




## SYMPOSIUM

# Glucocorticoid-Mediated Changes in Male Green Treefrog Vocalizations Alter Attractiveness to Females

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**Synopsis** Adrenal glucocorticoids (GCs) are increasingly recognized as important modulators of male courtship signals, suggesting that circulating levels of these steroids can play a central role in sexual selection. However, few studies have examined whether GC-mediated effects on male sexual signals actually impact mate choice by females. Here, we examine how corticosterone (CORT)-mediated changes in the vocalizations of male green treefrogs, *Dryophytes cinereus*, influence attractiveness to females. In this species, agonistic acoustic signaling between rival males competing for mates increases circulating CORT levels in contest losers. Acute elevations in CORT, in turn, decrease the duration of male advertisement calls and increase the latency between successive calls, resulting in a net reduction in vocal effort (the amount of signaling per unit time) that occurs independently of changes in circulating androgens. Based on known preferences for acoustic features in *D. cinereus*, and other anuran species, the direction of CORT-mediated effects on temporal call characteristics is expected to compromise attractiveness to females, but whether they are of sufficient magnitude to impact female mate choice decisions is unclear. To examine whether CORT-mediated effects on male advertisement calls reduce attractiveness to females, we broadcast vocalizations in dual speaker playback experiments approximating the mean and 1 SD above and below the mean call duration and vocal effort values (the two primary vocal features impacted by elevated CORT) of males with low and high CORT levels. Results revealed strong preferences by females for the calls characteristic of males with low CORT in tests using the approximate mean and 1 SD above the mean call duration and vocal effort values, but females did not show a preference for calls of males with low CORT in trials using call values approximating 1 SD below the mean. Overall, females preferred males with signal traits predictive of low CORT, however this effect was nonlinear with attenuated preferences when signal alternatives differed only marginally indicating a possible thresholding effect. Specifically, females appeared to discriminate between males with low versus high CORT based primarily on differences in call rates associated with CORT-mediated changes in call duration and vocal effort. Our results highlight that changes in circulating CORT during male–male vocal interactions can decrease attractiveness to females, suggesting that circulating levels of CORT can play a critical role in both intra- and intersexual selection.

## Introduction

Adrenal glucocorticoids (GCs) can dramatically affect the reproductive phenotype. For example, elevated GCs can suppress courtship behavior via negative effects on gonadal steroids or independently of changes in gonadal steroids (Moore and Miller 1984; Greenberg and Wingfield 1987; Sapolsky 1992; Von Holst 1998; Rose and Moore 1999; Sapolsky et al. 2000; Adkins-Regan 2005; Whirledge

and Cidlowski 2013). However, a growing body of work focuses on subtler effects of GCs. These studies are aimed at understanding how acute elevations in circulating GCs tentatively modulate the quality of sexual signals in courting males (reviewed by Moore et al. 2016; Leary and Baugh 2020). Such studies are rooted in the conventional view that acute elevations in circulating GCs shift the allocation of energy away from reproduction in favor of survival (Sapolsky

1992; Breuner et al. 2008; Bonier et al. 2009; Boonstra 2013; Dantzer et al. 2016), suggesting that transient elevations in circulating GCs could negatively impact the quality of energetically-costly courtship signals (Leary and Knapp 2014; Moore et al. 2016; Leary and Baugh 2020).

Indeed, there is growing evidence indicating that elevations in circulating GCs can affect both behavioral and structural aspects of male courtship signals in ways that are expected to decrease attractiveness (reviewed Moore et al. 2016; Leary and Baugh 2020). However, only a small fraction of these studies address whether females actually discriminate among males with varying GC levels (reviewed Moore et al. 2016; Leary and Baugh 2020). For example, in a recent review of 54 studies examining the relationship between GCs and behavioral and structural sexual male traits, only six studies directly examined whether GC-mediated effects on sexual signals/traits influence mate choice decisions by females (Leary and Baugh 2020). Four of those studies examined GC-mediated effects in an activational framework, including effects on vocal characteristics in toads (Leary et al. 2006), aggression and copulatory behaviors in lizards (Gonzalez-Jimena and Fitze 2012), and composite aspects of facial attributes in humans (Moore et al. 2011; Rantala et al. 2012). The two other studies were set within an organizational framework, examining the effects of exposure to high GCs during development on feather coloration (Roberts et al. 2007) and vocalization (Spencer et al. 2005) in birds. In all of these studies, females preferred males with low existing GCs or low GCs during development over males with high GCs (reviewed by Moore et al. 2016; Leary and Baugh 2020).

