

The acoustic expression of stress in a songbird: Does corticosterone drive isolation-induced modifications of zebra finch calls?

Emilie C. Perez^{a,*}, Julie E. Elie^{a,1}, Christophe O. Soulage^b, Hédi A. Soula^b,
Nicolas Mathevon^a, Clémentine Vignal^a

^a Université de Saint-Etienne, Université de Lyon, ENES/CNPS CNRS UMR8195, 42023 Saint-Etienne, France

^b Université de Lyon, INSA de Lyon, CarMeN, INSERM U1060, Univ Lyon-1, F-69621, Villeurbanne, France

ARTICLE INFO

Article history:

Received 16 December 2011

Revised 2 February 2012

Accepted 4 February 2012

Available online 24 February 2012

Keywords:

Acoustic communication

Distress

Emotion

Prosody

Playback

Social separation

Taeniopygia guttata

Vocal flexibility

ABSTRACT

Animal vocalizations convey multiple pieces of information about the sender. Some of them are stable, such as identity or sex, but others are labile like the emotional or motivational state. Only a few studies have examined the acoustic expression of emotional state in non-human animals and related vocal cues to physiological parameters. In this paper, we examined the vocal expression of isolation-induced stress in a songbird, the zebra finch (*Taeniopygia guttata*). Although songbirds use acoustic communication extensively, nothing is known to date on how they might encode physiological states in their vocalizations. We tested the hypothesis that social isolation in zebra finches induces a rise of plasma corticosterone that modifies the vocal behavior. We monitored plasma corticosterone, as well as call rate and acoustic structure of calls of males in response to the playback of female calls of varied saliences (familiar versus stranger) in two situations: social isolation and social housing. Social isolation induced both a rise in plasma corticosterone, and a range of modifications in males' vocal behavior. Isolated birds showed a lower vocal activity, an abolition of the difference of response between the two stimuli, and evoked calls with longer duration and higher pitch. Because some of these effects were mimicked after oral administration of corticosterone in socially housed subjects, we conclude that corticosterone could be partly responsible for the isolation-related modifications of calls in male zebra finches. To our knowledge, this is the first demonstration of the direct implication of glucocorticoids in the modulation of the structure of vocal sounds.

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Introduction

Animal vocalizations can convey information about context and events in the environment, as well as multiple pieces of information about the sender such as identity, physical characteristics, emotional or motivational state (Seyfarth and Cheney, 2003). Within a species, individuals often produce different context-specific types of calls that are characterized by different structures (amplitude, frequency, duration, etc). These calls generally carry stable characteristics related to the emitter's sex or individual identity, but also more instantaneous information such as the sender's motivation or physiological state. For instance, in the presence of a male, females of the South African clawed frog *Xenopus laevis* can produce two call types both consisting of repetitive click trains: a fast fertility call and a slower unreceptive call (Tobias et al., 1998). While these two call types differ in click rate, both are slower than male calls (Tobias et al., 1998). Thus, these calls encode stable information about the

sender's sex and social context, but also labile information about reproductive state (Tobias et al., 1998; Vignal and Kelley, 2007).

The emotional state of the emitter is an example of transient information conveyed in the vocalizations. Because of its importance in speech perception, several studies have focused on vocal expression of emotions in humans (Bachorowski, 1999; Bachorowski and Owren, 2003), and speech rhythm and intonation seem to be the main parameters affected by emotional state (Nygaard and Queen, 2008). In contrast, few studies have examined the acoustic expression of emotional state in non-human animals. One notable exception is the acoustic expression of distress during mother–young separation or during isolation from affiliated individuals in mammalian species. In infant rhesus monkeys, the isolation from the mother induces behavioral agitation and modification of the acoustic structure of vocalizations (Bayart et al., 1990; Levine et al., 1985). In several species of primates, isolation from the mother in young or separation from affiliated individuals in adults has been reported to elicit the emission of specific vocalizations named separation calls (Masataka and Symmes, 1986; Mendoza and Mason, 1986; Norcross and Newman, 1999; Scheumann et al., 2007). In rodents, isolated pups produce specific ultrasonic vocalizations that provoke a fast return of the mother to the nest (Ehret, 2005; Shair, 2007), suggesting that these separation calls convey information

* Corresponding author.

E-mail address: emilie.perez@univ-st-etienne.fr (E.C. Perez).

¹ Present address: 3210 Tolman Hall, University of California, Berkeley, CA 94720, USA.

about the emotional distress of the young and promote physical re-union with the mother (D'Amato et al., 2005).

Physiological stress is a good candidate as proximate mechanism of the acoustic expression of separation distress. Indeed, the perturbation of social interactions is one of the most efficient stressors in animals (DeVries et al., 2003). Social isolation in particular provokes a rise of plasma glucocorticoids in both young and adults of many mammalian and avian species. In infant squirrel monkeys and rhesus monkeys, isolation from the mother elicits a rise of plasma cortisol (Levine and Wiener, 1988; Levine et al., 1985). In marmosets (Norcross and Newman, 1999) and titi monkeys (Mendoza and Mason, 1986), separation of adult pair-mates elevates cortisol levels. In pigs, young females isolated from the social group show an increase of plasma cortisol (Ruis et al., 2001). In European starlings, isolation from group members increases plasma corticosterone, the main stress hormone in birds (Apfelbeck and Raess, 2008). Thus, social isolation may be perceived as a strong stressor, triggering a hypothalamic–pituitary–adrenal (HPA) axis response through a glucocorticoid release. This physiological stress response could provoke the emission of modulated vocalizations that could be considered as the vocal expression of stress. To the best of our knowledge, this hypothesis has not yet been directly tested.

