

Vocal plasticity at different time spans during exposures to biotic and abiotic natural sounds in an austral temperate forest frog

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ABSTRACT

Several instances of short-term plasticity of reproductive behaviors are known to modulate interactions among competing individuals. Habituation and sensitization processes result in decrements and increments, respectively, of agonistic behaviors in breeding areas. The occurrence of such phenomena in the long term have been reported to a quite restricted extent. Frogs communicating acoustically experience auditory activation along days of exposure to conspecific signals and thus we looked for vocal activation over such time span in male frogs *Batrachyla taeniata*. A total of 72 frogs were exposed during 8 or 10 days to either 5 hours of either broadcast of natural chorus sound, rain sound or left in silence, and subjected to stimulation with synthetic conspecific calls before and after such treatments. Results showed no significant variation of vocal activity between pre- and post- exposure days, indicating that no long-term plasticity phenomena occurred. However within each day of exposure an initial increment followed by a decrement in call rate were observed, pointing to processes of short-term sensitization and habituation or fatigue, respectively, but potential effects of photoperiod and energy depletion also need to be assessed. The lack

of long-term sensitization of vocal activity contrasts with the auditory system activation for such time span reported for other anurans. The mismatch between both phenomena could relate to constraints imposed by the high energy expenditure that characterizes anuran vocal activity.

Keywords: Abiotic noise, Biotic noise, Frogs, Habituation, Vocal responses, Sensitization

Summary statement

Male frogs exposed for various days to sounds of conspecific chorus, rain or silence do not change their vocal activity throughout this period, but exhibit stereotyped daily vocal temporal patterns

INTRODUCTION

Strategies for confronting acoustic interference often imply selective processes leading to adaptation along evolutionary time spans (e.g. Price et al., 2003; Snell-Rood, 2013; Potvin, 2016; Pigliucci, 2005). Evidence for selective responses to biotic sound environments has been extensively documented and formally addressed as the acoustic adaptation hypotheses (e.g. Wilkins et al., 2012; Okamoto and Grether, 2013). In addition, phenomena operating within the life term of organisms implying phenotypic plasticity, a process enabling individuals to express different phenotypes dependent on environmental conditions, have been extensively reported. A number of studies have documented responses to experimental exposures to biotic interference within short time spans, consisting in reductions of signal rate or alterations in signal timing in the presence of heterospecific signals in insects (Latimer and Broughton, 1984; Römer et al., 1989; Schatral and Yeon, 1990), anurans (Littlejohn and Martin, 1969; Wong et al., 2000; Penna and Velásquez, 2011; Penna and Meier, 2011) and birds (Popp et al., 1985; Brumm and Slabbekoorn, 2005; Brumm, 2006; Luther, 2008, 2009).

The characterization of changes in vocal output dependent on natural abiotic noises has been restricted relative to effects of biotic sounds and has mainly focused on torrent noise. The relevance of this acoustic component for evolutionary trends of vocal structure has been stressed by different studies (Goutte et al. 2013, 2016; Vargas-Salinas and Amézquita 2013) and even shifts of signal spectral structure to the ultrasound range have been reported for Asian torrent anurans (Feng et al., 2006). In the temperate austral forest of Chile, short-term stimulating effects of abiotic noises on frog vocal output have been reported (Penna et al., 2005; Penna and Zúñiga, 2014)

Responsiveness of animals to repetitive stimulation have been subjected to classical experimental psychology studies. Habituation is a behavioral manifestation subjected to studies since the first half of the twentieth century (Thompson and Spencer, 1966; Thompson, 2009). This process consists of a decrement of behavioral responses resulting from repeated presentation of a constant stimulus and differs from sensory adaptation or motor fatigue in its stimulus specificity, i.e. the responses are reinstated if the stimulus is modified (Rankin, 2009). A phenomenon concomitant with habituation but having a contrasting nature is sensitization, by means of which responses are enhanced throughout repetitive stimulation. Both processes run in parallel and their relative effects on the responses measured depend on the stimulation parameters used, namely presentation rate and intensity (Hinde, 1966; Groves and Thompson, 1970). Furthermore, habituation is stimulus specific, i.e. the response is reinstated in the presence of a different stimulus, but sensitization has generalization properties, as repetitive stimulation can increase responsiveness to stimuli similar to the one to which subjects have been exposed, due to an increase in motivation to respond (Peeke, 1982; Petrinovich, 1984).

Behavioral changes built up within minutes of repetitive stimulation are termed short-term habituation and sensitization, but long-term processes of both kinds also occur, persisting over training sessions during consecutive days of exposure to stimuli (Petrinovich, 1984; Montarolo, et al. 1986; Rankin et al., 2009; Glanzman, 2013).

The effect of sounds of biological significance such as conspecific calls on habituation of vocal responses has long been studied in birds. In this group of vertebrates, males and females exposed in nature to male songs, exhibit behavioral and concomitant neural habituation processes at the neurophysiological and early gene expression level (Mello et al., 1995; Dong and Clayton, 2009). In anurans, habituation

of aggressive displays in response to conspecific vocalizations occurs in the North American bullfrog *Lithobates catesbeianus*, in the short- and long-term span (Bee and Gerhardt, 2001a). Such plasticity underlies the occurrence of the dear enemy phenomenon in this species, by which territory owners behave less aggressively toward nearby neighbors as compared with unfamiliar conspecifics (Bee and Gerhardt, 2001b; Humfeld et al., 2009). Such behavior also occurs in birds and results in relatively stable social communities (Wilson, 1975; Temeles, 1994).

Decrements of aggressive calling in response to repetitive stimulation have also been reported in two other North American frog species (Brenowitz and Rose, 1994, Rose and Brenowitz, 1997; Marshall et al. 2003). In addition, neural correlates of habituation have been reported for *L. catesbeianus* using evoked potential recordings (Megela and Capranica, 1983).

Although habituation of aggressive behaviors to conspecific vocalizations predominates in birds, instances of sensitization also occur, and these are dependent on the presentation rate and amplitude level of the stimuli (Petrinovich and Patterson, 1981). Sensitization in the auditory system is evinced as an increase in the immediate early gene expression in two song-processing brain areas of female birds *Sturnus vulgaris* exposed for one week to preferred song patterns (Sockman et al., 2002, 2005). In anurans, sensitization of aggressive vocal responses has been reported in *L. catesbeianus* (Bee, 2001) and the neotropical *Dendropsophus ebbacatus* (Reichert, 2010).

Chorusing aggregations occur extensively in anurans (Gerhardt and Huber, 2002), originating background sound having highly dynamic spectral and temporal properties, potentially appropriate for sensitizing effects. Prolonged exposures to anuran chorusing sounds produce neural correlates of sensitization in the green treefrog *Hyla cinerea*, as increases in immediate early gene expression and decreases in thresholds of auditory evoked potentials are observed in the auditory midbrain of males and females after a 10-days exposure to conspecific chorusing noise, relative to subjects exposed to tones devoid of biological significance (Gall and Wilczynski 2014, 2015). Such prolonged exposure also reduces masking effects of chorus noise (Gall and Wilczynski, 2016). A likely benefit of sensitization of vocal behavior during exposure to conspecific chorusing noise is to predispose animals to confront acoustic interference while communicating amid variable sound environments.

There is no direct evidence that natural abiotic sounds generate habituation or sensitization effects on vocal behavior. But as previously mentioned, our studies have shown that within the genus *Eupsophus* and *Batrachyla* some species increase (Penna et al., 2005; Penna and Zuñiga, 2014) while others (Penna and Hamilton-West, 2007; Penna et al, 2017) do not alter their vocal responses during exposure to natural abiotic noises of creek and rain. *B. taeniata* in particular is highly responsive to rain sounds both in the laboratory and field (Penna and Zuñiga, 2014; Muñoz et al., 2020).