Determining whether females actually discriminate among males with varying GC levels is central to assessing whether the effects of GCs on male sexual signals are meaningful in the context of sexual selection and how sexual selection potentially shapes or contributes to variation in GC regulation (Leary and Baugh 2020). Here we investigated how variation in circulating GCs in male green treefrogs, *Dryophytes cinereus* (formerly *Hyla cinerea*), impacts mate choice decisions by females. The motivation for the current study stemmed from previous work showing that acoustic signals produced by males of this species can modulate circulating levels of corticosterone (CORT) that feedback to alter male courtship signals, implicating CORT in both intra- and intersexual selection. For example, this species communicates primarily through acoustic signals consisting of advertisement calls that are used to attract females and ward off rival conspecific males,

and aggressive calls that are used in the context of male–male agonistic interactions (Gerhardt and Huber 2002). Playback experiments revealed that broadcast isolated aggressive calls increase circulating CORT levels in male receivers while broadcast isolated advertisement calls sustain elevated CORT levels relative to males exposed to silence (Leary 2014; these findings were also replicated by Kennedy et al. 2021). Aggressive calls were more effective than advertisement calls in eliciting elevations in circulating CORT in signal receivers, but the magnitude of the CORT response was inversely related to body size for males exposed to isolated advertisement calls (Leary 2014). These acoustically-induced changes in circulating CORT are linked to variation in circulating CORT levels among male *D. cinereus* in natural choruses. For example, males that were recently engaged in naturally-occurring vocal contests, involving the exchange of aggressive and advertisement calls, had higher CORT levels than males that were not involved in vocal contests (Leary and Harris 2013). Moreover, vocal contest losers, which are characteristically the smallest males, had higher CORT levels than contest winners (Leary and Harris 2013). Increased chorus density, which is expected to increase the frequency of aggressive vocal interactions, was also related to higher CORT levels, providing additional support that intraspecific competition modulates CORT levels (Kennedy et al. 2021).

Acoustically-induced alterations in circulating CORT levels during male vocal contests in *D. cinereus* are associated with changes in the temporal characteristics of advertisement calls. For example, CORT administration to chorusing males, simulating CORT production in vocal contest losers, caused a rapid reduction in call duration and increased the latency between successive calls, resulting in a net reduction in vocal effort (i.e., the amount of calling per unit time), which occurred independently of changes in circulating androgen levels (Leary and Crocker-Buta 2018). These CORT-mediated effects on vocal attributes can be traced to variation in vocal behavior of males in natural choruses. For instance, CORT level was a better predictor of individual variation in call duration, intercall duration, and vocal effort among chorusing males than androgen level, body size, or body condition (Crocker-Buta and Leary 2018a).

The effects of elevated CORT on temporal vocal attributes of male *D. cinereus* tentatively have a negative impact on attractiveness to females. For example, in most anuran species studied, females prefer males with calls that are longer in duration and produced at more rapid rates, reflecting preferences for acoustic signals with more energy per unit time, or

males with higher vocal effort (see Gerhardt and Huber 2002). In *D. cinereus*, however, selection on call duration via mate choice by females may be more stabilizing than directional (reviewed in Gerhardt and Huber 2002). For example, females preferred calls 160 ms in duration over calls 120 ms in duration, but did not prefer extremely long 480 ms calls over 160 ms calls (Gerhardt 1987). In contrast, female *D. cinereus* generally preferred calls produced at more rapid rates (e.g., females preferred 86 calls/min and 150 calls/min over 75 calls/min) suggesting that this call parameter is under directional selection via mate choice by females (see Gerhardt 1987). However, whether CORT-mediated effects on temporal aspects of male *D. cinereus* advertisement calls impact female mate choice decisions is unclear. For example, parameterization of calls used in previous phonotaxis studies (Gerhardt 1987) included call duration and call rate values that do not reflect the mean differences in males with low and high CORT levels, and the difference in these parameters in dual speaker choice tests generally, but not always, exceeded the vocal differences of males with low and high CORT levels (Crocker-Buta and Leary 2018a; Leary and Crocker-Buta 2018). Appropriate parameterization of the calls is important because, while high CORT levels elicit changes in temporal call characteristics that are in the direction that is expected to decrease attractiveness, the ability of females to discriminate between calls will depend on the magnitude of the difference in vocal parameters (see Gerhardt 1991; reviewed in Gerhardt and Huber 2002). Similarly, extrapolating the potential impact of varying CORT levels on vocal attractiveness based on previous mate choice studies is tenuous because such studies typically vary only one acoustic property at a time in order to understand the importance of that particular acoustic attribute. Elevated CORT, however, alters multiple temporal acoustic properties that, when combined, are likely to influence the ability to discriminate between calls and the strength of female preferences.