The present study aimed at investigating the vocal expression of stress and its control by glucocorticoids using a songbird as a model system. Although songbirds use acoustic communication extensively during social interactions, nothing is known to date on how they encode emotional and physiological states like stress in their vocalizations. Our study focused on a songbird model species, the zebra finch (*Taeniopygia guttata*). This gregarious bird forms monogamous life-long pair bonds, and is thus highly social (Zann, 1996). Perturbations of social interactions in this bird such as separation from the mate (Remage-Healey et al., 2003) or isolation (Banerjee and Adkins-Regan, 2011) are already suspected to provoke physiological stress but nothing is known on the related modifications of acoustic communication. We hypothesized that social isolation in zebra finches provokes a rise of plasma corticosterone that modifies the acoustic structure of calls and the call rate of emission. We monitored plasma corticosterone concentration, as well as call rate and acoustic structure of calls of males in response to the playback of female calls of varied saliences (familiar versus stranger) in two situations: social isolation and social housing. To test whether the vocal modifications induced by social isolation are triggered by a physiological stress, we investigated whether oral administration of corticosterone can mimic the effects of isolation in socially housed subjects.

Materials and methods

Subjects and housing conditions

Birds used for this study were zebra finches (*T. guttata*) bred in our colony (ENES lab, University of Saint-Etienne). Thirty male subjects were used to study the kinetics of circulating level of corticosterone after oral administration of exogenous hormone (Experiment 1). Eighteen male subjects were used in the playback experiment (Experiment 2). All male subjects ($n=48$) were housed in individual cages (dimensions $24 \times 29 \times 39$ cm) for the duration of the experiments (light conditions: 14:10 h light:dark; temperature from 22 to 24 °C) to acclimate them to the experimental condition. Food (mixed seeds) and water were provided ad libitum. Birds had free access to a water pool for bathing and were also supplemented with fresh salad once a week.

For at least two weeks before the playback experiment, subjects shared the room with 10 breeding pairs (referred to as “audience birds” in the protocol; each breeding pair was housed in an individual cage) and six females (whose calls were used as familiar acoustic stimuli during playback experiments; each female was housed in an individual cage). Because all cages were in the same room allowing

visual and acoustic contacts between birds, all birds in the room can be considered as familiar to each other.

Six females unknown by the other birds were housed in individual cages and kept in a second room without any acoustic or visual contact with the subjects. Distance calls from these females were used as stranger acoustic stimuli during the playback experiment. The corticosterone baseline of our birds (see results, mean = 3.50 ng/mL) was not different from corticosterone baseline of birds living in aviaries of other studies (Remage-Healey et al., 2003, 3.80 ng/mL for pair housed birds, 3.50 ng/mL for group housed birds). Thus, we assumed that our housing conditions did not modify the baseline stress level and thus the basal behavior of our birds.

Experiments were performed under the authorization no. 42-218-0901-38 SV 09 (ENES Lab, Direction Départementale des Services Vétérinaires de la Loire) according to the guidelines laid down by the French Ministère de l'Agriculture (no. 87-848) and the E.U. Council Directive for the Care and Use of Laboratory Animals of November 24th, 1986 (86/609/EEC).

Corticosterone manipulation

To non-invasively induce an acute increase of plasma corticosterone, a procedure of oral administration was used. The subjects were fed with 300 mg of seeds sprinkled either with corticosterone dissolved in peanut oil (CORT condition in Experiment 1 and 2) or peanut oil alone (Control condition in Experiment 1, NOCORT and ISOLATION conditions in Experiment 2). To elicit a huge increase of plasma corticosterone in few minutes (Spencer and Verhulst, 2007), a dose of 0.0125 mg of exogenous corticosterone was used (50 µL, concentration: 0.25 mg/mL; Sigma Aldrich ref: 27840). Seeds were presented via a trap-door at the bottom of the experimental cage. The cage was inside an acoustically-isolated chamber (internal dimensions: 2.00 m H $\times$ 1.35 m W $\times$ 1.00 m D; Silence Box model, Tip-top Wood, Saint-Etienne, France). To make sure that subjects ate the entire dose of seeds in less than 5 min, any food was removed from the cage the day before the experiment (15 h30 $\pm$ 30 min before the experiment). To monitor when the subject started eating the seeds (denoted as $t=0$) and the time spent eating, the experimenter observed the bird through a one-way mirror and was thus outside the chamber.

Blood sample collection and hormone assay

All blood samples (<150 µL) were obtained by puncturing the alar vein with a 25-gauge needle. Blood was collected in heparinized tips, and plasma was separated under centrifugation (15 min, 3800 $\times$ g) and stored at -80 °C until hormone assay. Blood samples were taken within 3 min so as to measure corticosterone baseline and not corticosterone due to handling (Wingfield et al., 1982). No bird was bled more than once per week to ensure that they had sufficiently replenished their blood volume between bleeds and HPA axis function had recovered from previous sampling (Romero and Reed, 2008).

Because circulating level of endogenous corticosterone shows circadian variations in many bird species with a peak just before the active period and a rapid decrease during the first hours of activity, all experiments were performed between 8 and 11 am (Remage-Healey and Romero, 2000; Tarlow et al., 2003).

Hormone assays were performed using the ELISA method (Kit Corticosterone EIA, Cayman, #5000561) according to the manufacturer's recommendations. All samples were run in duplicate in three different assays: all samples from Experiment 1 were run in one assay and samples from Experiment 2 in two other assays. The detection limit was 0.2 ng/mL, intra-assay coefficient of variation was 0.14, and inter-assay coefficient of variation was 0.09.