In the current study we exposed males of *B. taeniata* to sounds of conspecific choruses or rain during 8-10 days, and control subjects were left in silence, to test for changes in vocal activity dependent on the acoustic exposures. It is hypothesized that prolonged experience with conspecific chorusing and rain sounds will produce sensitization, i.e. an enhancement of the vocal responses of male frogs *B. taeniata*, as both kinds of noises have dynamic temporal patterns, and the spectra of rain noise contains energy within the range of conspecific vocalizations, promoting calling and breeding activity in this species. Long-term sensitization of vocal activity is likely to occur, as auditory responses have been shown to undergo a corresponding activation in these vertebrates (Gall and Wilczynski, 2014, 2015, 2016).

MATERIALS AND METHODS

Acoustic stimuli

The *B. taeniata* chorus sounds used for experimental playbacks were employed in a former study (Muñoz et al. 2020, Fig. 1), and built from natural field recordings devoid of other biotic or abiotic noises, conducted on the shore of Tinquilco lake (39°07' S, 71°46' W), near Huerquehue National Park in southern Chile. These recordings were conducted in February 1993, 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 10-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-min duration choruses had linear fade-in and fade-out times of 2 s (Fig. 1a).

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 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 10-min rain sounds were created using the same procedures as for the chorus sounds (Fig. 1b).

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 & Zúñiga 2014; Muñoz et al. 2020).

An imitation of the advertisement call of *B. taeniata* consisting of 25 pulses built with a 2 kHz sinusoidal carrier was synthesized. Pulses had a duration of 5 ms (rise-fall times 1 and 4 ms, respectively) and were repeated with an inter-pulse period of 20 ms (pulse rate: 50 pulses/s), resulting in a call duration of 505 ms. The three initial and final two pulses within each call had a graded increment and decrease in amplitude, respectively, mimicking amplitude modulation of natural calls. Synthetic calls were organized in bouts of 20 calls presented with an inter-call period of 1.5 s (call rate: 40 calls /min), resulting in a bout duration of 29 s (Fig. 1). Twenty successive bouts were presented leaving 60-s silent intervals between bouts, resulting in a total presentation time of 29 min. The entire sequence of synthetic calls was embedded in a continuous chorus sound having an amplitude of -12 dB relative to the stimuli. This sound started 3 min before the onset of the first bout of synthetic calls and ended 1 min after the end of the last bout of synthetic calls, resulting in a total time of exposure of 33 min (Fig. 2). The synthetic stimuli were embedded in chorus sound at a low amplitude because preliminary experiments have shown that synthetic calls alone did not evoke readily vocal responses from male *B. taeniata*.

Field and maintenance procedures

Experiments were conducted during March 2018, 2022 and 2023. During these years a total of 24, 32 and 16 male *B. taeniata* were captured, respectively, at their

native locality. 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). Two groups of experimental subjects were formed during 2018 and 2022, early and late in the breeding season, respectively. During 2023, a single group captured early in the breeding season was used. During 2018, the subjects were exposed during 8 days to either chorus sound or no broadcast and in 2022 and 2023 the exposures lasted 10 days and the subjects were exposed to chorus sound, rain sound or no broadcast. The dates of capture, exposure times and experimental treatments are listed in Table 1. Data of vocal activity recorded during 8 days of exposure to chorus sound during 2018 (16 individuals) were used for a study on call repeatability of the study species (Moreno-Gómez et al. 2024).

Frogs were transported from their native locality to the laboratory in the Faculty of Medicine of the University of Chile in Santiago, Chile, and housed individually inside acoustically transparent cylinder-shaped plastic containers lined with fine plastic mesh (container diameter: 15 cm, height: 23 cm) and kept at 12 ± 1 °C inside a low-noise 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 or crickets once per week and the containers were rinsed with filtered, dechlorinated water twice per week.

On each day the frogs were exposed to the sequences of choruses or rain sounds described above during 5 hours, starting one hour after the beginning of the dark phase. The sounds were delivered through an active loudspeaker (Optimus AMX-21) at an amplitude of 50 dB SPL RMS (A frequency weighting, slow time weighting). The Z frequency weighting was used to measure this sound level, because the Z level of background noise in this room was 52 dB SPL RMS. The 55 dB SPL amplitude at which the chorus sound was set corresponds to the amplitude of distant *B. taeniata* chorusing aggregations at the site where frogs were captured (Muñoz et al. 2020). The animals were kept in these conditions for one week before starting the experimental phase.

Experimental procedures

For the acoustic recordings, each plastic container housing an individual male *B. taeniata* was transferred from the common refrigerated room to individual acoustically-isolated wooden chambers (80 x 20 x 35 cm) placed inside another refrigerated room at 12 ± 1 °C, where the frogs were also kept under an inverted 14:10 light:dark cycle. Each individual 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 light emitting diodes positioned on the ceiling of each chamber provided illumination.

After one week of maintenance at low levels of chorus broadcast, frogs were presented during the initial experimental day with the bouts synthetic advertisement calls described in a preceding section. Three of such sequences were delivered, leaving 30 min of interval between successive sequences. On the final experimental day, that is one day after completing the eight (2018) or ten (2022-2023) days of exposure to chorus, rain sound or silence, the frogs were presented with the same experimental schedule to test for changes in vocal activity dependent on the prolonged acoustic treatment.

During eight (2018) or ten (2022-2023) consecutive nights, frogs were either exposed to chorus or rain sound or left unexposed to broadcast sound. The chorus and rain sound was played back with a Mac minicomputer (Apple inc.) connected to each of 12 (2018) or 16 (2022-2023) attenuators (Tucker-Davis Technologies PA4, PA5 or Hewlett-Packard 350D) and to one channel of six (2018) or eight (2022-2023) integrated stereo amplifiers (Topaz AM5, NAD3020i or NAD C352).

The nine 10-min choruses and rain sounds were presented in random succession, leaving 3 min of silence in between emissions. On each night these sequences were presented during 5 hours starting one hour after the beginning of the dark phase.

The exposure level used for chorus and rain sounds was 67 dB SPL RMS (Z frequency weighting, slow time weighting) and was set by adjusting manually the attenuator levels in 1-dB steps. The chorus and rain sound level at the position of the subjects inside each of the 16 experimental chambers was measured with a sound level meter (Brüel and Kjaer 2238). The amplitude used has been employed in former studies as it is within the range of amplitudes of calls of nearest neighbors at the position of

focal subjects at the study site and evokes consistent vocal activity of male *B. taeniata* in field and laboratory settings (Muñoz et al. 2020).

In 2018, each frog was recorded continuously throughout eight days with a small omnidirectional tie-clip microphone (AKG C417) placed inside each plastic container and connected to a channel of two six-channel digital recorders (Tascam DR-680). Recordings were restricted to the 5-hours period of chorus sound presentation or an equivalent time for animals left in absence of sound exposure. In 2022 and 2023, 16 microphones were plugged into the two 8-channel audio interfaces (Focusrite Scarlett 18i20) connected to two PC computers fitted with Pro Tools 2022.9 and 2022.10 software. A schematic diagram of the experimental schedule is provided in Fig. S1.

For two experimental groups (16 frogs from the second 2022 group and 16 frogs from the 2023 group) additional exposures to rain sound at different amplitudes were applied. This was done two days after the post-exposure experimental day in which the subjects were presented with the synthetic calls embedded in chorus noise. On the day after the post-exposure experimental day frogs were left in silence and on the next day they were exposed to five ten-minute exposures to rain noise presented at increasing amplitude level of 61, 67, 73, 79 and 85 dB SPL. Silent 3-min intervals separated the successive exposures.

Acoustic analysis of vocal responses

During the 5 hours of daily exposure to chorus or rain sounds, 24 10-min chorus separated by 3-min silent intervals were delivered. The total number of calls produced during the 5-hour exposures to chorus or rain sounds (or the corresponding time interval for the groups for which no sound was broadcast) 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 15 % of the calls) were manually selected with RavenPro 1.4 (Cornell Laboratory of Ornithology, Ithaca, NY, USA).