Here we examine female preferences for the calls characteristic of male *D. cinereus* with low and high CORT levels in dual speaker phonotaxis tests. Given the direction of the effects of elevated CORT on temporal aspects of vocalization, we predicted that females would prefer the calls characteristic of males with low over high circulating CORT.

## Materials and methods

We hand-captured a total of 83 gravid female *D. cinereus* in amplexus with males during peak periods

of breeding activity (May–June) from 2018 to 2020 at the University of Mississippi Field Station (UMFS), Springdale Wildlife Refuge, and a privately-owned pond, all located in Lafayette County, MS. Upon capture, females were separated from males, immediately placed in damp cloth pillow cases, and transported to the UMFS (within 1 h) for phonotaxis trials. We assessed female preferences for the calls characteristic of males with low versus high CORT levels in dual speaker playback experiments performed in a custom built phonotaxis chamber measuring 2.44 m (length)  $\times$  1.22 m (width)  $\times$  1.22 m (height) equipped with small plexiglass viewing windows and low red-incandescent lighting.

Unlike temporal call properties, there are no clear CORT-mediated effects on spectral call features in *D. cinereus* (Leary and Crocker-Buta 2018). Hence, stimuli used in playback experiments were acquired from isolated recordings of advertisement calls from three individual males possessing spectral characteristics that approximated the mean values for the study populations (dominant carrier frequencies  $\approx$  800 and 2500 Hz) based on analysis of calls from 67 individual males recorded at temperatures ranging from 18°C to 27°C (Crocker-Buta and Leary 2018b). We then manipulated the temporal characteristics of the calls using Raven software (Cornell Bioacoustics Laboratory) to generate three pairs of calls, with each pair possessing temporal call characteristics of males with low and high CORT levels.

The first pair of calls was altered to approximate the mean call duration values of males injected with saline only (= low CORT) and an 8  $\mu$ g dose of CORT in saline vehicle (= high CORT) based on data from 11 individuals in each treatment group provided in Leary and Crocker-Buta (2018) (see Table 1 and Fig. 1). We then inserted silent intervals between successive calls to approximate the mean vocal effort of males with low and high CORT (see Table 1 and Fig. 1). We parameterized the calls around call duration and vocal effort because CORT administration primarily impacted these call parameters (as indicated by a significant time  $\times$  CORT/saline treatment effect, see Leary and Crocker-Buta 2018). CORT administration procedures in our previous work (Leary and Crocker-Buta 2018) elevated CORT level within the upper physiological range for this species (mean low CORT level = 1.8 ng/mL, mean high CORT level = 60.3 ng/mL, see Leary and Crocker-Buta 2018) and the effects of CORT administration on call characteristics aligned with the differences in call characteristics of males with low and high CORT levels in

natural choruses (see Crocker-Buta and Leary 2018a).