Experimental setup and test procedures

Experiment 1: Study of the kinetics of circulating level of corticosterone after oral administration of exogenous hormone

To assess the effects of exogenous corticosterone administration on circulating levels of hormone over time, 30 males were used. The day before the experiment ($15 \text{ h}30 \pm 30 \text{ min}$ before the experiment), the subject was placed without any food in an individual cage next to a pair of audience birds (placed in another individual cage). Both cages were inside an acoustically-isolated chamber. On the morning of the experiment, seeds with corticosterone (CORT condition, $n=13$) or without corticosterone (control condition, $n=13$) were presented to the subject via the trap-door. Blood was then collected either at 15 min ($n=4$ and $n=5$ for the control and the CORT conditions, respectively), or 25 min ($n=4$ for the control and the CORT conditions, respectively) after the subject started eating the seeds. Baseline corticosterone ($t=0$) was assessed using other subjects ($n=4$) placed in the same experimental conditions (placed without any food and with the presence of an audience) but without the presentation of the seeds before blood sampling.

Experiment 2: Playback experiments

Experimental groups. Eighteen male subjects were used in playback experiments in three different conditions:

- NOCORT condition: subjects were placed with a pair of audience birds and received seeds with peanut oil.
- CORT condition: subjects were placed with a pair of audience birds and received seeds with corticosterone.
- ISOLATION condition: subjects were alone and received seeds with peanut oil.

Twelve males (group 1) experienced a NOCORT condition followed by a CORT condition a week later. 6 males (group 2) experienced a CORT, then a NOCORT and finally an ISOLATION condition, with one week between two conditions. Birds of group 2 were exposed to CORT and NOCORT conditions in the reverse order as group 1 so as to control for a potential order effect. Results did not differ between groups, so data were pooled and analyzed together.

Repertoire choice for playback stimuli and analysis of the subject's vocal response. While zebra finches have ten distinct calls in addition to the song (Zann 1996), we decided to focus our study on distance calls: first, these calls are usually used by sexual partners when they lose visual contact, and secondly, it has been demonstrated that distance calls carry the identity of the sender (Vignal et al. 2008, Forstmeier et al. 2009), supposedly allowing the birds to distinguish between calls from familiar females and calls from stranger females. Distance calls were thus supposed to have a stable and not easily alterable acoustic structure.

Preparation of the subjects. The day before the experiment (at least 12 h prior to the start of stimulus presentation), the subject was deprived of food in the experimental cage (Fig. 1) either in the presence of a pair of audience birds (CORT and NOCORT conditions) or alone (ISOLATION condition). The experimental cage was inside an acoustically-isolated chamber. Both the subject and the audience birds were placed in the lower part of the cage, but the subject was physically separated from the audience by a wire barrier that allowed acoustic and visual contact (Fig. 1). On the morning of the experiment, seeds were presented to the subject via the trap-door. Five minutes after the subject started eating the seeds, the experimenter opened the door between the lower part and the upper part of the experimental cage, so that only the subject was able to visit this part of the cage. It was important not to open the door between

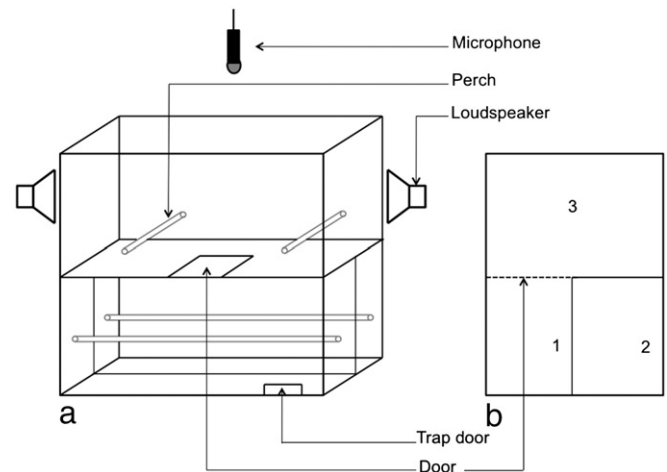


Fig. 1. Experimental cage used in Experiment 2. a) Front view, b) Side view. The experimental cage is composed of three parts: the subject was placed in part 1. This area communicated with the upper part of the cage (part 3) when the door was open. The audience was placed in part 2. This area did not communicate with the other parts of the cage, so that only the subject could visit the upper part of the cage. The two lower parts of the cage were separated by wire mesh allowing visual and acoustic contacts between the subject and the audience but preventing physical contacts. A trap-door allowed the experimenter to give seeds to the subject.

the two parts of the cage before the subject started eating, so as to make sure that the bird would easily find the seeds and eat them quickly.

Recording and preparation of acoustic stimuli. Distance calls from six stranger and six familiar females were recorded in an acoustically isolated room. The day before the recording session, the female subject was placed in an individual cage in sight of another cage containing two females to provide a social context and to avoid social isolation. Recording started on the following morning. Subjects were stimulated with distance calls of males, played by a computer using Goldwave software (GoldWave Inc. St. John's, NL Canada) through an amplifier (Yamaha AX 396) connected to a loudspeaker (Audiopro Bravo Allroom sat E7140044). Evoked calls were recorded using a microphone (Sennheiser MD42) located 50 cm above the subject and connected to a recorder (Marantz PMD 670, sampling rate: 44.1 kHz).

Playbacks. Fifteen minutes after the beginning of ingestion, a playback session started in the upper part of the cage. This delay was determined based on the results of Experiment 1. Each playback session consisted of four sets of five calls. Each set lasted five seconds (one call per second) and sets were separated by at least 1 min and 55 s. The next set started only if the subject remained silent for at least 10 s, considered as a criterion of basal activity level. Thus, a playback session lasted at least 8 min. Among the four sets, 2 sets contained different calls from one familiar female and 2 sets contained different calls from one stranger female. Sets were randomly played. The females used as stranger or familiar stimuli were never used in two different conditions for the same subject. So, the stranger/familiar stimuli used in one condition (e.g. NOCORT) were different from the stranger/familiar stimuli used in the following condition (e.g. CORT).