Statistical analyses

Analyses for the two-year assemblies were performed separately, corresponding to the individuals subjected to experimentation during 2018 and 2022-2023, respectively, as during 2018 prolonged exposure of individuals to noise and silent controls lasted 8

days, while in 2022-2023 the animals were exposed for 10 days to the experimental conditions. Furthermore, during 2018 the experimental animals were subjected to two treatments: chorus noise and silence, and during 2022-2023 an additional treatment of rain noise was applied. During 2018, two experimental groups were studied, one at the beginning of the breeding season and one at an advanced stage of the breeding season, During 2022-2023, three experimental groups were studied, one at the beginning of the 2022 breeding season, one at an advanced stage of the 2022 breeding season and one at the beginning of the 2023 breeding season (Table 1).

The variation in number of calls produced by individuals was evaluated by Zero-inflated Generalized Linear Mixed Models (ZIGLMM) as implemented by the R library glmmTMB (Brooks et al., 2017). The individual was included as random effect to account for data dependence and a single inflation parameter was included. The proper family distribution to fit the models, i.e., Poisson, Negative binomial 1 and Negative binomial 2, was evaluated by Akaike information criteria using the function AICtab from the bbmle library (Bolker & R Development Core Team, 2023).

To analyze the difference in calls produced between the pre- and post- exposure experimental day, the model included as fixed effects day, treatment, interaction between day and treatment, group and body size. This analysis was intended to evaluate the occurrence of long-term sensitization or habituation. These analyses were intended to assess the variation between 8 or 10 days of prolonged exposure, where the model included as fixed effects the day, block, treatment and their paired interactions. This analysis was intended to evaluate the occurrence of short-term sensitization or habituation. A block corresponded to a within day interval encompassing six 10-min bouts of chorus sound presentation. In addition, body size and experimental group were also included as fixed effects. The significance of the fixed effects was evaluated by a type III test using the function Anova.glmmTMB. Then, a model including only significant factors was fitted and *a posteriori* analyses with Tukey adjustment to obtain p-values were performed using the library emmeans (Lenth, 2024), these analyses were visualized using procedures available in the same library. In addition, the association between the daily mean of calls and the difference between the number of calls produced in the pre- and post- exposure day was assessed by Spearman correlation, and this last analysis was performed separately for year assemblies and treatments. These

analyses were intended to unveil relationships between daily vocal activity and processes of long-term sensitization or habituation.

For the exposures to rain noise at different amplitudes, similar procedures were followed by fitting ZIGLMMs, however in this case the dB SPL of stimuli and the body size were included as fixed effects. The *a posteriori* analyses considered only the 67 dB SPL stimulus as a reference for the comparisons. This analysis was intended to determine the influence of abiotic acoustic stimulation level on vocal activity, as the calling rate of the frogs subjected to prolonged exposure to rain noise at 67 dB SPL during the entire experimental seemed to produce a low output relative to the frogs exposed to chorus noise at the same level. Such difference could depend on the spreading of energy over the audible range of rain noise relative to the chorus noise concentrated in the 2 kHz zone. The exposure to rain noise at higher amplitudes would compensate for such spectral difference.

RESULTS

Calling activity in the pre- and post-exposure day

For the comparison of number of calls between the pre- and post-exposure day to prolonged exposure of the subjects to noises or left in silence, a significant effect of day occurred for both year assemblies (i.e., 2018 and 2022-2023), and no effects of treatment, group, body size and interaction between day and treatment were observed. However, opposite trends occurred for the effect of day between both datasets, as a decrease and an increase in the number of calls was observed in 2018 and in 2022-2023, respectively (Fig. 2, Table 2), as averages $\pm$ SD for number of calls during the pre- and post-exposure day were 491 ± 760 and 377 ± 597 in 2018 and 915 ± 811 and 1029 ± 924 in 2022-2023.

Calling activity throughout the days of prolonged exposure

For number of calls among days, significant effects were found in both year assemblies for day, block, treatment and paired interactions among these factors. No effects for experimental group and body size occurred (Table 3).

A posteriori analyses of the interaction between day and treatment indicated that for the 2018 assembly, the number of calls was higher in the subjects exposed to chorus

noise than in the subjects left in silence during the 8 days of treatment (Fig. 3a, Table S1). Among animals exposed to chorus noise, a trend to an increase in number of calls emitted throughout days was observed, as the number of calls for days 1, 3 and 4 was lower than for days 5-8, and the number of calls for day 2 was lower than for days 5-7. Only for one comparison an opposite result occurred, as the number of calls for day 6 was higher than for day 8 (Fig. 3b, Table 2018 treatment day). Among animals left in silence, a trend to a decrease in number of calls emitted throughout days was observed, as the number of calls for days 1-6 was higher than for days 7 and 8 (Fig. 3b Table S1).

For the 2022-2023 assembly, the number of calls was higher in the subjects exposed to chorus noise than in the subjects exposed to rain noise and to those left in silence during the 10 days of treatment, but no differences occurred between subjects exposed to rain and those left in silence (Fig. 3c Table S2). Among animals exposed to chorus noise, a trend to a decrease in number of calls emitted throughout days was observed, as the number of calls for days 1, 2 and 3 was higher than for days 4-10. Also, the number of calls for days 4 and 5 was higher than for day 8. Only three comparisons yielded an opposite result, as the number of calls for day 6 was lower than the number of calls for day 9, and the calls for day 8 was lower than for day 9 and 10 (Fig. 3d, Table S2). Among animals exposed to rain noise, no clear trend of change of number of calls throughout days was observed, as only 4 out of 45 comparisons yielded significant differences: the numbers of calls was higher for day 2 relative to day 4, and the numbers of calls were lower for day 4 relative to days 6,7 and 10 (Fig. 3d, Table S2). Among animals left in silence, no clear trend was observed either, as only 6 out of 45 comparisons yielded significant differences: the number of calls was higher for day 1 relative to days 4 and 9, and for day 2 relative to day 4. In addition, the number of calls for day 4 was lower than for days 7 and 10, and for day 9 relative to day 10 (Fig. 3d, Table 2022-2023 treatment day).

A posteriori analyses of the interaction between block and treatment indicated that for the 2018 assembly, the number of calls was higher in the subjects exposed to chorus noise than in the subjects left in silence during the 4 blocks within a day (Fig. 4a, Table S3). When the two treatment groups of the 2018 assembly were analyzed separately, the number of calls produced by animals exposed to the chorus noise showed a tendency to increase during an experimental day, as call number was lower

during block 1 relative to blocks 2, 3 and 4, but no significant difference occurred for animals kept in silence (Fig. 4b, Table S3).

For the 2022-2023 assembly, the number of calls was higher in the subjects exposed to chorus noise than in the subjects exposed to rain noise and in those left in silence during the 4 blocks within a day, but no differences occurred between subjects exposed to rain and those left in silence (Fig. 4c, Table S4). When the three treatment groups of the 2022-2023 assembly were analyzed separately, the number of calls produced by animals exposed to chorus noise showed a tendency to increase initially during an experimental day, as call number was lower during block 1 relative to block 2, 3 and 4. Thereafter the number of calls tended to decrease, as it was higher in blocks 2 and 3 relative to block 4, but the vocal activity remained high in block 4 relative to block 1. For the subjects exposed to rain noise and in those left in silence, call number was also lower during block 1 relative to block 2 and higher in block 1 relative to block 4, the paired differences showed significant decrements from block 2, 3 to 4 (Fig. 4d; Table S4).

A posteriori analyses of the interaction between day and block were conducted to formally complete the GLMM including the fixed effects of day, block, treatment and their paired interactions. However, since the aim of the study was to compare effects of acoustic exposure treatments on vocal activity, the analysis of the effects of block and day independent of acoustic treatment do not provide significant support for our tenets and thus the analyses and the description of these results are provided as supplementary material (see Fig. S2 and Supplementary Materials and Methods for further details, respectively).

Correlations between calling activity during prolonged exposure and difference in calling activity between the pre- and post-exposure day

We explored whether there was a relationship between the calling activity during the exposure period and the difference between the calls produced on the pre- and post-exposure day. There were no significant correlations between the mean number of calls produced during 8 or 10 days of acoustic exposure and the difference between the number of calls produced in the pre- and post-exposure day of stimulation with synthetic calls for any the experimental groups in the 2018 and 2022-2023 assemblies. The association between the number of calls produced in the last day of acoustic

exposure and the difference between the number of calls produced in the pre- and post-exposure day was also explored and a significant correlation occurred for frogs exposed to chorus noise in the year assembly 2022-2023, as frogs that gave lower and higher number of calls in the tenth day tended to decrease and increase their calls in the post-relative to the pre-exposure day of stimulation with synthetic calls, respectively (Table 4).