The second call pair approximated 1 SD above the mean call duration and vocal effort values for males with low and high CORT while the third call pair approximated 1 SD below the mean call duration and vocal effort values, but parameterization of the calls was asymmetrical around the mean to probe how variation in temporal aspects of the calls impact mate choice by females (see Table 1 and Fig. 1). For example, for the second pair of calls the magnitude of the difference in call duration was relatively small (8 ms) while the magnitude of the difference in call rate was relatively large (16 calls/min; see Table 1 and Fig. 1). In contrast, for the third pair of calls the magnitude of the difference in call duration was relatively large (20 ms) while the magnitude of the difference in call rate was relatively small (4 calls/min; see Table 1 and Fig. 1). This asymmetry arose, in part, from differences in the distributions of temporal call characteristics of males with low and high CORT levels (see Fig. 1). The three pairs of calls represented realistic differences in the calls of males with low and high CORT levels and were used in three separate phonotaxis trials. Calls were acquired from three different males but calls used in each of the three phonotaxis trials were acquired from the same male. Hence, paired calls used in each of the phonotaxis trials were spectrally indistinguishable (see Table 1).

At the onset of individual phonotaxis tests, females were placed under a perforated plastic funnel located in the center of the chamber. Acoustic stimuli were then broadcast from speakers placed equidistant from the funnel. Calls were broadcast at an amplitude of ~90 dB SPL (see Gerhardt 1987), measured with an Ivie Technologies sound pressure level meter (model IE-45, RMS fast weighting setting), from SME-AFS Amplified Field Speakers (Saul Mineroff Electronics, Inc.) placed at opposite ends of the phonotaxis chamber (~2.1 m apart). Speakers were connected to Marantz PMD222 recorders that played continuous recordings of calls. Because paired stimuli differed with respect to temporal call characteristics (see Table 1), they drifted in and out of phase over the course of the trials. After 2–3 min, the funnel was lifted with a string pulley attached to the outside of the chamber. Females were randomly assigned to one of the three trials and each female was run in a single phonotaxis trial. We alternated stimuli between speakers across successive trials to control for side biases. Positive phonotactic scores included female orientation, approach, and direct contact with the speaker which occurred in

**Table 1** Call parameters for the six stimuli used in dual speaker phonotaxis tests

Stimuli	Low CORT stimuli			High CORT stimuli		
	1a	2a	3a	1b	2b	3b
Call duration (ms)	158	181	130	142	173	110
Vocal effort	0.25	0.30	0.21	0.20	0.24	0.17
Intercall duration (ms)	472	422	489	565	551	537
Call rate (calls/min)	95	99	97	85	83	93
Low carrier frequency (kHz)	0.80	0.80	0.80	0.80	0.80	0.80
High carrier frequency (kHz)	2.60	2.50	2.50	2.60	2.50	2.50

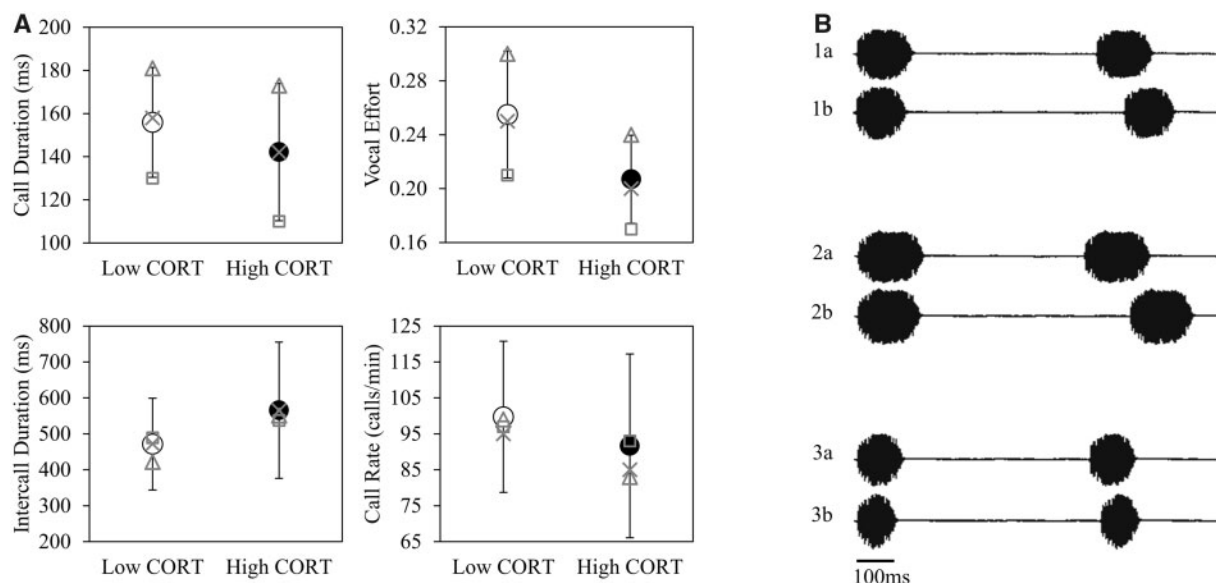
Calls were paired in trials such that females were given the choice between the approximate mean call duration and vocal effort values of males with low and high CORT (stimuli 1a and 1b), ~1 SD above these mean values (stimuli 2a and 2b), and ~1 SD below these mean values (stimuli 3a and 3b). Vocal effort is an estimate of the amount of signaling per unit time and can be calculated by dividing call duration by the sum of call duration and intercall duration. See text for parameterization of the calls.

