frogs to different acoustic stimuli were studied in the laboratory and field exposing the subjects to the same set of audio files in both settings. *B. taeniata* choruses were selected from field recordings conducted in the locality of Tinquilco (39°07' S, 71°46' W) in February 1993, using 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 on 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 seconds. The experimental subjects were exposed to the nine 3-min choruses

leaving 30 s of silence in between successive choruses in order to prevent fatigue of vocally active individuals.

Rain noise was 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 x 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. 2) and 3-min rain noises were created using the same procedure as for the chorus sounds.

The temporal and spectral characteristics of the *B. taeniata* choruses and rain noises used for stimuli synthesis were judged to be representative of these signals and were devoid of other biotic or abiotic sounds. A similar procedure has been previously used to create files of abiotic noise of different kinds to study the vocal behavior of native frogs (Penna et al. 2005; Penna & Zúñiga 2014). The order of presentation of the nine 3-min choruses and rain noises to the experimental subjects followed a random sequence and the exposure level used for both kinds of sound was 67 dB SPL RMS (linear frequency weighting, slow time weighting). Rain and other abiotic noises at this amplitude have been previously shown to elicit vocal responses in *B. taeniata* males (Penna & Zúñiga 2014).

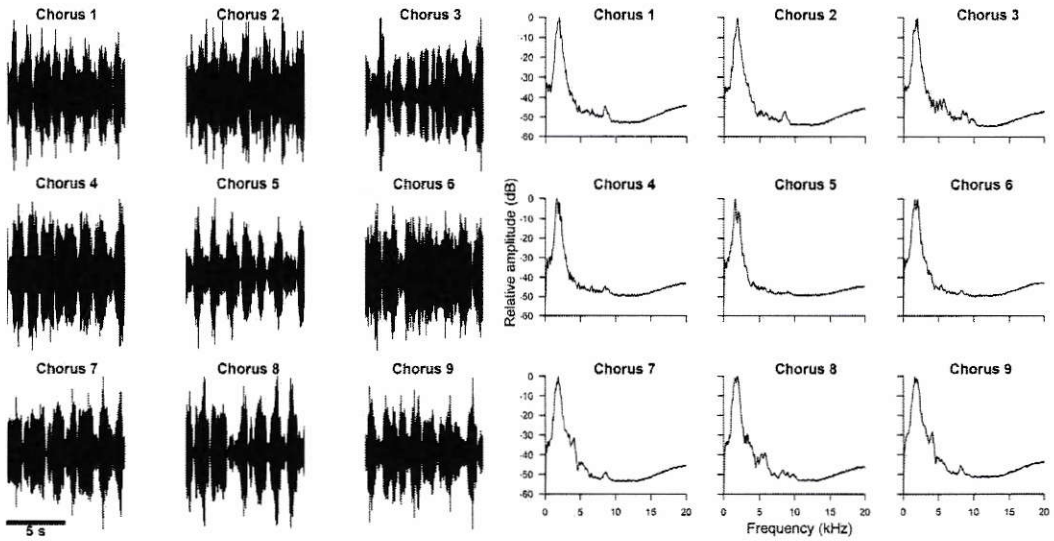

Figure 1: A) Oscillograms and B) average power spectra of the nine 10-second duration *B. taeniata* chorus sounds used for stimuli edition. Power spectra were computed with a Hanning window of 1024 points and 0% overlap.

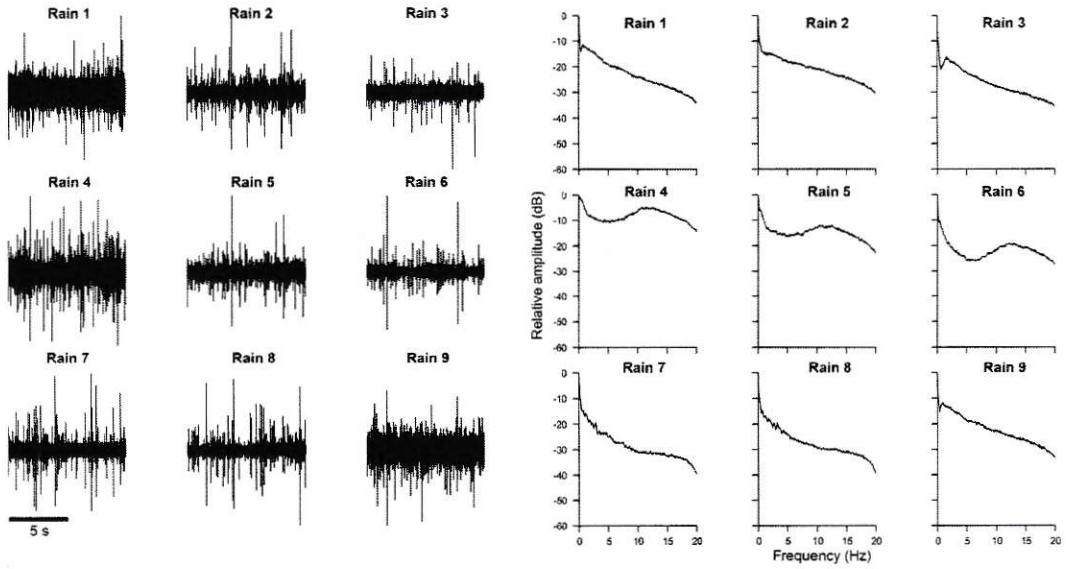

Figure 2: A) Oscillograms and B) average power spectra of the nine 10-second duration rain noises used for stimuli edition. Power spectra were computed with a Hanning window of 1024 points and 0% overlap.