Calling activity in exposures to rain sound at different amplitudes

Fourteen out of 32 frogs tested responded to a series of rain noise presented at different, increasing amplitudes. The results show that the number of calls depended on stimulus amplitude ($X^2_{(5)} = 131.82$, $p < 0.001$), the *a posteriori* analysis showed that when considering the stimulus at 67 dB SPL as a reference, stimuli having higher amplitudes elicited larger numbers of calls (Table 5).

DISCUSSION

Long-term changes in vocal activity

Results of the comparison of vocal activity between the initial and final day of the experimental schedule, i.e, immediately before and after days of prolonged exposure to environmental sounds or silence, do not show a consistent pattern, as a decrease and an increase in number of calls was observed for the 2018 and 2022-2023 assemblies, respectively. This outcome indicates that prolonged exposures to sound environments are not effective in producing changes in sensitivity of vocal responses to acoustic stimulation in *B. taeniata*. Furthermore, acoustic treatment, experimental group and size of the subjects do not affect the direction of the changes observed.

The lack of consistent patterns of change in vocal activity between the initial day and final day when the different experimental groups were exposed to synthetic calls, indicates that long-term vocal sensitization processes do not occur in our experimental settings. This result contrast with instances of sensitization that occur in the anuran auditory system, as documented in the green treefrog *Hyla cinerea* at different levels of analysis (Gall and Wilczynski, 2014, 2015, 2016). However, these studies have not explored activation effects of such experimental interventions on motor vocal centers. Short-term stimulation with biologically significant acoustic signals has shown to

activate both sensory auditory and motor vocal centers (Hoke et al., 2007) and therefore to extend research efforts to investigate effects of prolonged sound exposures on vocal centers would identify structural substrates for eventual effects of long-term exposures on vocal output.

In addition, it could be argued that the different outcome in the long-term sensitization of auditory activity in *H. cinerea* and the lack of long-term sensitization of vocal responses in *B. taeniata* is attributable to inter-specific differences in the susceptibility to prolonged exposures, as inter-specific differences in short-term habituation have been reported in neotropical frogs *Anomaloglossus* and *Allobates* (Tumulty et al., 2021; Tumulty and Bee, 2021). Further research in closely related species comparing the same kind of responses to prolonged exposures to sound of biological relevance would help to clarify the issue. For instance, in the genus *Batrachyla*, inter-specific differences in responses to short-term exposures to biotic (Penna and Meier, 2011; Penna and Velásquez, 2011; Penna and Toloza, 2014) and abiotic (Penna and Zúñiga, 2014; Penna et al., 2017) natural noises do occur, and it may be expected that different responses to prolonged noise exposures also happen among these frogs.

Daily changes in vocal activity

Measurements of vocal activity during the days of prolonged exposure indicate a clear effect of acoustic treatment, as subjects exposed to chorus sound had higher activity relative to those left in silence during 2018 and to those exposed to rain sound and left in silence in 2022-2023. No differences occurred between the vocal activity of frogs exposed to these two last environmental conditions. However vocal activity measured throughout the days of exposure does not show a uniform pattern, as for frogs exposed to chorus sound, trends to an increase and a decrease in vocal output occurred for the 2018 and 2022-2023 assemblies, respectively. Furthermore, for frogs left in silence, a decrease in vocal activity occurred in the 2018 assembly and no change in vocal activity occurred for this treatment and for exposure to rain sound in the 2022-2023 assembly.

In contrast with the non-systematic variation in vocal activity observed in the long term, the number of calls within days yielded a relatively uniform pattern. In 2018 an initial increase in vocal activity followed by a subsequent maintenance of vocal

output occurred in animals exposed to chorus sound, whereas no change within days was observed for animals left in silence, a difference between treatments consistent with a short-term sensitization process. In 2022-2023 an initial increase and a further maintenance of a high daily vocal activity occurred in subjects exposed to chorus sound, but in subjects exposed to rain sound and silence, the initial increase was followed by a further decrease in vocal activity. The difference in the patterns of change of daily vocal activity with the maintenance of a high vocal activity in response to the sound exposure having the highest effectiveness in vocal output also conveys support for a short-term sensitization process, a phenomenon that is known to affect vocal activity of anurans (Bee, 2001; Reichert, 2010). The subsequent decrement could be due to either a habituation process or a limitation in energy expenditure, as calling activity has a high metabolic rate in anurans (Prestwich et al., 1989), and in tropical frogs the nightly vocal output is positively correlated with trunk muscle glycogen depletion and amount of lipid reserves (Bevier, 1997).

A result that needs to be explained in frogs kept in silence in the 2022-2023 assembly is the initial augmentation in daily vocal activity observed in these subjects. Such outcome is likely due to the effect of light/dark schedule under which the experiments were conducted, as measurement of vocal activity started one hour after lights went off and ended when six hours of darkness were completed. Vocal activation after the onset of darkness occurs regularly in nocturnal frogs, and in *B. taeniata*, a maximum of vocal activity occurs after about one hour of darkness for individuals kept chronically in absence of sound broadcast in our low-noise refrigerated room (Bidart-Enríquez et al., unpublished data). Photoperiod is a main determinant of seasonal vocal activity in nocturnal frogs (Canavero and Arim, 2009; Pouso et al., 2026), as typically a marked increase in vocal activity occurs at dusk, decreasing thereafter (Akmentins et al., 2014; de Souza et al., 2020; Guerra et al., 2020), although in diurnal species temperature and humidity are main determinants of vocal activity (Cui et al. 2011; McCallum and McCallum, 2022; Zigler et al. 2023). Furthermore, artificial light alters calling rhythms in nocturnal anurans (Baker and Richardson, 2006; Secondi et al. 2021). As such, the daily vocal patterns observed in the experimental groups of the current study could result in part from entrainment to the photoperiod and not to daily processes of initial sensitization and subsequent habituation. Further experiments altering the light-dark schedule would shed precision on the factors involved in the daily vocal

patterns observed, but to the best of our knowledge, few studies have systematically evaluated anuran circadian rhythms in laboratory-controlled conditions (Li et al., 2026).

The analysis carried out for interactions between day and block, irrespective of the acoustic treatment yielded similar results for daily activity, with values in the initial block lower than in the subsequent blocks. As for the tendencies observed throughout days, the decrease of activity in the last two days of exposure in the 2018 assembly are likely due to the decrease observed in the frogs kept in silence during this year. The tendency to a stable activity pattern throughout days in the 2022-2023 assembly is likely due to the constant pattern obtained for the groups exposed to rain sound and left in silence.

The lack of correlation between average call numbers emitted along days of prolonged acoustic exposure and the difference in calls evoked by the stimulation with synthetic calls in the pre- and post-exposure day indicates that the level of overall vocal output is not involved in potential changes in vocal activation during prolonged acoustic exposures. However, the relationship found in the 2022-2023 assembly between the vocal output during the last day of vocal exposure and the change in vocal activity between pre- and post- exposure days last day suggests that vocal output is related to the calling level achieved at a given time of the experimental progression.

Vocal activity during exposure to rain sound

Another result that deserves consideration is the relatively low vocal activity elicited by the rain sound in the experimental subjects, as their vocal activity did not differ from subjects left in silence. This result is somehow consistent with a former study, frogs in the field respond with lower vocal activity to short-term exposure to rain relative to conspecific chorus sound (Muñoz et al. 2020). Also, in that study the vocal activity elicited by those sounds in the laboratory is low relative to responses evoked in the field, and thus the relative low calling rate in response to rain sound observed in the current study is likely related to constraints of laboratory settings for vocal activation. The exposures to rain noise at different amplitude levels conducted in the current study show that frogs respond consistently when 67 dB SPL was used, but higher call rates can be evoked by raising the amplitude of the exposure sound. The lower vocal activity elicited by rain relative to chorus sound presented at the same 67 dB SPL RMS Z level could be due to the energy dispersion of rain noise into high frequencies, beyond the

auditory sensitivity of this species, restricted to frequencies below about 3 kHz (Penna et al., 2023).