65/79 (82%) of the successful trials, or approach and close proximity (<10 cm) to the speaker for at least 60 s (the remaining 14 trials). Trials ended when either of these two scenarios occurred, which never took longer than 6.2 min (= 372 s). Trials were terminated if females did not respond to the broadcast stimuli within a 12 min period. Phonotaxis experiments were run at temperatures ranging from 21°C to 28°C (mean = 25.5°C) reflecting the ambient temperatures on the nights in which females were captured.

We recorded stimulus choice and the latency of phonotactic responses (the time elapsed from when the funnel was raised until a choice was made) during each trial. Response latency was only recorded in cases in which females made direct contact with one of the speakers. Latency times are often used in phonotaxis tests in anurans to gauge a female's motivation to mate (Wilczynski and Lynch 2011; Baugh et al. 2021). Because all females were gravid and in amplexus when captured, and were randomly assigned to one of the three sets of phonotaxis trials within ~1 h, we predicted that motivational state would vary minimally. However, we also considered response latency as a possible indicator of a female's ability to discriminate between alternative stimuli. For instance, longer latency times for one or more of the three phonotaxis trials potentially conveyed a reduced ability of females to distinguish between the two broadcast signals.

Body condition values could influence various aspects of female mate choice behavior, especially if





**Fig. 1** (a) Means (circles) and standard deviations (whiskers) for call duration, vocal effort, intercall duration, and call rate of males injected with CORT in saline vehicle (high CORT) and saline only (low CORT) based on data provided in [Leary and Crocker-Buta \(2018\)](#). Call values used in phonotaxis trials representing the mean, 1 SD above the mean, and 1 SD below the mean call duration and vocal effort values are depicted by X's, triangles, and squares, respectively. (b) Waveform representations of stimuli used in each of the three phonotaxis trials where low CORT calls are designated by 1a, 2a, and 3a and high CORT calls are designated by 1b, 2b, and 3b. See [Table 1](#) for specific call values.

females in poor condition have higher CORT levels ([Davis and Leary 2015](#); [Leary and Baugh 2020](#); [Baugh et al. 2021](#); see [Leary and Harris \[2013\]](#) for evidence that body condition is inversely related to CORT levels in male *D. cinereus*). Hence, following each trial, we weighed each female with a portable OHAUS digital scale and measured snout–ischial length (SIL) with a ruler. These two measures were used to calculate body condition estimates using the residual values from a linear regression of the cubed root body weight on SIL divided by SIL ([Baker 1992](#)). Following phonotaxis trials, and prior to release on the same night at the site of capture, females were tattooed on the venter using a portable tattoo device (Tattoo-A-Pet, Fort Lauderdale, FL, USA) to avoid sampling the same individuals on subsequent nights of data collection. Tattoos are clearly visible for the duration of a single breeding season, but not across years. Hence, it is possible that some females were sampled more than once across the three years of the study.

Female preferences were examined using Chi square goodness of fit for a single categorical variable. We compared phonotaxis latency, body size, temperature, and body condition of females across the three sets of phonotaxis trials using ANOVA. We then examined whether phonotaxis latency was related to body size, temperature, and body condition using simple linear regression.