Calls were played by a computer using Goldwave software, through an amplifier (Yamaha AX 396) connected to two loudspeakers (Audiopro Bravo Allroom sat E7140044). Loudspeakers were placed on both sides of the experimental cage, so that sets of calls could be randomly played either on the right or on the left side of the cage. The intensity of calls given by audience birds placed in the test room was used as a sound intensity reference for the playback. The root mean square (RMS) sound pressure of the stimuli was set at half the RMS value of the audience's calls using Goldwave software, to mimic a distant bird.

Vocal response of the subjects was recorded during the entire session using a microphone (Sennheiser MD46) connected to a recorder (Marantz PMD 670, sampling rate: 44.1 kHz).

At the end of the playback session, the subject was rapidly caught (less than 2 min after the end of the last playback set) and bled within 3 min after entering the acoustically-isolated chamber to measure the level of plasma corticosterone.

Analysis of the vocal response. For each playback test, the vocal response of the subject was assessed using two behavioral parameters:

- the latency to the first call (in seconds): the time between the start of the first call of a stimulus set and the first distance call emitted by the subject.
- the call density: the number of calls emitted by the subject during the first minute of each stimulus set was first quantified using Pratt software (Boersma, 2001). Since calling activity during one playback session was highly variable across individuals (mean number of calls = 41 ± 20 (SD), min = 8, max = 81), normalization procedures were used. First, the number of calls emitted during each stimulus set was divided by the total number of calls emitted by the subject during the entire playback session (4 stimulus sets, 2 stranger and 2 familiar sets). This procedure produced a parameter called an allocation factor “AF STIM” that reflected the proportion of calling activity allocated to each stimulus set in a given playback session. Second, the number of calls emitted during one playback session was divided by the total number of calls emitted by the subject during the different playback sessions (2 sessions in group 1, 3 sessions in group 2). This procedure produced an allocation factor called “AF COND” that reflected the proportion of calling activity allocated to each condition (NOCORT versus CORT versus ISOLATION). Because birds underwent either 3 playback sessions (group 2) or 2 playback sessions (group 1), “AF COND” was scaled by a coefficient 2/3 in group 1 to allow for comparisons of all birds.

Distance calls used by the subjects in response to the stimuli were isolated using Pratt software and analyzed. Seven acoustic parameters were measured for each call. Using a home-made computer program, the call duration and the call mean frequency were quantified as follows:

- call duration (in seconds): the energy was first computed from the signal envelope and the peak maximum was used as an energy reference. The beginning and the end of the call were then defined as the points where the signal reached 10% of this reference before and after the peak maximum.
- call mean frequency (in Hertz): the mean frequency was first calculated between 0 and 6000 Hertz using the power spectrum of the signal (Fast-Fourier-Transform). A high-pitched call was characterized by a high mean frequency, whereas a low-pitched call showed a low mean frequency. An index of variation “I” was then calculated for each call as follows: $I = FX - FM$ where FX is the mean frequency of the call analyzed, and FM is the average of the mean frequencies of the 10 lowest-pitched calls emitted by the subject during the whole experiment. This index thus represented a measure of the pitch variation relative to the average pitch of the subject's lower-pitched calls.

Using the Seewave package (Sueur et al., 2008) implemented in R software (R Development Core Team, 2007, www.r-project.org), we measured the following spectral parameters:

- the call dominant frequency (in Hertz) is the average over the duration of the call of the frequencies of highest amplitude (obtained via the ‘dfreq’ package function). The frequency of highest amplitude is first assessed on time bins using a window length of 512, and the series of values obtained over the duration of the call is then averaged.

- the call fundamental frequency (in Hertz) is the average over the duration of the call of the fundamental frequency values (these are tracks of the fundamental frequency through a short-term cepstral transform obtained via the ‘fund’ package function).
- the median, the mode and the inter-quartile range of the call spectrum (using ‘specprop’ package function).

Statistical analysis

All statistical tests were performed using R software. Normal distribution was tested using the Shapiro test and homoscedasticity was tested using the Bartlett test. When at least one of these two conditions was violated, non-parametric statistical tests were used.

In Experiment 1 (kinetic study), data were independent so the results were analyzed using t-tests.

In Experiment 2, hormone data were analyzed using a Linear Mixed-effects Model (LMM, using lme function of the R package nlme) with the condition (NOCORT, CORT or ISOLATION) as a fixed factor, and subject identity as a random factor. This allowed us to control for potential confounding effects of the number and order of conditions experienced by each subject. All post hoc tests were done using paired t-tests with Bonferroni correction.

Call density (AF STIM and AF COND) and fundamental frequency were all analyzed using LMM with the condition (NOCORT, CORT or ISOLATION) and the type of stimulus (familiar calls or stranger calls) as fixed factors, and subject identity and order of presentation of the stimulus as random factors. All post hoc tests were done using paired t-tests with Bonferroni correction.

Latency to the first call, call duration, index of variation of call mean frequency, dominant frequency, median, mode and inter-quartile range values were analyzed using a Kruskal–Wallis ANOVA. Posthoc tests were done using Wilcoxon tests with Bonferroni correction.

Values displayed are means are given $\pm$ SE. All significant differences are reported for $P \leq 0.05$.