Concluding remarks

Overall, this study shows no evidence of long-term sensitization. Nevertheless, a daily temporal pattern with an initial increase and subsequent decrease or maintenance of vocal activity consistent with processes of short-term sensitization and habituation, respectively is observed, but potential influences of photoperiod and energy depletion also need to be assessed. Such daily plasticity would help to boost the vocal activity at the beginning of individual breeding nights with a subsequent decrement or maintenance of the vocal output. In contrast, call rate does not experience clear tendencies of change across nights, and thus no evidence of prolonged sensitization processes. The long-term sensitization of auditory responses observed in North American frogs (Gall and Wilczynski 2014, 2015, 2016) has not been shown to result in a concomitant activation of motor vocal centers, thus restricting costly energy expenditure along the prolonged breeding period. Alternatively, activation of motor centers may occur but the expression of related behaviors could remain unmodified, a mismatch that has been reported to occur in other vertebrates (Fischer et al., 2018).

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Competing interests

The authors declare no competing interests

Author contributions

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Data availability

The data associated with this study are available from the supplementary material

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Figures and Tables

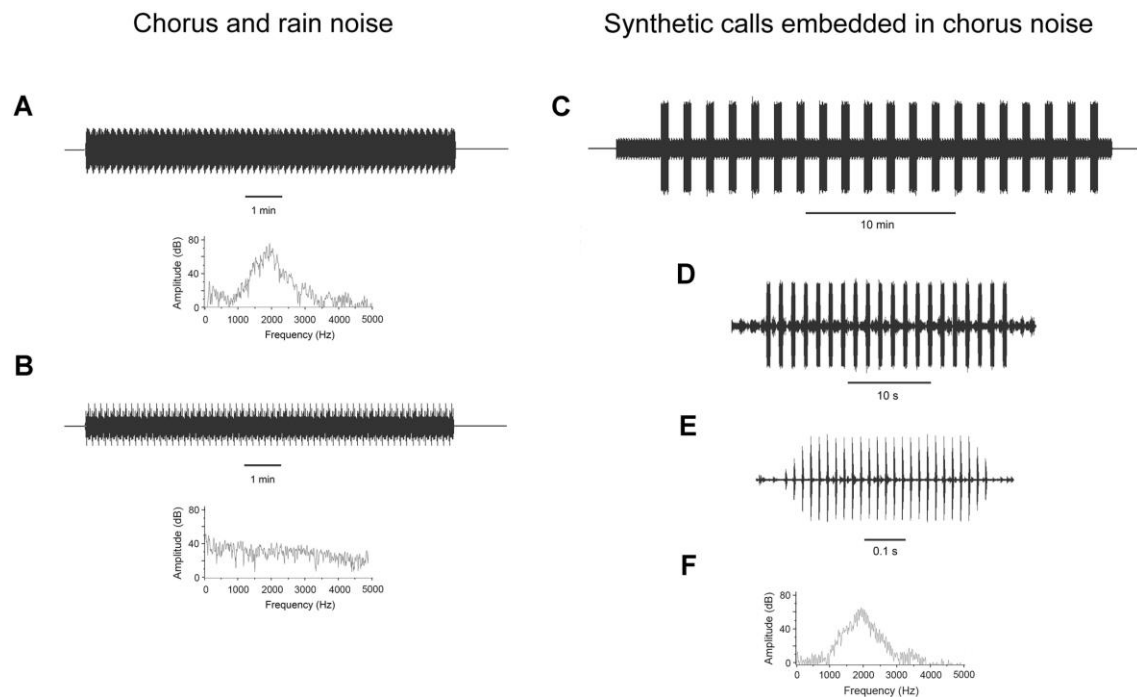


Fig. 1. Oscillograms and power spectra (3174 points Hanning window, 20-Hz filter bandwidth) of different stimuli. Left: (A) 10-min chorus sound and (B) 10-min rain sound, preceded and followed by 3-min silent intervals. Right: Oscillograms and power spectrum of bouts of synthetic calls of *B. taeniata* embedded in conspecific natural chorus sound at an amplitude of -12 dB relative to the synthetic calls. (C) 20 bouts of synthetic calls with 1 min intervals between successive bouts, preceded and followed by 3 min and 1 min of chorus sound, respectively (D) A single bout composed of 20 synthetic calls at an inter-call period of 1.5 s (E) A single synthetic call composed of 25 5-ms duration pulses (F) Power spectrum of synthetic call embedded in chorus sound (3174 points Hanning window, 20-Hz filter bandwidth)

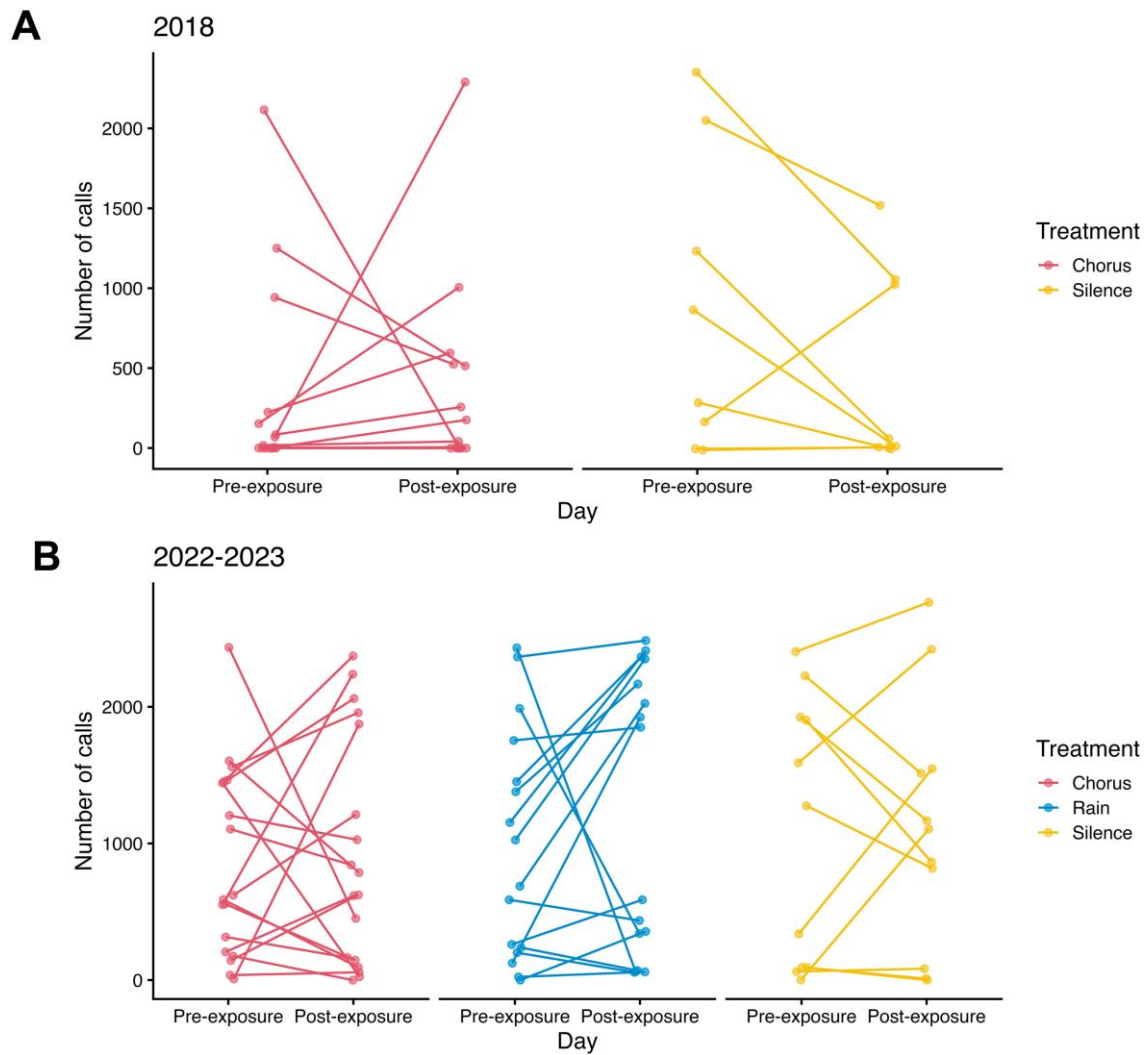


Fig. 2. Number of calls produced during the pre- and post-exposure experimental day. (A) individuals of the 2018 assembly and (B) individuals of the 2022-2023 assembly subjected to two and three acoustic treatments, respectively.