### Ethical note

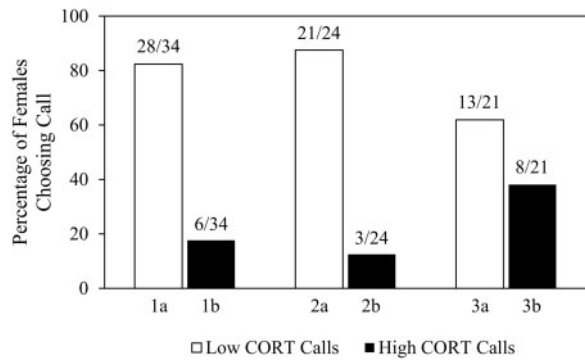
Scientific collecting permits were provided by the Mississippi Department of Wildlife, Fisheries and Parks. All procedures were approved by the University of Mississippi Animal Care and Use Committee (protocol #18-023).

### Results

Four of the 83 captured females were either unresponsive for at least 12 min or attempted to escape during phonotaxis tests and were thus removed from the analysis. Results for the 79 successful phonotaxis trials (involving 79 females) are presented below separately for each of the three trials.

#### Trials involving approximate mean call duration and vocal effort values for males with low and high CORT

Thirty-four females were used in these trials, and 28 approached the speaker broadcasting calls characteristic of males with low CORT levels ([Fig. 2](#)). The remaining six females approached the speaker broadcasting calls characteristic of males with high CORT levels ([Fig. 2](#)). In 25/34 (74%) of the trials, females made direct contact with the speaker. In the remaining nine trials females did not come in contact with the speaker but remained in close proximity (within 10 cm) of the speaker for at least 60 s; all but one of these nine females selected the calls characteristic of



**Fig. 2** Percentage of females choosing calls representative of males with low and high CORT levels for each of the three phonotaxis trials. The proportion of females choosing the calls is depicted above each bar. Results indicated a significantly greater probability of choosing the calls characteristic of males with low CORT levels in the first two trials but not the third trial (see the “Results” section). Call parameters used in phonotaxis trials are provided in Table 1 and Fig. 1.

males with low CORT. Chi-square analysis revealed that females were more likely to choose the speaker broadcasting the calls characteristic of males with low CORT level ( $\chi^2 = 14.2$ ,  $P = 0.0002$ ).

#### **Trials involving $\sim 1$ SD above the mean call duration and vocal effort values for males with low and high CORT**

Of the 24 females used in these trials, 21 approached the speaker broadcasting calls characteristic of males with low CORT levels and 3 approached the speaker broadcasting calls characteristic of males with high CORT levels (Fig. 2). In 23/24 (96%) of the trials, females made direct contact with the speaker and one female remained in close proximity of the speaker. Chi-square analysis revealed that females were more likely to choose the speaker broadcasting the calls characteristic of males with low CORT levels ( $\chi^2 = 13.5$ ,  $P = 0.0002$ ).

#### **Trials involving $\sim 1$ SD below the mean call duration and vocal effort values for males with low and high CORT**

Of the 21 females used in these trials, 13 approached the speaker broadcasting calls characteristic of males with low CORT levels and eight approached the speaker broadcasting calls characteristic of males with high CORT levels (Fig. 2). In 17/21 (81%) of the trials, females made direct contact with the speaker. In the remaining four trials, females remained in close proximity of the speaker but did not make contact with it, and in all of these cases females selected the calls of males with high CORT. Chi-square analysis revealed that females were not

more likely to choose calls characteristic of males with low CORT levels ( $\chi^2 = 1.2$ ,  $P = 0.28$ ).

#### **Comparisons across phonotaxis trials**

There were no significant differences among the three phonotaxis trials with respect to latency of female phonotactic responses ( $F_{2,62} = 0.001$ ,  $P = 0.99$ ), body condition estimates ( $F_{2,76} = 0.217$ ,  $P = 0.81$ ), or temperature ( $F_{2,76} = 0.156$ ,  $P = 0.86$ ) (degrees of freedom differ for response latency because this measure was not tabulated in the 14 cases in which females approached but did not make direct contact with the speaker). However, there were differences in body size ( $F_{2,76} = 30.5$ ,  $P < 0.001$ ); females used in the trials involving the mean call values were smaller (mean =  $51.9 \text{ mm} \pm 5.2 \text{ SD}$ , range = 42–61 mm) than those used in trials involving  $\sim 1$  SD above the mean call values (mean =  $59.4 \text{ mm} \pm 2.98 \text{ SD}$ , range = 52–64 mm) and  $\sim 1$  SD below the mean call values (mean =  $59.4 \text{ mm} \pm 3.6 \text{ SD}$ , range = 52–68 mm) (Tukey–Kramer,  $P < 0.05$ ). Linear regression analysis for all the data combined indicated, however, that there was no significant relationship between the latency of phonotaxis responses and body size ( $r^2_{1,63} = 0.003$ ,  $P = 0.67$ ). Phonotaxis latency was also unrelated to body condition estimates ( $r^2_{1,63} = 0.001$ ,  $P = 0.77$ ) and temperature ( $r^2_{1,63} = 0.002$ ,  $P = 0.75$ ).