Results

Experiment 1: Circulating levels of corticosterone after oral administration of exogenous hormone

Plasma corticosterone levels increased significantly following oral administration while levels of corticosterone in the control group did

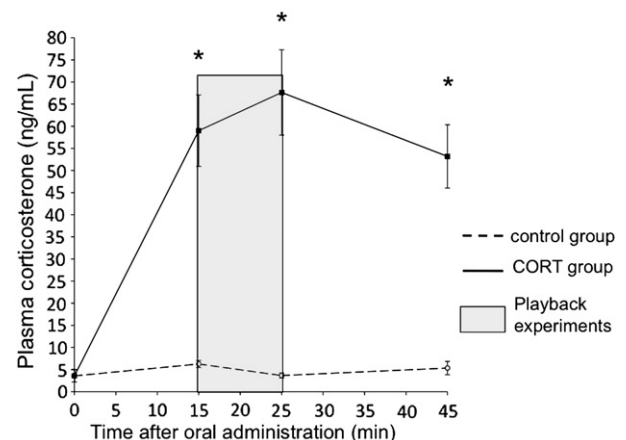


Fig. 2. Effect of oral administration of exogenous hormone on plasma corticosterone levels: Kinetic study (Experiment 1). $n = 13$ for both CORT group and control group. The zero time-point represents baseline corticosterone level ($n = 4$). The gray rectangle highlights the choice of the beginning and the end of playback experiments (experiment 2). Data are presented as mean $\pm$ SE. *: significant differences between the two treatment groups at the $P < 0.05$ level.

not change over time and remained at baseline with a mean value around $3.57 \text{ ng/mL} \pm 0.97$ (t-tests, CORT group vs control group: $P=0.008$ at 15 min, $P=0.035$ at 25 min and $P=0.018$ at 45 min, Fig. 2). Corticosterone levels peaked 15 min after oral administration and remained high until 45 min. Playback experiments (Experiment 2) were thus performed during this peak ranging from 15 to 25 min after oral administration — as shown with a gray time range on Fig. 2.

Experiment 2: Modifications of behavioral response to playback with elevation of circulating levels of corticosterone

Modification of circulating levels of corticosterone with experimental conditions

Plasma corticosterone was measured at the end of the playback session, i.e. 25 min after the ingestion of seeds. In line with the results obtained in Experiment 1, oral administration of exogenous corticosterone provoked a huge increase of circulating hormone reaching 1700% of basal values (LMM: $F_{2,19}=40.80$, $P<0.001$, paired t-tests with Bonferroni correction: NOCORT versus CORT: $P<0.001$, ISOLATION versus CORT $P<0.001$). Isolation also significantly increased circulating levels of corticosterone (164% of basal values) that did not reach the levels obtained in CORT condition (paired t-test with Bonferroni correction: ISOLATION versus NOCORT: $P=0.002$, Fig. 3).

Exogenous administration of corticosterone as well as isolation abolished the difference of response between the two stimuli

Whereas subjects in NOCORT condition showed higher calling activity in response to the “stranger” stimulus compared to the “familiar” stimulus (LMM: $F_{5,63}=2.87$, $P=0.02$, paired t-test with Bonferroni correction: $P<0.001$, Fig. 4), ISOLATION and CORT conditions abolished this difference (paired t-tests with Bonferroni correction: $P=0.98$ and $P=0.69$ respectively). There was no significant difference in response latency in any of the three conditions (Kruskal–Wallis ANOVA: $P=0.07$ for all comparisons).

Isolation, but not exogenous administration of corticosterone, decreased total calling activity

Subjects in ISOLATION condition showed a significantly lower total calling activity than subjects in experimental conditions with an audience (LMM: $F_{2,23}=8.25$, $P=0.002$, paired t-tests with Bonferroni correction: ISOLATION versus NOCORT $P=0.002$, ISOLATION versus CORT $P=0.05$, Fig. 5). There was no significant difference between CORT and NOCORT conditions (paired t-test with Bonferroni correction: $P=0.33$).

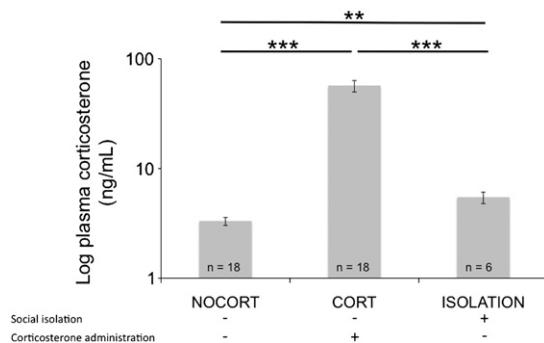


Fig. 3. Modifications of circulating level of corticosterone with experimental conditions (Experiment 2). Data are presented as mean $\pm$ SE. **: $P=0.002$; ***: $P<0.001$. NOCORT ($n=18$): subjects were placed with a pair of audience birds and received seeds with peanut oil; CORT ($n=18$): subjects were placed with a pair of audience birds and received seeds with corticosterone; ISOLATION ($n=6$): subjects were alone and received seeds with peanut oil. «+» and «-» indicate specific context for each condition: birds in social isolation (+) or in social context (-), administration of exogenous corticosterone (+) or no administration (-).

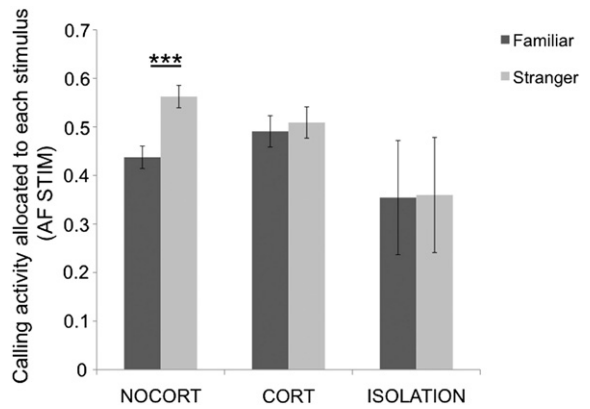


Fig. 4. Vocal activity allocated to each stimulus in response to the playback (Experiment 2). ***: $P<0.001$. Note the high variability of AF in ISOLATION condition that caused the sum of the 2 group means to be below 1.