Conditioning stimuli synthesis

Because the long-term previous acoustic experience may modulate the androgen levels of frogs (Burmeister & Wilczynski 2000, Chu & Wilczynski 2001), all the individuals tested in the laboratory underwent an acoustic conditioning phase. For 7 or 8 consecutive nights all the frogs were exposed to conspecific choruses, to homogenize the auditory experience of the individuals before conducting the noise exposure experiments. The same nine 10-s duration *B. taeniata* chorus segments used for the synthesis of the test stimuli were pasted consecutively to create 10-minute duration audio files. Each 10-min duration chorus sound had a fade-in and fade-out of 5 seconds. The nine 10 min choruses were presented in random succession leaving 3 min of silence in between choruses. During each night this sequence of choruses was presented during 5 hours starting one hour 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).

Field experiments procedure

Study site

Field experiments were conducted at night, between 21:00 and 04:00 hrs, 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 call from the substrate, hidden among grasses and underneath fallen leaves and branches in a forest where *Drymis winteri* was the predominant tree.

Field noise exposure experiments

Vocally active male *B. taeniata* were located in the field and exposed to conspecific chorus (N = 5), natural rain noise (N = 5) or silence (N = 5). The snout-vent lengths (Kruskal-Wallis rank sum test, $\chi^2 = 3.8343$, $df = 2$, $P = 0.147$) and body weights (Kruskal-Wallis rank sum test, $\chi^2 = 1.1492$, $df = 2$, $P = 0.5629$) of frogs were similar across acoustic treatments. Acoustic stimuli were played back from 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 to 67 dB SPL RMS with the attenuators during brief sound broadcasts before proceeding with the experiment. Following stimulation, all the experimental individuals were left in silence for 30 additional minutes. During two minutes before stimuli onset, and throughout the experiment (31.5

minutes of stimulation + 30 minutes of silence), the vocal activity of focal individuals was recorded 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). Figure 3 shows a schematic diagram of the stimulation protocol used for the playback experiments. Stimuli delivered thorough the Ipod nano were recorded on the right channel of the same digital recorder. The spontaneous vocal activity of individuals not exposed to any broadcast sound was recorded a time equaling the duration of the playback experiments. To avoid acoustic interferences during the experiments, neighboring frogs were either captured or silenced by gently tapping the substrate.

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).

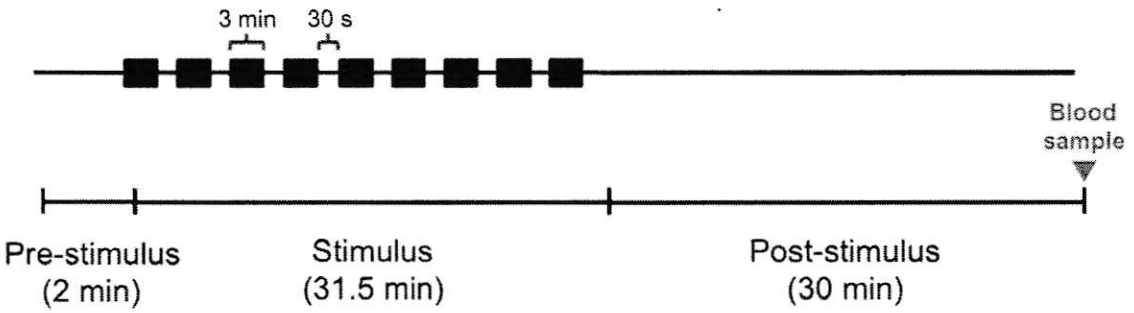

Figure 3: Schematic diagram of the stimulation protocol. The experimental procedure included two initial minutes of spontaneous activity, followed by 31.5 minutes of acoustic stimulation and finally 30 minutes of post-stimulation vocal activity. After completing the stimulation protocol males were rapidly captured and a blood sample was obtained. The vocal activity of males was recorded through out 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 sound or rain noise 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.

Laboratory experiments procedure

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 the 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 sac). 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 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.

Laboratory acoustic conditioning phase

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 x 20 x 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 nine chorus conditioning stimuli played back in a randomized order 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 the loudspeakers placed inside each of the chambers. 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 noise exposure experiment"* subsection). Following the 7 or 8 nights of conspecific chorus exposure, stimulation was discontinued and individuals were left in silence for one day before testing. All the experiments were conducted within 3 weeks of the arrival of the frogs in the laboratory.

Throughout the acoustic conditioning phase 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-hours period of conditioning chorus presentation.

Laboratory noise exposure experiment

The overall experimental protocol used for the noise exposure experiments in the laboratory was similar to the one used in the field. The day of the test frogs were exposed to *B. taeniata* chorus sound (N = 8), natural rain noise (N = 8) or silence (N = 8). The snout-vent lengths (Kruskal-Wallis rank sum test, $\chi^2 = 0.34917$, $df = 2$, $P = 0.8398$) and body weights (Kruskal-Wallis rank sum test, $\chi^2 = 0.\ 0.39335$, $df = 2$, $P = 0.\ 8215$) of frogs were similar across acoustic treatments. Due to a failure of the recording system during the noise 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. Noise exposure experiments were conducted within the interval of time during which the conditioning sound was played back, that is, within five hours starting one hour after lights went out. 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 two minutes before the onset of the stimuli playback and the post-stimulation vocal activity was recorded for additional 30 minutes. The spontaneous 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.

Body measurements and blood sampling

The same blood sampling protocol was used for frogs tested in the laboratory and the field. Once the noise 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 at least 3 minutes and blood samples were collected through cardiac puncture 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. After obtaining the blood samples, individuals were transcardially perfused with 0.75% saline solution and 4% paraformaldehyde and brains stored for a concurrent immunohistochemical study.