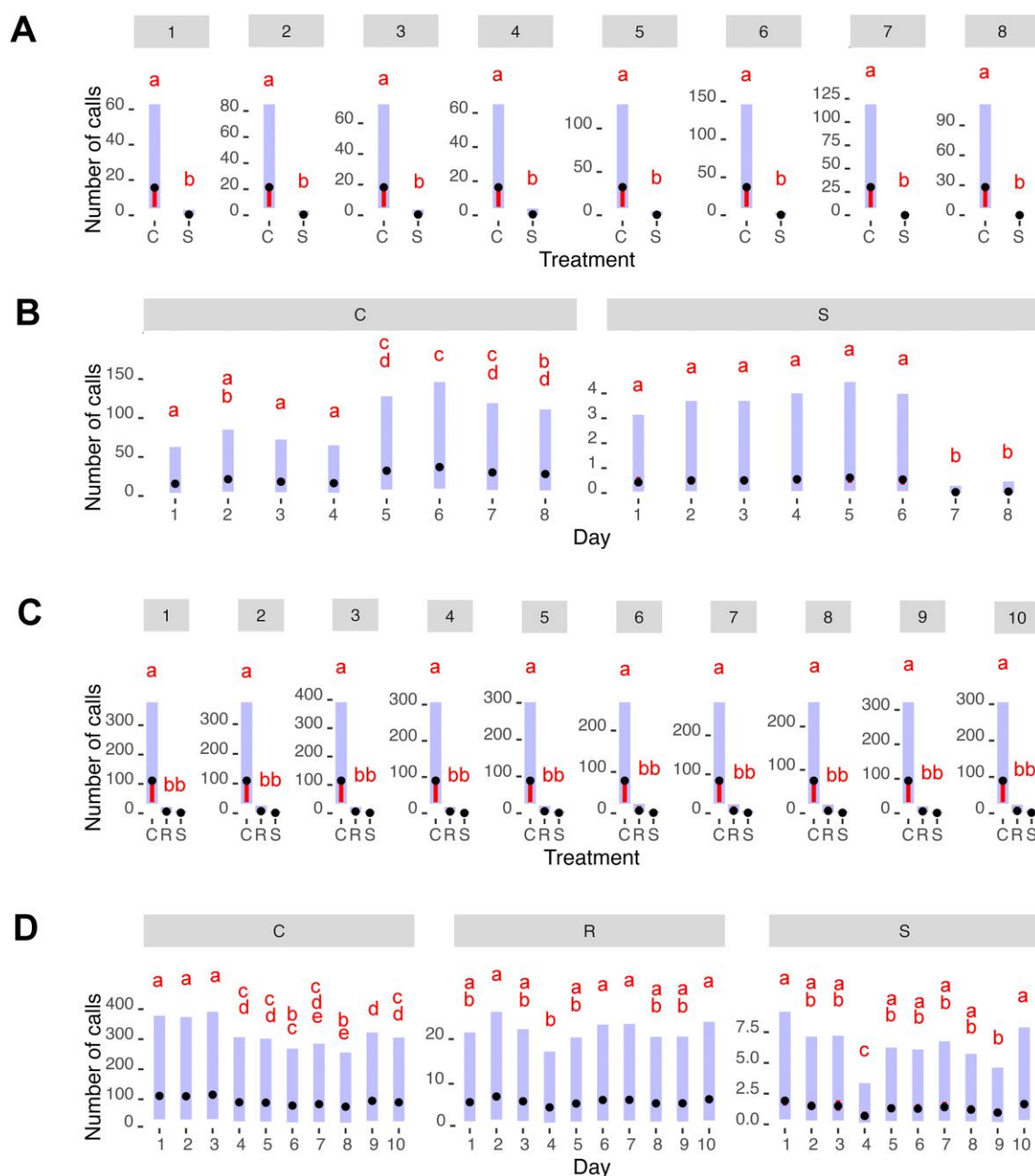


Fig. 3. A posteriori analyses of the interaction between day and treatment. (A, B) Number of calls produced by individuals of the 2018 assembly throughout 8 days of exposure to two treatments: chorus noise and silence. (C, D) Number of calls produced by individuals of the 2022-2023 assembly throughout 10 days of exposure to three treatments: chorus noise, rain noise and silence. Blue bars: 95% confidence intervals. Red bars: comparison bars that indicate significant differences when no overlap occurs among them. Dots: means, C: chorus, S: silence, R: rain. Red lower-case letters above bars indicate similarities and differences for paired comparisons.