Exogenous administration of corticosterone as well as isolation modified acoustic parameters of evoked calls

Subjects emitted higher-pitched and longer calls in ISOLATION than in the two other conditions (Table 1). Indeed, the index of variation of call's mean frequency and the duration showed significant differences between ISOLATION and the two other conditions (Tables 2 and 3). Albeit significant (see Tables 2 and 3), the differences in duration between conditions are probably too small (up to 6 ms) to be of biological importance. On the other hand, the index of variation of call's mean frequency not only showed significant differences but also amounted to easily discernable variation (Table 1).

In addition, the pitch of the calls in CORT condition can be described as intermediate between NOCORT and ISOLATION conditions since the dominant frequency, the mode and the median were all ordered decreasingly as ISOLATION > CORT > NOCORT (Tables 1, 2 and 3). These differences can be observed on a spectrogram of the calls (Fig. 6 and Supplementary sound file).

Calls in NOCORT condition showed a more broadband spectral composition than calls in CORT and ISOLATION. Indeed, the inter-quartile range (IQR) is significantly higher in NOCORT condition (Tables 1, 2 and 3).

The index of variation of call's mean frequency, the median and the mode of the spectrum as well as the fundamental frequency were all affected by the stimulus type: “stranger” stimuli evoked higher-pitched calls and calls of higher fundamental frequency than “familiar” stimuli (Tables 1, 2 and 3).

Finally, interactions between conditions and stimuli yielded no significant differences.

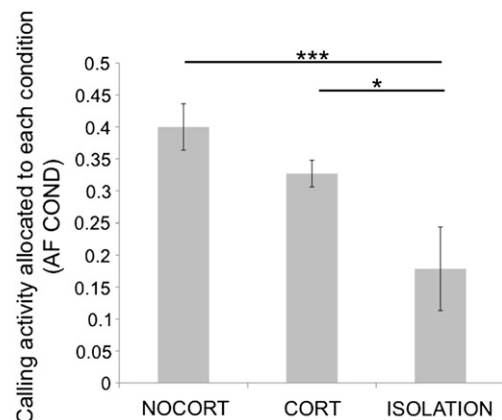


Fig. 5. Vocal activity allocated to each condition in response to the playback (Experiment 2). ***: $P<0.001$; *: $P=0.05$.

Table 1

Measure of seven acoustic parameters on the calls emitted during the playback experiment. Values are given as mean $\pm$ SE. Duration is given in seconds, whereas other parameters are given in Hz. (Index = index of variation of call mean frequency; IQR = inter-quartile range).

	Condition			Stimulus	
	NOCORT	CORT	ISOLATION	Familiar	Stranger
Duration	0.106 $\pm$ 1.6E–3	0.108 $\pm$ 2E–3	0.112 $\pm$ 2.2E–3	0.107 $\pm$ 1.6E–3	0.109 $\pm$ 1.5E–3
Index	259.6 $\pm$ 11.1	295.9 $\pm$ 13.0	380.9 $\pm$ 24.6	269.8 $\pm$ 11.7	309.0 $\pm$ 11.2
Dominant frequency	3876.5 $\pm$ 20.6	4057.7 $\pm$ 13.5	4193.1 $\pm$ 19.1	3957.5 $\pm$ 18.9	4018.8 $\pm$ 15.3
Fundamental frequency	781.3 $\pm$ 6.6	773.6 $\pm$ 7.8	860.7 $\pm$ 10.7	774.9 $\pm$ 6.5	802.1 $\pm$ 6.7
Median	4062.8 $\pm$ 12.4	4149.8 $\pm$ 11.1	4301.9 $\pm$ 18.1	4105.5 $\pm$ 12.5	4150.2 $\pm$ 10.5
Mode	4023.2 $\pm$ 27.2	4196.3 $\pm$ 28.3	4542.1 $\pm$ 41.9	4118.8 $\pm$ 27.8	4198.3 $\pm$ 25.8
IQR	1389.6 $\pm$ 16.9	1295.5 $\pm$ 13.6	1294.2 $\pm$ 19.4	1342.3 $\pm$ 15.2	1338.5 $\pm$ 13.5

Discussion

In our experiment, social isolation induced in male zebra finches both an acute physiological stress, evidenced through a notable rise in plasma corticosterone concentration, and a range of modifications in their vocal behavior in response to playback. Indeed, isolated birds showed an overall decline of their vocal activity, an abolition of the difference of response between the two stimuli, and evoked calls with longer duration and higher pitch. Because some of these effects were mimicked after oral administration of corticosterone in social context (CORT group), we conclude that corticosterone could be partly responsible for the isolation-related modifications of vocal response to playback in male zebra finches.

Implication of corticosterone in isolation-related modifications of vocal response to playback

To our knowledge, our evidence is the first demonstration of the direct effect of glucocorticoids in the modulation of the acoustic structure of vocal sounds on the short term. Indeed, natural elevations of glucocorticoids induced by both acute and chronic stress are known to modify or impair a huge range of behaviors such as locomotor activity (Breuner et al., 1998; Cox et al., 2011; Landys et al., 2006), maternal behavior (Saltzman and Abbott, 2009; Yamada et al., 2002) or aggressiveness (Meddle et al., 2002). However, very few studies have looked at the short-term impact of glucocorticoids on the vocal behavior of animals and apparently, no one has ever shown the implication of corticosterone in the modification of the fine structures of the acoustic signals such as spectral modifications. Indeed, previous studies have only dealt with modifications of temporal cues such as the rate of emission of vocalizations (Kitaysky et al., 2001), and long-term effects such as the impact of developmental stress on adult song structure (Buchanan et al., 2003, 2004; Spencer

et al., 2005). Inversely, modification of the finer structures of the vocal signals was observed in mammalian as well as avian species during stressful situations but the direct effect of glucocorticoids has not been previously demonstrated. For instance, female bottlenose dolphins with dependent calves produced whistles with higher maximum frequencies during capture–release sessions (Carter et al., 2009). Here we show for the first time that the isolation-induced modifications of the acoustic structure of vocal sounds are at least partly linked to an elevation in plasma corticosterone.