Plasma testosterone analysis

Plasma levels of testosterone were measured using a competitive binding radioimmunoassay kit (DIA source ImmunoAssays, DIAsource TESTO-RIA-CT kit). The radioimmunoassay kit used has a high specificity for testosterone, having a cross reactivity with dihydrotestosterone of 0.31%. 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). The plasma testosterone levels of two individuals fell below the detection threshold of the assay and therefore they were assigned 0 ng/ml.

Samples were run in duplicate when enough plasma was collected. Average intra-assay variation was 22.99% and 8.55% for plasma samples collected in the laboratory and in the field, respectively.

Acoustic analysis of vocal responses

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

Calls emitted by male *B. taeniata* frogs during the laboratory conditioning phase were automatically counted using 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. Because of the large number of calls emitted by the frogs during the acoustic conditioning phase, and the unreliability of the automatic detection script to measure call duration precisely, other acoustic properties of these calls were not measured. For each individual we computed the average number of calls emitted daily and used this value for further statistical analyses (see below, "Statistical analysis" section). 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.

Statistical analysis

All the statistical analyses and figures were performed using R (version 3.3.3, R Core Team 2017).

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 call rate and call duration of frogs were included as dependent variables in two different models that included the fixed-effect of recording period (categorical variable with 3 levels: pre, during and post exposure). 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 recording period 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) multiple comparisons performed with the library 'lsmeans' (version 2.26-3, Lenth 2016).

Analysis of covariance (ANCOVA) was used to investigate whether plasma testosterone level was related to the different acoustic treatments (chorus, rain or silence). Because a former study found that the evoked vocal activity and testosterone levels were correlated in male *B. taeniata* (Solís & Penna 1997), the call rate of individuals during stimuli presentation was included as a covariate.

Laboratory experiments

Similarly to field experiments, the vocal responsiveness of frogs to the different acoustic treatments was investigated by fitting LMMs for call rate and call duration. Temperature was stable in the laboratory settings were frogs were housed and tested and therefore it was not included as a covariate. Individuals were included in the models as random-effects and the significance of the recording period was evaluated by means of LRT.

We used ANCOVA to compare testosterone levels of frogs exposed to the different acoustic treatments (chorus, rain or silence). Three continuous covariates were included in the model to assess their possible influence on individual testosterone levels: (1) the evoked call rate during the 30 min of noise exposure; (2) the average number of calls emitted during the acoustic conditioning phase and (3) the number of calls produced by the subjects during the day of silence prior to testing.

A large number of individuals tested in laboratory conditions did not call during the playback experiments (See results section), and thus 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.

The assumptions of all the linear models fitted (ANCOVAs and LMMs) were evaluated by visually inspecting the model residuals, and if necessary, dependent variables were log(x+1) or square-root-transformed to attain the normality assumption of parametric tests.

RESULTS

Field experiments

Field vocal activity

In the field, the two acoustic noises tested evoked vocal responses of male *B. taeniata* effectively. The linear mixed model analyses revealed a significant effect of the recording period (pre, during and post exposure) on the call rate of frogs exposed to conspecific chorus (Fig. 4A, $\chi^2 = 14.136$, $df = 2$, $P = 0.0008518$) and rain noise (Fig. 4B, $\chi^2 = 11.008$, $df = 2$, $P = 0.004071$). Post hoc analyses showed that for both acoustic treatments the call rate was higher during stimuli presentation as compared to the pre- and post-stimulation periods (Tukey HSD, $P < 0.05$ for all comparisons). There were no differences in the call rate of frogs before and after the acoustic rain and chorus treatments (Tukey HSD, $P > 0.1$)

The call duration of frogs treated with chorus showed a significant effect of the recording period (Fig. 5A, $\chi^2 = 19.248$, $df = 2$, $P < 0.0001$). Call duration was longer during chorus presentation relative to calls emitted before (Tukey HSD, $P < 0.0001$) and after (Tukey HSD, $P = 0.0004$) the playback. Also, there was a significant increase in the post-playback. call duration relative to calls emitted before stimuli presentation (Tukey HSD, $P = 0.0275$). Recording period had a non-significant effect on the call duration of individuals exposed to rain

noise (Fig. 5B, χ^2 = 2.8573, *df* = 2, *P* = 0.2396). In contrast to the vocal output of frogs under playback treatment, the call rate (Fig. 4C, χ^2 = 1.423, *df* = 2, *P* = 0.4909) and call duration (Fig. 5C, χ^2 = 5.3778, *df* = 2, *P* = 0.06796) of frogs in silence was stable across recording periods.