#### **Discussion**

Our results reveal preferences by females for males with low CORT in tests using the mean call duration and vocal effort values for males with low and high CORT. Females also preferred the calls of males with low CORT in trials using call duration and vocal effort values approximating 1 SD above the mean wherein the magnitude of the difference in intercall duration (and hence call rate) was relatively large and the difference in call duration was relatively small. However, females did not prefer the calls of males with low CORT in trials using call duration and vocal effort values approximating 1 SD below the mean wherein the magnitude of the difference in intercall duration/call rate was relatively small and the difference in call duration was relatively large. There was no evidence to suggest that variation in female preferences across the three sets of phonotaxis trials was related to differences in body condition or temperature of females, but females used in trials involving the mean call values were smaller on average than females used in the other two trials. However, body size was not related to phonotaxis

response latency, nor did response latency differ among the three phonotaxis trials.

Together, our results indicate that variation in circulating CORT levels in male *D. cinereus* can impact mate choice by females; calls characteristic of males with high CORT levels are generally less attractive. When combined with our previous work, the results suggest that elevations in circulating CORT level during male–male contests compromise attractiveness to females. Agonistic interactions between males competing for mates are well known to influence circulating GC levels (Goymann and Wingfield 2004; Creel et al. 2013) and variation in stress responsiveness can be a key determinant of contest outcomes (Von Holst 1998; Pottinger and Carrick 2001; Hsu et al. 2006; Briffa and Sneddon 2007), but we know of no other studies that have shown that acute elevations in CORT levels during male–male contests negatively impact subsequent attractiveness to females.

In *D. cinereus*, the magnitude of the CORT response to broadcast calls is generally greater for small males who typically lose vocal contests (Leary 2014). Body size is usually inversely related to call carrier frequency in anurans (reviewed by Gerhardt and Huber 2002). Consistent with this pattern, larger chorusing male *D. cinereus* had lower call carrier frequencies than smaller males (see Crocker-Buta and Leary 2018a). In the current study we did not account for spectral differences in small vocal contest losers versus large winners in playback experiments, which is potentially important because, in some anuran species, larger males with lower carrier frequencies are more attractive to females (reviewed by Gerhardt and Huber 2002). However, Gerhardt (1987) found no evidence that larger male *D. cinereus* with lower carrier frequencies are more attractive to females based on extensive playback studies examining female preferences for spectral call features. Hence, incorporating potential spectral differences of large contest winners and small contest losers is unlikely to impact the findings reported in the current study.

Previous phonotaxis experiments also help explain why there were not strong preferences for calls characteristic of males with low CORT across all three phonotaxis trials. For instance, by independently manipulating both call duration and call rate, Gerhardt (1987) determined that call rate was more important in mate choice by female *D. cinereus*. In the current study, call duration differed by 16 ms while call rate differed by 10 calls/min in trials involving the approximate mean call duration and vocal effort values of males with low and high CORT. For trials

involving  $\sim 1$  SD above the mean call duration and vocal effort values, these same parameters differed by 8 ms and 16 calls/min respectively, while for  $\sim 1$  SD below the mean they differed by 20 ms and 4 calls/min (see Table 1). The lack of female preferences in the latter trials likely reflected the minimal difference in call rate for the two stimuli despite relatively large differences in call duration. Our parameterization of the calls used in phonotaxis trials captured realistic variation in the call characteristics of males with low and high CORT levels. In doing so, our results emphasize that the strength of female preferences for males with low CORT depend on the magnitude of the difference in temporal aspects of the calls, which is likely to vary in any particular dyadic comparison of males with low and high CORT (i.e., contest winners and losers), especially considering the variation in vocal attributes for males with low and high CORT levels (see Fig. 1).