In the present experiment, oral administration of exogenous corticosterone in social condition provoked a huge rise of plasma corticosterone while isolation provoked a more moderate elevation compared to social condition. This range of variation is comparable to already described population variability in response to restraint stress: Evans et al. (2006) observed that peak corticosterone production showed considerable individual variation after 20 min holding in a cloth bag, a standardized capture/restraint technique. Indeed, corticosterone concentrations in their experiment ranged from 3.06 to 183.29 nmol/L, i.e. 1.06 to 63.5 ng/mL (Evans et al., 2006).

Nevertheless, our results show that the oral administration of exogenous corticosterone (CORT group) only partly mimicked the effect of social isolation (ISOLATION group). Several hypotheses can be envisaged. First, corticosterone has a non-monotonic dose-dependent effect on many behaviors: for instance, white-crowned sparrows that received an intermediate dose of exogenous corticosterone (10 ng/mL) show a higher motor activity than those that received a significantly higher dose (60 ng/mL) (Breuner et al., 1998). Such a non-monotonic effect of corticosterone could explain why the CORT condition did not totally mimic the ISOLATION condition.

Secondly, the audience could act as a social buffer on birds of the CORT group and could thus lessen the effect of the physiological stress. This buffering effect has been reported in rhesus and saimiri monkeys, in which infants separated from their mother emit less vocalizations in the presence of conspecifics than when totally isolated (Levine and Wiener, 1988).

Table 2

Statistical tests on the seven acoustic parameters measured on the calls emitted during the playback experiment. Only significant effects are presented. Call duration, Index (= index of variation of call mean frequency), dominant frequency, median, mode and IQR (= inter-quartile range) values were analyzed using a Kruskal–Wallis ANOVA. Fundamental frequency values were analyzed using a linear Mixed-effects Model.

Parameter	Factors	Chi-squared	df	P-value	
Duration	Condition	14.52	2	0.0007	
Index	Condition	21.55	2	<0.0001	
	Stimulus	6.53	1	0.0106	
Dominant frequency	Condition	94.40	2	<0.0001	
Median	Condition	95.41	2	<0.0001	
	Stimulus	4.75	1	0.0292	
Mode	Condition	79.72	2	<0.0001	
	Stimulus	5.94	1	0.0148	
IQR	Condition	9.44	2	0.0089	
Parameter	Factors	numDF	denDF	F-value	P-value
Fundamental frequency	Condition	2	846	2.097	0.1234
	Stimulus	1	846	7.493	0.0063

Table 3

Posthoc tests on the seven acoustic parameters measured on the calls emitted during the playback experiment. Posthoc tests were run only when there was a significant effect of the factor (condition and stimulus). Posthoc tests were done using Wilcoxon tests with Bonferroni correction for call duration, Index (= index of variation of call mean frequency), dominant frequency, median, mode and IQR (= inter-quartile range) values. Fundamental frequency values were analyzed using paired t-tests with Bonferroni correction.

	Condition			Stimulus
	NOCORT vs CORT	NOCORT vs ISOLATION	ISOLATION vs CORT	FAMILIAR vs STRANGER
Duration	1.0000	0.0010	0.0020	–
Index	0.1570	<0.0001	0.0040	0.0110
Dominant frequency	<0.0001	<0.0001	<0.0001	–
Fundamental frequency	–	–	–	0.0380
Median	<0.0001	<0.0001	<0.0001	0.0290
Mode	0.0001	<0.0001	<0.0001	0.0150
IQR	0.0210	0.0370	1.0000	–