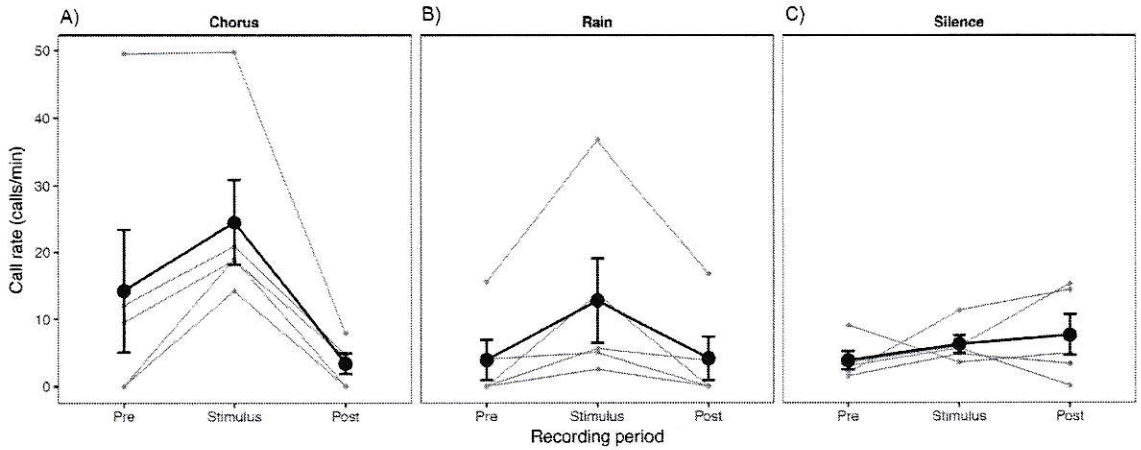

Figure 4: Call rates of male *B. taeniata* exposed to A) conspecific chorus noise (N = 5), B) rain noise (N = 5) and C) silence (N = 5) in the field. Grey lines and dots indicate responses of individual males. Black dots and error bars indicate the mean ± s.e.m.

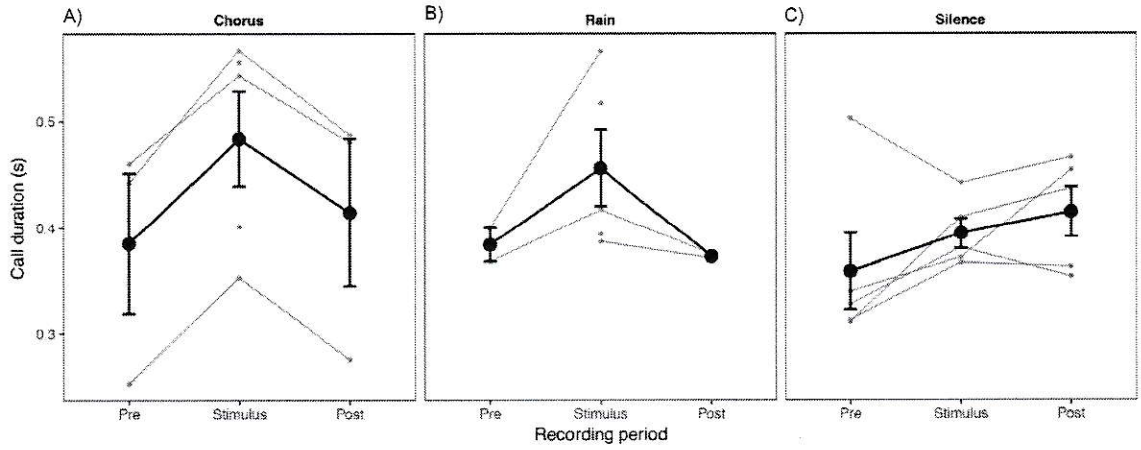

Figure 5: Call durations of male *B. taeniata* exposed to A) conspecific chorus noise (N = 5), B) rain noise (N = 5) and C) silence (N = 5) in the field. Grey lines and dots indicate individual responses. Black dots and error bars indicate the mean ± s.e.m.

Field testosterone levels

The ANCOVA showed that testosterone levels did not differ between the frogs exposed to conspecific chorus noise, rain noise and silence (Fig. 6, F = 0.4323, df = 2, P = 0.6618) and that the calling activity of males was unrelated to their plasma testosterone levels (F = 0.7446, df = 1, P = 0.4106). The interaction between acoustic treatment and call rate was not significant (F = 1.4865, df = 2, P = 0.2768).

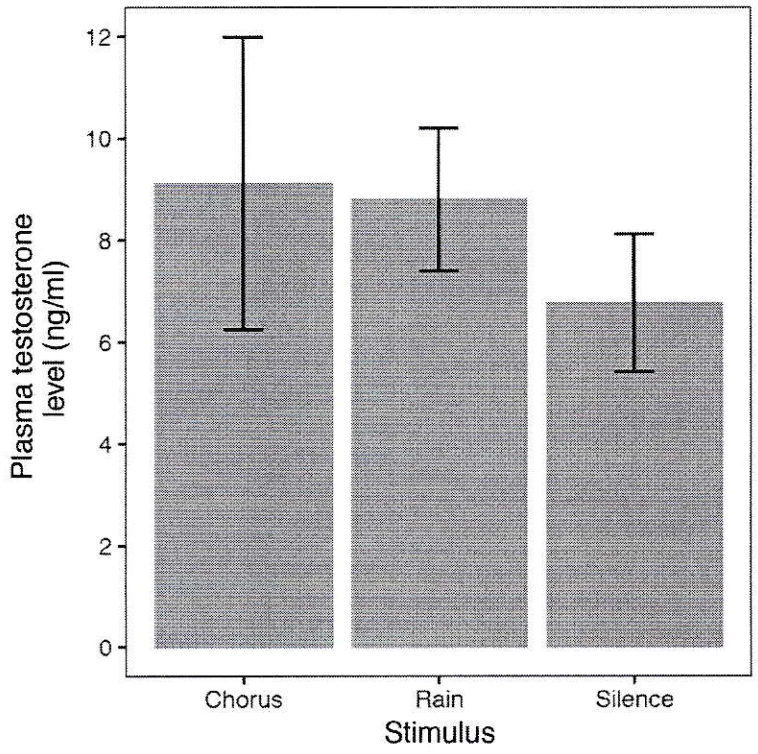

Figure 6: Plasma testosterone levels of male *B. taeniata* exposed to conspecific chorus (N = 5), rain noise (N = 5) and silence (N = 5) in the field. Data are expressed as mean ± s.e.m.