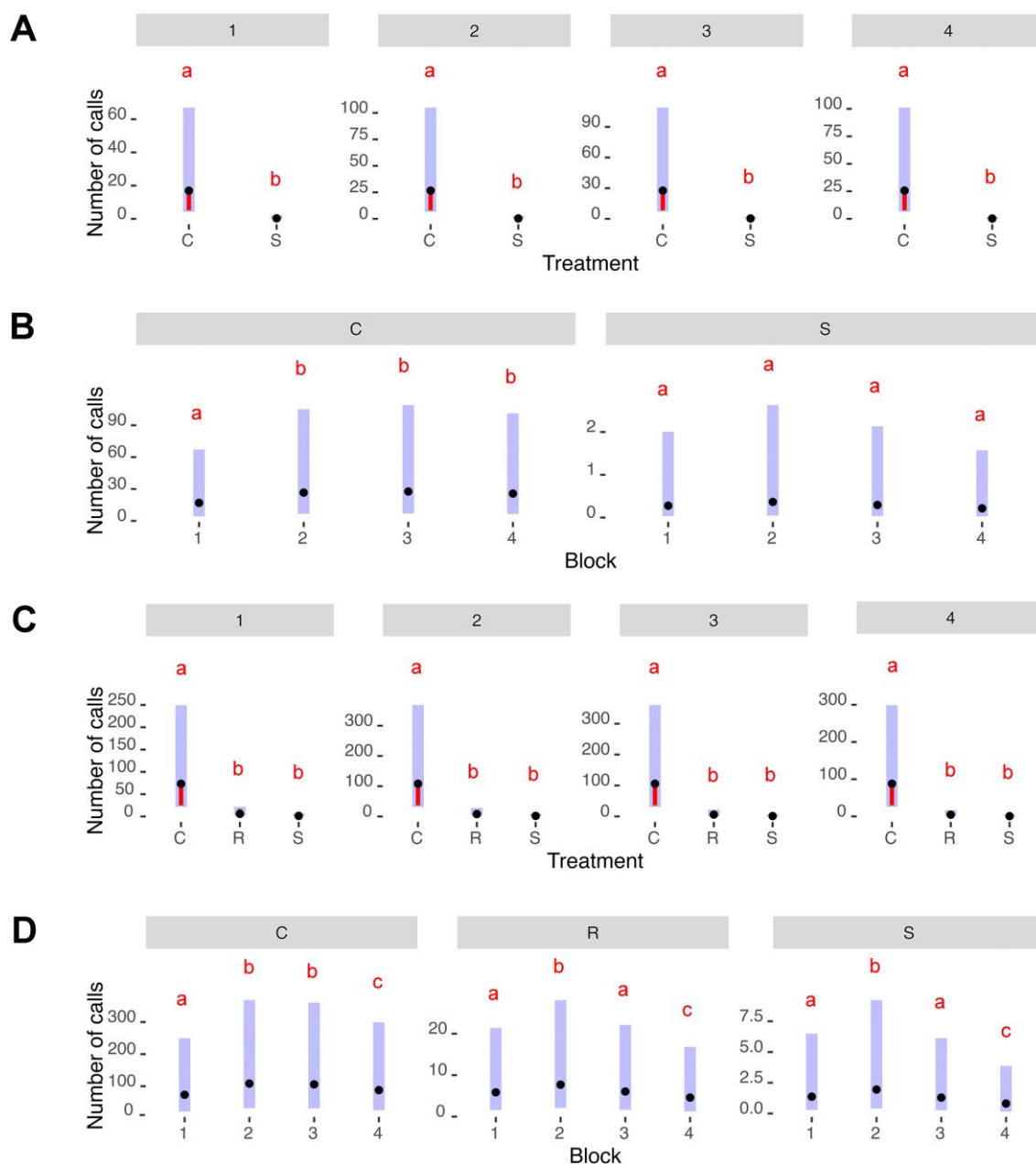


Fig. 4. A posteriori analyses of the interaction between block and treatment. (A, B) Number of calls produced by individuals of the 2018 assembly throughout four successive daily time blocks during exposure to two treatments: chorus noise and silence. (C, D) Number of calls produced by individuals of the 2022-2023 assembly throughout four successive daily time blocks during exposure to three treatments: chorus noise, rain noise and silence. Blue bars: 95% confidence intervals. Red bars: comparison bars that indicate significant differences when no overlap occurs among them. Dots: means, C: chorus, S: silence, R: rain. Red lower-case letters above bars indicate similarities and differences for paired comparisons.

Table 1. Dates, acoustic exposure times and treatments for *Batrachyla taeniata* males in this study

Year	Capture date	Pre-exposure day	Exposure days	Post-exposure day	Exposure period (days)	Individuals exposed		
						Chorus	Rain	Silence
2018	February 27	March 7	March 8-15	March 16	8	8		4
2018	March 15	March 23	March 24-31	April 1	8	8		4
2022	February 24	March 4	March 5-14	March 15	10	6	6	4
2022	March 17	March 26	March 27-April 25	April 6	10	6	6	4
2023	February 16	February 26	February 27-March 8	March 9	10	6	6	4

Table 2. Zero-inflated Generalized Linear Mixed Models for number of calls in the pre- and post-exposure day to acoustic treatments. Bold characters indicate significant P values

Year assembly	Factor	Chisq	Df	P value
2018	Day	56.302	2	<0.0001
	Treatment	2.144	1	0.1431
	Day:Treatment	2.847	1	0.0916
	Group	0.034	1	0.8536
	Body size	0.034	1	0.8533
2022-2023	Day	739.375	2	<0.0001
	Treatment	0.671	2	0.7150
	Day:Treatment	0.130	2	0.9373
	Group	0.975	2	0.6143
	Body size	3.477	1	0.0622

Table 3. Zero-inflated Generalized Linear Mixed Models for number of calls during days of prolonged exposures to chorus, rain noise or silence. Bold characters indicate significant P values

Year assembly	Factor	Chisq	Df	P value
2018	Day	36.797	8	<0.0001
	Block	17.623	3	0.0005
	Treatment	6.710	1	0.0096
	Day:Block	53.626	21	0.0001
	Day:Treatment	99.773	7	<0.0001
	Block:Treatment	8.122	3	0.0436
	Group	0.148	1	0.7008
	Body size	0.033	1	0.8550
2022-2023	Day	182.349	10	<0.0001
	Block	37.149	3	<0.0001
	Treatment	16.517	2	0.0003
	Day:Block	102.610	27	<0.0001
	Day:Treatment	84.259	18	<0.0001
	Block:Treatment	97.341	6	<0.0001
	Group	2.636	2	0.2676
	Body size	0.301	1	0.5832

Table 4. Spearman correlations for the difference in number of calls between the pre- and post-exposure day and average number of calls emitted during 8 and 10 days of prolonged exposure to acoustic treatments in the 2018 and in 2022-2023 assemblies, respectively (top), and calls emitted during the last day of prolonged exposure for the same two assemblies (bottom). Bold characters indicate significant P values

Variable analyzed	Year assembly	Treatment	S	rho	P value
Average number of calls during days of prolonged exposure	2018	Silence	116.150	-0.383	0.3493
		Chorus	182.000	0.364	0.2463
	2022-2023	Silence	172.000	-0.042	0.9186
		Chorus	874.000	0.098	0.6986
		Rain	642.970	0.054	0.8413
Number of calls during the last day of prolonged exposure	2018	Silence	90.970	-0.083	0.8451
		Chorus	184	0.357	0.256
	2022-2023	Silence	159.670	0.032	0.9294
		Chorus	474.000	0.511	0.0321
		Rain	461.300	0.322	0.2245

Table 5. *A posteriori* contrasts for the effect of rain noise amplitude.

Values in the first column indicate dB SPL.

The stimulus broadcast at 67 dB SPL was the reference for comparisons

Contrast	ratio	SE	Z ratio	P value
61 / 67	0.65	0.14	-2.05	0.13322
73 / 67	1.82	0.34	3.17	0.00582
79 / 67	2.12	0.41	3.93	0.00033
85 / 67	2.12	0.44	3.64	0.00107

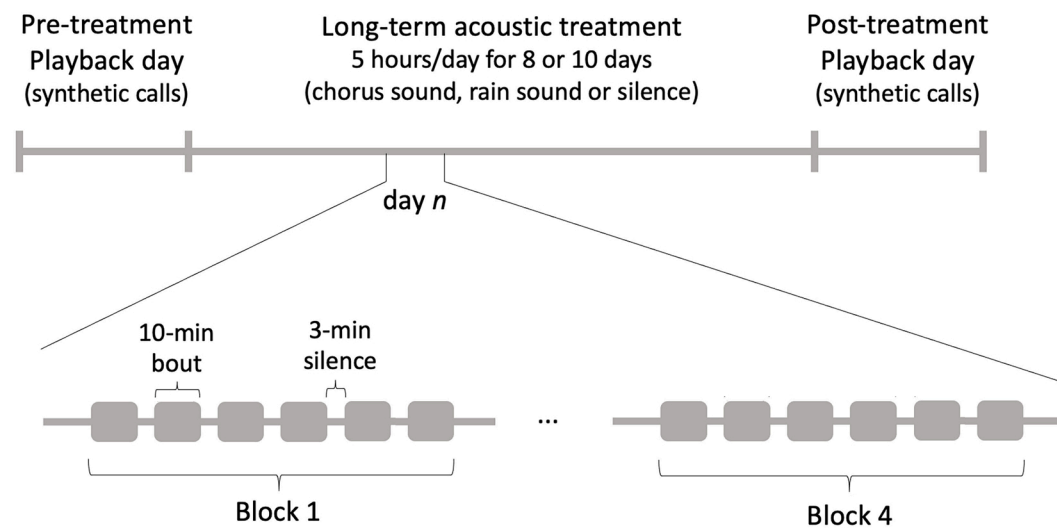


Fig. S1 Diagram showing the experimental protocol used to study short- and long-term habituation and sensitization processes in *Batrachyla taeniata*. Frogs' vocal responses to conspecific synthetic calls were measured on the first day of the experimental sequence. Thereafter the individuals were exposed during 8 or 10 days (5 hours/day) to either chorus sound, rain sound or silence. After this long-term exposure, their vocal responses to synthetic calls were measured again. Blocks 1 and 4 presented on a given day of prolonged exposure, each containing six stimuli bouts are shown