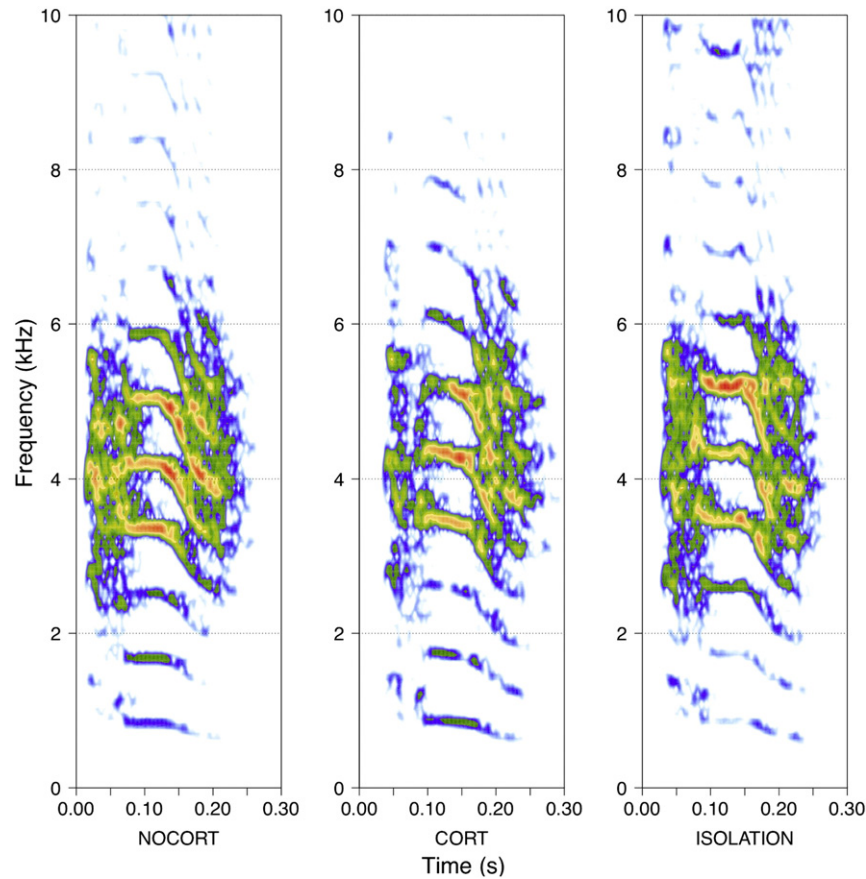


Fig. 6. Spectrograms of the calls of the same subject in the three experimental conditions. Note that the intensity of the third harmonic (around 3.4 kHz) is ordered decreasingly as $\text{NOCORT} > \text{CORT} > \text{ISOLATION}$, whereas the intensity of the fifth harmonic (around 5 kHz) is ordered increasingly as $\text{NOCORT} < \text{CORT} < \text{ISOLATION}$. These variations are consistent with a pitch ordered increasingly as $\text{NOCORT} < \text{CORT} < \text{ISOLATION}$. Spectrograms are calculated using the Seewave package implemented in R software (overlap = 90, window length = 700, color scale = -40 dB – 0 dB). See Supplementary sound file.

Finally, others hormones such as catecholamines could contribute to the modulation of isolation-induced vocalizations. In rodent pups, the injection of a dopamine receptor agonist reduces the rate of emission of isolation-induced vocalizations (Muller et al., 2005). Nevertheless, this hypothesis remains to be tested in birds.

Isolation-related modifications of zebra finch calls and their potential role in communication

To the best of our knowledge, this is the first demonstration in a bird of a vocal flexibility induced by the social context. While it has been demonstrated that the presence of an audience as well as social isolation modifies the vocal activity of male zebra finches in response to playback stimuli (Vignal et al., 2004, 2005), here we show that the acoustic structure of calls is altered in response to the social context. Moreover, isolation-related modifications of the vocal response to playback were mimicked using an experimental increase of plasma corticosterone without any variation in the social context of the bird. Thus, physiological stress could be an underlying mechanism of the audience effect in zebra finches.

The behavioral modifications triggered by the elevation of plasma corticosterone could allow the birds to respond to the stressful situation. This function has been previously demonstrated in rodents, in which the emission of modulated ultrasonic calls by isolated pups elicits reunion with the mother (D'Amato et al., 2005). An equivalent function is highly possible in zebra finches, which use distance calls when social partners lose visual contact. The adjustment of behavior could then explain why stressed birds (CORT and ISOLATION groups) do not show any difference of response to either familiar or stranger stimuli, while

they do so when they are not stressed (NOCORT group). This loss of discrimination could reveal the urgent need to elicit physical reunion with other individuals. Another explanation could be that birds are no longer able to discriminate between stranger and familiar stimuli in stressful situations. This hypothesis remains to be explored.

Our results suggest that distance calls of male zebra finches are more flexible than previously thought and can be modified in relation to an increase in plasma corticosterone indicating that the bird is experiencing stress (Cockrem, 2007), that is to say in relation to the emotion of the emitter. Corticosterone is known to elevate basal metabolism and muscular activity. A recent study has also demonstrated that the mean frequency and the duration of vocalizations are directed by basal metabolism and muscular activity in many animals (Gillooly and Ophir, 2010). Thus, the effect of corticosterone on vocalizations could be mediated by its action on the muscular activity of the emitter. This leads to an important question: do modified vocalizations represent only a by-product of the bird's physiological stress, or do they carry information about the emotion of the emitter that could be perceived by conspecifics? Zebra finches are able to perceive fine changes in the spectral components of sounds (Dooling et al., 1995; Ohms et al., 2012; Weisman et al., 1998), so they could potentially discriminate stress-induced modifications in calls. Whether stress-induced modifications of calls have a role in zebra finches' communication remains a question to investigate. Moreover, none of the birds have ever emitted distance calls spontaneously during our experiment: consequently, we do not know if spontaneous calls are modified by stress in the same way as evoked calls and thus also carry information about the emotion of the emitter.

Modifications of duration and spectrum of vocalizations in relation to the emotional state of the emitter seem to represent a conserved effect in mammals and birds. In elephants, low-ranking females interacting with dominant individuals produce rumbles with increased and more variable fundamental frequency, as well as increased duration and amplitude (Soltis et al., 2009). In humans, stressful situations such as fear or anxiety modify not only the rhythm but also the mean fundamental frequency and the pitch of the speech (Banse and Scherer, 1996). In rodents, modifications in frequency modulations and call duration of isolation-calls correlate with the distress of the pup (Ehret, 2005; Hodgson et al., 2008). Finally, the calls of primates can also be modified by the emotional state of the emitter: for instance, separated infants of squirrel monkeys give longer calls at greater separation distances from their natal group members, and responding adults and juveniles similarly extend the length of their vocalizations (Masataka and Symmes, 1986). Our study demonstrates that birds are also able to modify their vocalizations in relation to their emotional state, using similar prosodic rules as mammals.

Supplementary materials related to this article can be found online at doi:10.1016/j.yhbeh.2012.02.004.

Acknowledgments

The authors warmly thank Colette Bouchut and Nicolas Boyer for all the technical support in the ENES laboratory. We are also grateful to the BVpam laboratory, who kindly let us use their centrifuge. Thanks to Simon Griffith for proof-reading the English.

This study is funded by the French Agence Nationale de la Recherche (A.N.R., project “Acoustic partnership”). C.V. is supported by the Institut Universitaire de France (IUF). E.C.P. is supported by the Région Rhône-Alpes (CMIRA fund).

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