Laboratory experiments

Acoustic conditioning phase

The vocal activity of males during the 7 – 8 days of acoustic conditioning 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 conditioning phase (Fig. 7A). Similarly, the vocal activity of males was variable during the day of silence before conducting the noise exposure experiments (Fig. 7B). A within-individual comparison showed that the number of calls emitted during the day of silence was significantly lower as compared to the number of vocalizations emitted during the last day of conditioning chorus (Wilcoxon signed rank test, $V = 75$, $P = 0.005355$).

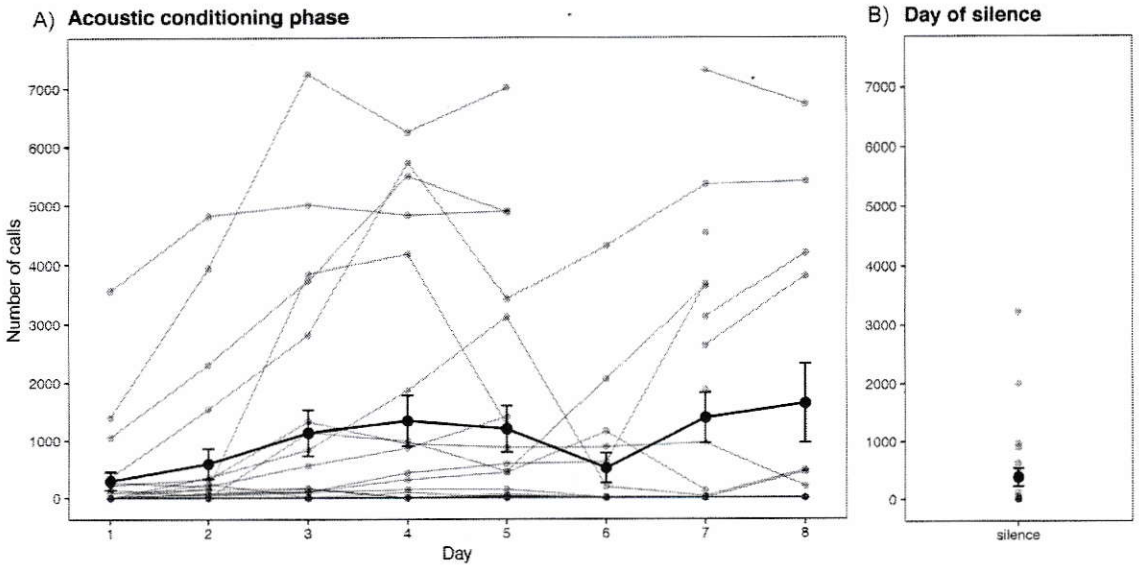

Figure 7: A) Number of calls emitted by male *B. taeniata* (N = 24) during 7 – 8 consecutive days of acoustic conditioning with conspecific chorus. B) Number of calls emitted during the day of silence prior to conducting the noise exposure experiments. Due to a failure of the recording system, the vocal activity of six individuals could not be measured during the day 6 of the conditioning phase. Grey lines and dots depict individual responses. Black dots and error bars indicate the mean ± s.e.m.

Laboratory vocal activity

Unlike field experiments, individuals tested in the laboratory showed limited responsiveness to noise exposure (Fig. 8). Two out of seven and two out of eight experimental subjects emitted advertisement calls in response to conspecific chorus and rain noise, respectively (Fig. 8A, B). Four out of eight frogs treated with silence showed spontaneous vocal activity (Fig. 8C). The effect of recording period was not significant for frogs exposed to chorus (χ^2 = 4.9584, *df* = 2, *P* = 0.08381), rain (χ^2 = 4.0321, *df* = 2, *P* = 0.1332) or silence (χ^2 = 2.7075, *df* = 2, *P* = 0.2583). The restricted vocal activity of frogs tested in the laboratory precluded any analysis of call duration.

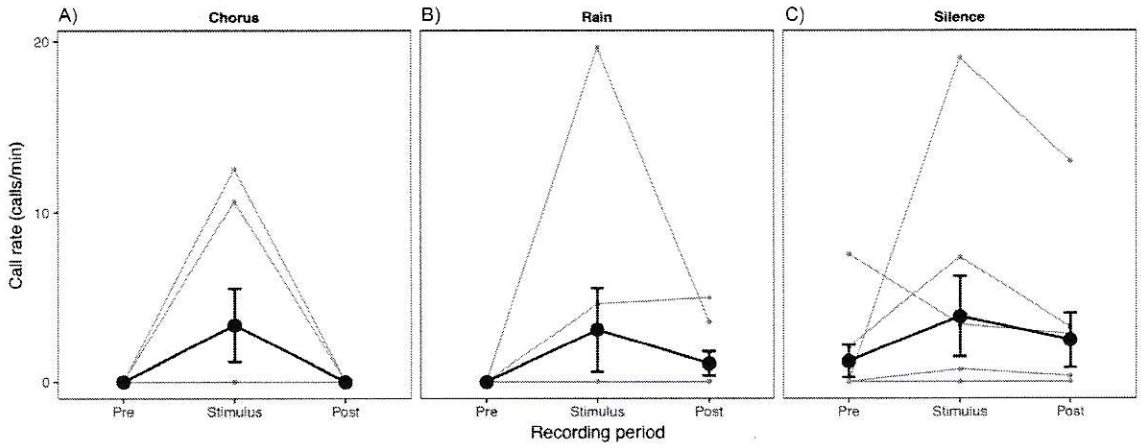

Figure 8: Call rate of male *B. taeniata* exposed to A) conspecific chorus (N = 7), B) rain noise (N = 8) and C) silence (N = 8) in the laboratory. Two, two and four individuals called during A) chorus sound, B) rain noise and C) silence exposure, respectively. Grey lines and dots depict individual responses. Black dots and error bars indicate the mean ± s.e.m.