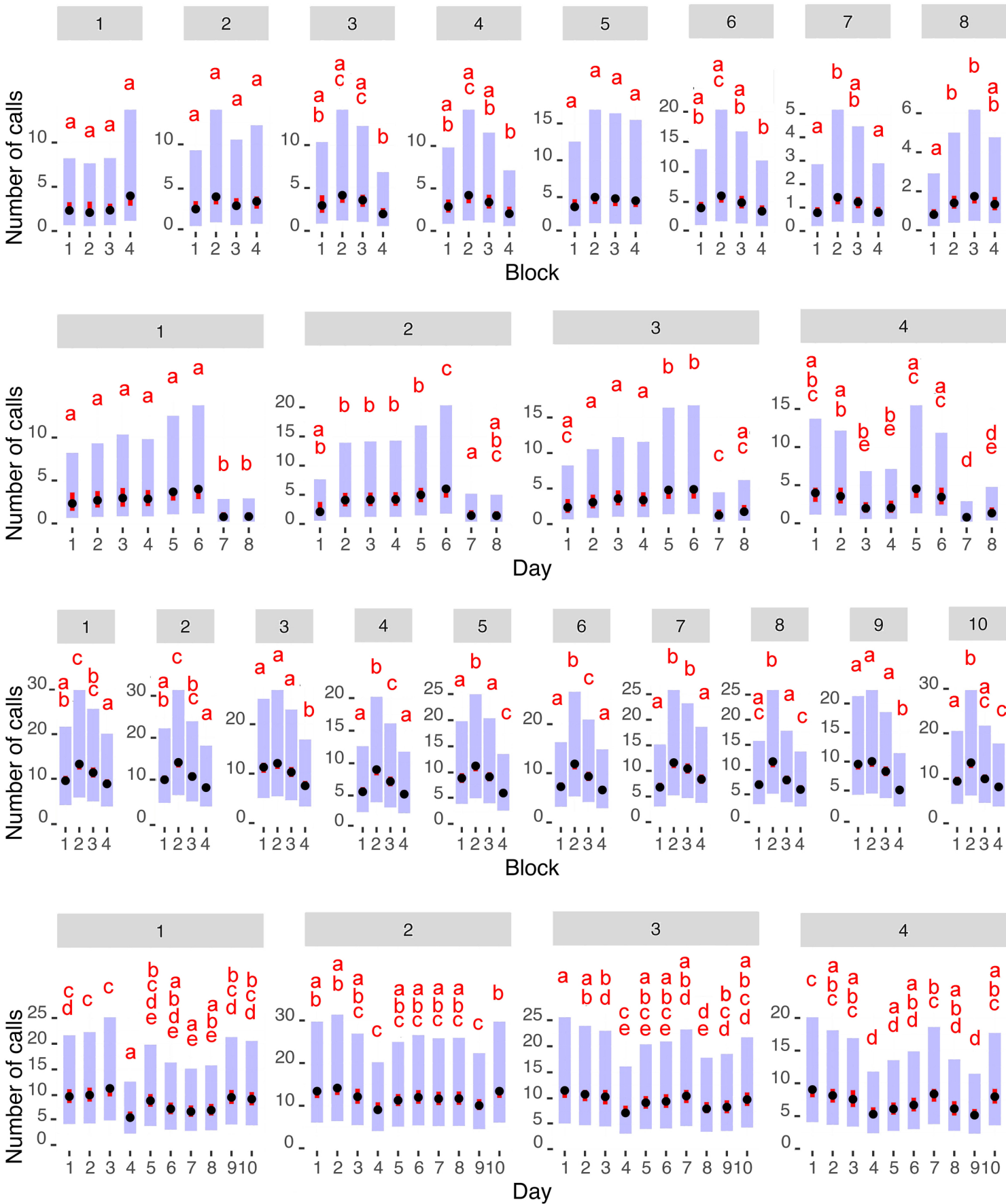


Fig. S2

Fig. S2. *A posteriori* analyses of the interaction between day and block. (A, B)

Number of calls produced by individuals of the 2018 assembly throughout 8 days of exposure and four successive daily time blocks (C, D) Number of calls produced by individuals of the 2022-2023 assembly throughout 10 days of exposure and during four successive daily time blocks. Blue bars: 95% confidence intervals. Red bars: comparison bars that indicate significant differences when no overlap occurs among them. Dots: means, C: chorus, S: silence, R: rain. Red lower-case letters above bars indicate similarities and differences for paired comparisons.

Table S1. *A posteriori* contrasts of the interaction between day and treatment for the 2018 assembly. Abbreviations: C: Chorus. S: silence. Bold numbers indicate significant differences

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Table S2. *A posteriori* contrasts of the interaction between day and treatment for the 2022-2023 assembly. Abbreviations: C: Chorus. S: silence. Bold numbers indicate significant differences

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Table S3. *A posteriori* contrasts of the interaction between block and treatment for the 2018 assembly. Abbreviations: C: Chorus. S: silence. Bold numbers indicate significant differences

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Table S4. *A posteriori* contrasts of the interaction between block and treatment for the 2022-2023 assembly. Abbreviations: C: Chorus. S: silence. Bold numbers indicate significant differences

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Table S5. *A posteriori* contrasts of the interaction between day and block for the 2018 assembly. Abbreviations: C: Chorus. S: silence. Bold numbers indicate significant differences

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Table S6. *A posteriori* contrasts of the interaction between day and block for the 2022- 2023 assembly. Abbreviations: C: Chorus. S: silence. Bold numbers indicate significant differences

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SUPPLEMENTARY MATERIALS AND METHODS

A posteriori analyses of the interaction between day and block

A posteriori analyses of the interaction between day and block indicated that for the 2018 assembly, number of calls in block 2 was higher than in block 4 on days 3, 4, 6 and 7; number of calls in block 3 was higher than in block 4 on day 3; number of calls in block 1 was lower than in block 2 on days 7 and 8 and number of calls in block 1 was lower than in block 3 on day 8, indicating an overall tendency to a decrease in calling activity within days (Fig. S2a, Table S5). The analyses of each block throughout days showed that in general, a lower number of calls was observed in the last two days of experimentation. For block 1, higher number of calls were produced during days 1- 6 relative to days 7 and 8. For block 2, lower number of calls were produced during day 1 relative to day 6 and higher numbers of calls were produced during days 2-6 relative to days 7 and 8. For block 3, lower number of calls were produced during day 1 relative to days 5 and 6, and higher number of calls were produced during days 2-6 relative to day 7, and also higher number of calls were produced during days 5 and 6 relative to day 8. Finally for block 4, the trend was more variable, as a higher number of calls were produced during day 1 relative to days 3, 7 and 8, during day 2 relative to days 7 and 8, during days 3 and 4 relative to day 7, during days 5 and 6 relative to days 7 and 8, but in contrast lower numbers of calls were produced during days 3 and 4 relative to day 5 (Fig. S2b Table S5).

For the 2022-2023 assembly, the general trend throughout days showed an increment in number of calls from block 1 to block 2, and then a progressive decrease towards block 4. The number of calls in block 1 was lower relative to block 2 on days 1, 2, 4-8 and 10, and lower in block 1 relative to block 3 on days 4, 6 and 7; number of

calls was higher in block 1 than in block 4 on days 3, 5 and 9; number of calls was higher in block 2 than in block 3 on days 2, 4-6, 8 and 10; number of calls was higher in block 2 than in block 4 on all days; number of calls was higher in block 3 than in block 4 on days 1-9 (Fig. S2c, Table S6). The analyses of each block throughout days showed a more variable trend, but in general a lower number of calls were produced on the fourth day of experimentation. Number of calls was lower on day 4 than on days 1 and 2 in the four blocks. Also, number of calls was lower on day 4 than on day 3 for blocks 1 and 3, and lower on day 4 than on day 7 for blocks 3 and 4, lower on day 4 than on day 10 for blocks 1, 2 and 4, and lower on day 4 than on days 5 and 9 for block 1. Other differences for block 1 comprised higher number of calls on days 1-3 relative to days 7 and 8, on days 2 and 3 relative to day 6, and lower number of calls on day 7 relative to days 9 and 10. For block 2, lower number of calls occurred on day 9 relative to days 1, 2 and 10. For block 3, higher number of calls occurred on day 1 relative to days 8 and 9, and on day 2 relative to day 8. For block 4, higher number of calls occurred on day 1 relative to days 5, 6 and 9, higher number of calls occurred on days 2, 3, 7 and 10 relative to day 9, and higher number of calls on day 5 relative to day 7 (Fig. S2d, Table S6).