Our work with *D. cinereus* suggests that circulating levels of CORT can play a role in mediating trade-offs between signal components that govern attractiveness to females. For example, in Cope's gray treefrog, *Dryophytes (Hyla) chrysoscelis*, females prefer males with higher vocal effort, which is the product of call duration and call rate (see Ward et al. 2013). These two call parameters negatively covary, indicating that the production of one signal component constrains the production of the other (Ward et al. 2013). For example, males of this species produce longer duration calls at slower rates during competitive interactions with other males. Yet, despite socially-related modifications in the vocalizations in this species, these alterations in temporal call characteristics maintain similar vocal effort, resulting in little or no impact on attractiveness to females (Ward et al. 2013). In contrast, male eastern gray treefrogs, *D. (Hyla) versicolor*, can “break” the trade-off between call duration and call rate during competitive interactions with other males, effectively increasing vocal effort by producing longer duration calls, which increases attractiveness to females (Reichert and Gerhardt 2012). In *D. cinereus*, males also increase call duration during simulated territorial intrusions (i.e., broadcast advertisement calls), and the magnitude of the increase in call duration was not statistically different between males injected with saline vehicle only and males injected with CORT in saline (see Leary and Crocker-Buta 2018). However, CORT-injected males experienced a rapid reduction in call duration that was followed by a longer latency increase in the interval between successive calls during simulated territorial intrusions (see Leary and Crocker-Buta 2018). The net effect

was a dramatic reduction in call rate and vocal effort in CORT-injected males compared with saline injected males. These results suggest that only males with low CORT sustain high enough call rates to maintain roughly equivalent levels of vocal effort when longer duration calls are produced. Call rate, in turn, appears to be of primary importance in mate choice by females, which translates into selection for males with low circulating CORT levels. How circulating CORT levels potentially influence the negative covariance between call duration and call rate should be investigated more thoroughly to determine how individual differences in CORT levels impact trade-offs between these two vocal parameters and attractiveness to females.

Despite evidence that CORT level is an important factor in both intra- and intersexual selection in *D. cinereus*, it is still not clear if or how sexual selection shapes GC regulation (see also review by Leary and Baugh 2020). In *D. cinereus*, this stems from the fact that, while small males are more responsive to broadcast calls (Leary 2014), contest winners and losers may not differ in heritable components of GC regulation but may simply vary in circulating GC levels. For instance, increased GC responsiveness in small males during male–male contests may arise from the perception of competing with larger males, which could be conveyed by size-related variation in call carrier frequencies. Moreover, while circulating GCs are modulated by male–male interactions, they are undoubtedly modulated by a host of other biotic and abiotic factors that may randomly impact males (e.g., predator encounters and resource availability), which is likely to introduce considerable “noise” into the system. However, our results highlight considerations that may be important in understanding selection on GC regulation. For instance, if increased GC responsiveness of small males during male–male contests arises from the perception of competing with larger males, sexual selection may act on body size to indirectly impact GC regulation during male–male contests rather than GC regulation *per se*. But if intra- and intersexual selection favor traits that reduce GC responsiveness, such modifications potentially compromise survival (Breuner et al. 2008; Bonier et al. 2009). Reduced survival in male *D. cinereus* that maintain low levels of GCs seems highly probable because increased conspicuous signaling is likely to increase predation. Hence, rather than favoring a single GC phenotype, sexual selection could actually promote variation in GC regulation (i.e., alternative strategies involving low/high survival versus high/low reproductive investment, see Leary and Baugh 2020).

Given that females prefer males with low CORT levels in all studies done thus far (see the “Introduction” section) the next step is to understand what impact, if any, sexual selection has on GC regulation. Such studies will indeed be challenging but a formidable approach would include half-sib crosses using contest winners and losers to examine heritable aspects of offspring growth, performance, and GC regulation.

### Data availability statement

The data underlying this article are available in the Dryad Digital Repository at doi:10.5061/dryad.1zcrjdfv.

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### Conflict of interest

The authors declare that they have no conflicts of interest.

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