Laboratory testosterone level

The ANCOVA showed that testosterone levels did not differ between the frogs exposed to conspecific chorus noise, rain noise and silence (Fig. 9, Table 1, F = 0.4995, df = 2, P = 0.6154). In addition, plasma testosterone was not related to the average calling activity during the conditioning phase, or the number of calls emitted during the day of silence (Table 1). In contrast, the call rate of frogs during the 30 min noise exposure was significantly related to testosterone plasma levels (Table 1). Indeed, 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 (Fig. 10, F = 19.61, df = (1, 21), P = 0.0002334). Testosterone levels were higher in calling individuals as compared with non-calling males (Fig. 11, Wilcoxon rank sum test, W = 7, P = 0. 0006933).

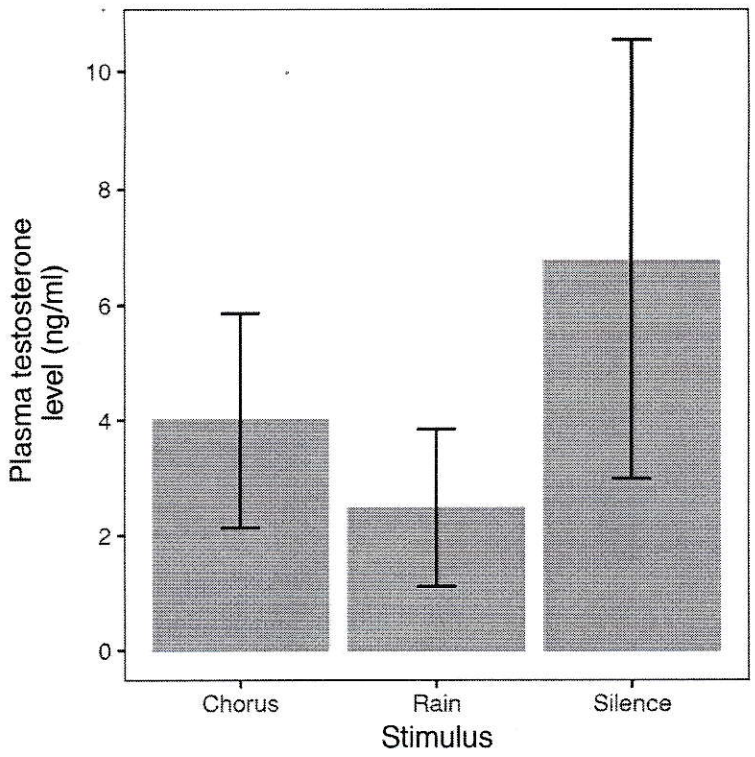

Figure 9: Plasma testosterone levels of male *B. taeniata* exposed to conspecific chorus noise (N = 7), rain noise (N = 8) and silence (N = 8) in the laboratory. Data are expressed as mean ± s.e.m.

Table 1: ANCOVA showing the effects of stimulus type and variables associated to the vocal activity of frogs on the plasma testosterone levels of male *B. taeniata* in the laboratory.

Dependent variable	Factor	*F*	*df*	*P*
log(Testosterone + 1)	Stimulus type	0.4995	2	0.61545
	Stimulus evoked call rate	9.3552	1	**0.00711**
	Average call number during the acoustic conditioning	0.1819	1	0.67512
	Call number during the day of silence	0.1153	1	0.73834

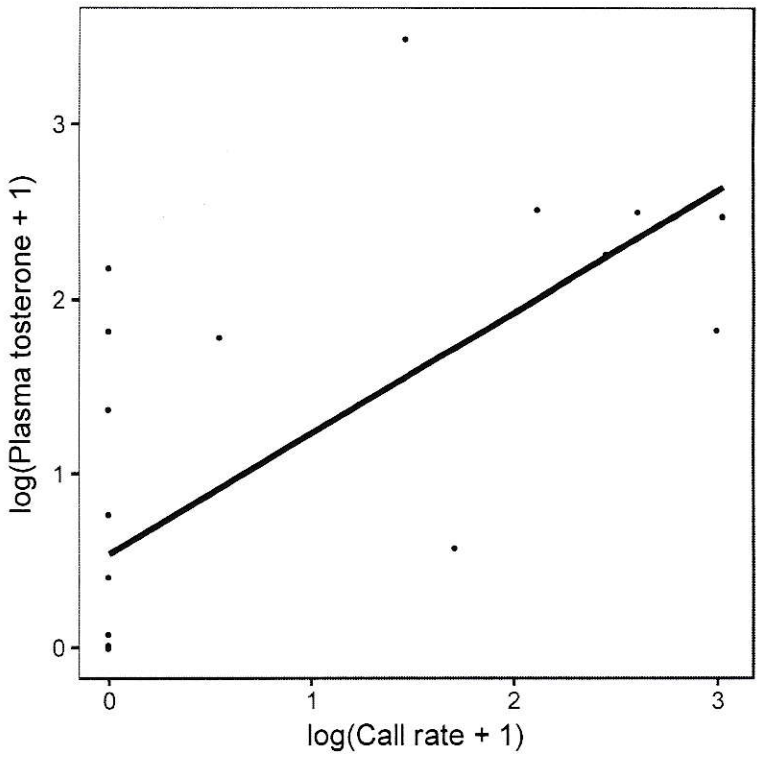

Figure 10: Linear regression between plasma testosterone levels and call rate of frogs (N = 23) tested in laboratory settings. The data from individuals exposed to conspecific chorus noise, rain noise and silence were pooled for the analysis. Variables were transformed to improve the normality of the residuals.

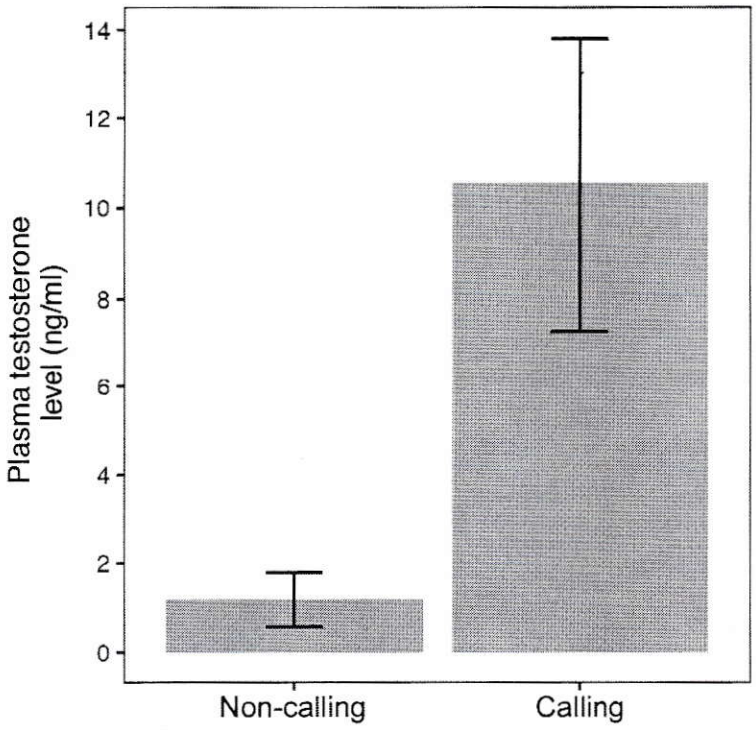

Figure 11: Plasma testosterone levels of non-calling (N = 15) and calling (N = 8) male *B. taeniata* tested in the laboratory. The same data are presented in Figure 10. Data are expressed as mean ± s.e.m.

DISCUSSION

In the present study, male *B. taeniata* frogs were exposed to different acoustic stimuli to determine 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 chorus and rainfall noise do not elevate testosterone above the levels of frogs that were not exposed to any broadcast sound. 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 for various consecutive days as compared to frogs hearing control sounds (Burmeister & Wilczynski 2000, Chu & Wilczynski 2001) and in males of *Rana temporaria* exposed to conspecific advertisement calls, testicular size and interstitial cell size does not decrease as in non exposed animals (Brzoska & Obert 1980). These studies suggest that the short duration of the acoustic exposure used in the present study (31.5 minutes) may explain the similar testosterone levels of male *B. taeniata* tested with different acoustic stimuli. This is a plausible explanation, as in spotted antbirds, *Hylophylax n. naevioides*, testosterone levels elevate only after two hours of song playback (Wikelski et al. 1999).

Although rainfall noise effectively evokes vocal responses in male *B. taeniata* (Penna & Zúñiga 2014, this study), testosterone levels do not increase in frogs exposed to this sound. As discussed above, longer playback experiments may be necessary to induce testosterone production in frogs. However, the secretion of other hormones in response to this sound is an alternative outcome. Although the influence of 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). In consideration of these studies, and the similar spectral composition of anthropogenic and natural abiotic noises, containing the main frequency components in the low-frequency range, measuring the secretion of glucocorticoids following abiotic noise exposure stands as a promising avenue for future research. 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 noise of rainfall, or other environmental factor 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.

Overall, the results of this study do not support a dose-dependent association between testosterone levels and the calling activity of frogs. In the field, *B. taeniata* increased their call rate and call duration in response to chorus sound and rain noise but not in response to control silence, and testosterone levels were unrelated to the vocal output of frogs. In contrast, in laboratory settings frogs exhibited limited responsiveness to acoustic stimulation, yet the calling activity evoked by these sounds was positively associated with plasma testosterone levels. Laboratory conditions allowed the homogenization of the prior acoustic experience, and the quantification of the vocal activity of frogs before conducting the experiments. Additionally, laboratory settings facilitated the careful control of the acoustic environment in which playbacks were performed. Thus, experiments conducted in this context have the potential of revealing androgens-behavior relationships that remain obscured amid the complexity of the social and environmental conditions present in natural field settings. However, the apparent graded association between calling and testosterone should be interpreted cautiously, as the vocal activity of frogs in the laboratory was scarce and does not reflect the response pattern of individuals tested under natural field conditions. The physiological stress associated to captivity may explain 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 under 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.

Social isolation is a method commonly used in behavioral endocrinology to standardize the previous experience of experimental subjects and to reach baseline hormonal levels (e.g., Oliveira et al. 2005, Galhardo & Oliveira 2014), however, it is possible that exposing male *B. taeniata* to a day of silence before conducting the playback experiments in the laboratory decreased the testosterone levels and inhibited the vocal behavior of frogs. Indeed, the vocal output of frogs decreased during the day of silence as compared to their calling activity during the last day of conditioning chorus playback. Comparing the present behavioral and endocrine measurements with results obtained from individuals not exposed to a day of silence prior to testing would help to clarify the potential effect of this time interval on our results.

The "Energetics-Hormone Vocalization" model (EHV, Emerson 2001) states that the high energetic demand associated to calling in frogs increases corticosterone levels, and that at some threshold level, elevated corticosterone inhibits androgens release, thus decreasing the vocal activity of males. According to the model, calling is resumed once the energetic reserves are restored, circulating corticosterone decreases and androgen levels increase back again. The EHV provides a useful framework for testing the interplay of hormones and the vocal behavior of frogs, and experimental studies have reported limited support for the predictions derived from this model (Leary 2004,

Leary & Harris 2013, Leary et al. 2015, Joshi et al. 2017), suggesting that other hormonal factor 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). Thus, 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 multiple different hormones. Unfortunately, the small blood samples obtained from *B. taeniata* frogs precluded measuring other hormones in the same experimental males.

The lack of association between testosterone and the vocal output of *B. taeniata* found in field conditions in the present study is in consonance with other studies that have failed to find correlations between androgens and vocal effort in a number of frog species (Burmeister & Wilczynski 2000, Leary et al. 2008, Leary et al. 2015, Joshi et al. 2017). Nevertheless, this result is in contrast with a previous field study in which the call rate elicited by synthetic calls was positively correlated with testosterone levels in the same species (Solís & Penna 1997). A series of methodological differences could account for this discrepancy. In the current study acoustic stimuli were broadcast at a constant amplitude of 67 dB SPL RMS, while the study by Solís & Penna 1997 played back the synthetic calls at variable amplitudes ranging from about 84 – 95 dB peak SPL, which corresponds to an estimated amplitude of about 67 – 79 dB SPL RMS. Therefore, the frogs tested by Solís & Penna 1997 were exposed to louder

stimuli as compared to the amplitude of the stimuli used in the current study. As noted by Leary 2014, differences in the sounds used for stimulation may also explain discrepancies in the association between acoustic stimulation and circulating hormones in frogs. In the present study natural chorus sounds were edited for stimuli synthesis while Solís & Penna 1997 employed synthetic individual calls. Thus, the calls of a single nearby rival male, as simulated by Solís & Penna 1997 through playback, are likely to be perceived as a more threatening challenge as compared to the sound of chorusing aggregations, and may relate to the contrasting results between both studies.

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 noise playback, and their vocal activity was unrelated to testosterone levels. Similar results have been obtained from experiments in free-ranging dark-eyed juncos (*Junco hyemalis carolinensis*). In this species song playback drastically increases the song rate of experimental subjects without any apparent increase in circulating plasma testosterone (Rosvall et al. 2012). The overall vocal activity of males tested in the laboratory was low, and males did not respond to acoustic playback. In spite of this limited responsiveness, a positive association between the call rate and testosterone levels was found. Also, calling males had higher testosterone levels than non-calling males. This hormone-behavior relationship should be interpreted cautiously, as the vocal activity of males in the laboratory largely underrepresents the normal vocal activity of males in natural settings,

and is likely explained by the stress-induced secretion of corticosterone associated with captivity. Undoubtedly further work is required in order to elucidate how different environmental acoustic cues, beyond the largely studied conspecific social signals, influence the behavior and hormone secretion in animals.

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.

Alward, B.A., Balthazart, J., Ball, G.F. 2013. Differential effects of global versus local testosterone on singing behavior and its underlying neural substrate. P. Natl. Acad. Sci. USA. 128:19573-19578.

Alward, B.A., Rouse Jr., M.L., Balthazart, J., Ball, G.F. 2017. Testosterone regulates birdsong in an anatomically specific manner. Anim. Behav. 124:291-298.

Assis, V.R., Titon, S.C.M., Barstotti, A.M.G., Titon, B. Jr., 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.

Bass, A.H., Remage-Healey, L. 2008. Central pattern generatons for social vocalization: Androgen-dependent neurophysiological mechanisms. Horm. Behav. 53:659-672.

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.

Brumm, H. 2013. Animal communication and noise. Springer-Verlag, Berlin.

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.

Chu, J., Wlczynski, 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.

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 M.J., Ryan (ed), Anuran communication, pp. 36-44. Smithsonian Institution Press, Washington.

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.

Galhardo, L., Oliveira, R.F. 2014 The effects of social isolation on steroid hormone levels are modulated by previous social status and context in a cichlid fish. Horm. Behav. 65:1-5.

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: 436-432.

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.

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. 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., 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., 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., 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.

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.

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.

Orchinik, M., Licht, P., Crews, D. 1988. Plasma steroid concentrations change in response to sexual behavior in *Bufo marinus.* Horm. Behav. 22:338-350.

Oliveira, R.F., Carneiro, L.A., Canario, A.V.M. 2005. No hormonal response in tied fights. Nature 437:207–208.

Penna, M. Selectivity of evoked vocal responses in the time domain by frogs *Batrachyla* (Leptodactylidae). J. Herpetol. 31:30-45.

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., Velásquez, N. 2011. Heterospecific vocal interactions in a frog from the southern temperate forest, *Batrachyla taeniata*. Ethology 117:63-71.

Penna, M., Veloso, A. 1990. Vocal diversity in frogs of the South American temperate forest. J. Herpetol. 24:23-32.

Penna, M., Zúñiga, D. 2014. Strong responsiveness to noise interference in an anuran from the southern temperate forest. Behav. Ecol. Sociobiol. 68:85-97.

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/.

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.

Shevchouk, O.T., Ghorbanpor, S., Ball, G.F., Cornil, C.A., Balthazart, J. 2017. Testosterone-induced neuroendocrine changes in the medial preoptic area precede song activation and plasticity in song control nuclei of female canaries. Eur. J. Neurosci. 45: 886-900.

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, B. Jr., 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. doi: 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. doi: 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. 8:310-219.

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.

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., Hegner, R.E., Dufty, A.M., Ball, G.F. 1990. The "Challenge Hypothesis": Theoretical implications for patterns of testosterone secretion, mating systems, and breeding strategies. Am. Nat. 136:829-846.

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.

Yamaguchi, A., Kelley, D.B. 2003. Hormonal mechanisms in acoustic communication. In Simmons, A.M., Popper, A.N., Fay, R.R. (eds), Acoustic communication. pp. 275-323. Springer-Verlag, New York.

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.