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Moderately elevated corticosterone levels increase mate choosiness in female Cope's gray treefrogs without impacting sexual proceptivity or preferences

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Abstract

Female mate choice and its effects on sexual selection have largely been considered with regard to sex steroids; however, an increasing body of literature suggests that glucocorticoids have important effects on female mate choice behavior. Females must balance current reproductive efforts against survival and future reproductive efforts, thereby incurring substantial tradeoffs while breeding. Glucocorticoid levels are elevated during the breeding season and are also produced in response to environmental stressors. To test the hypothesis that elevated glucocorticoids degrade female mate choice by interrupting the energetic demands required for reproduction, I assessed the impact of elevated corticosterone (CORT) on aspects of female mate choice in wild gray tree frogs (*Hyla chrysoscelis*). Those aspects consisted of proceptivity, preference for high pulse number, and choosiness. Female frogs were collected in amplexus and tested using two-choice phonotaxis assays. A thirty-minute rest period shortened approach latencies and injection *per se* appeared to weaken one measure of preference for high pulse number calls. However, elevated CORT levels did not impact either proceptivity or species-typical preferences, providing evidence that seasonal breeders are buffered against high concentrations of CORT that might jeopardize reproductive investment. On the other hand, a medium CORT dose significantly increased choosiness in females, though this effect was not seen with either higher or lower CORT doses. This suggests that CORT modulates energetic tradeoffs in a non-linear dose response fashion.

Introduction

Wild animals may incur significant tradeoffs at major life history stages, such as the tradeoff between survival and reproduction during the breeding season (Stearns, 1989, 2000). Endocrine products modulate animals' responses to both external and internal stimuli and regulate tradeoffs during and between life history stages. Glucocorticoids (GC) are the major product of the HPA endocrine axis and known for their role in modulating animals' stress response. GCs are essential to animals' regulation of energy balance and response to stressors at important life history stages due to their pleiotropic actions (Breuner, Patterson, & Hahn, 2008; Leary & Baugh, 2020; Ricklefs & Wikelski, 2002; John C Wingfield et al., 1998). The particular compromise between survival and reproduction invokes the CORT tradeoff hypothesis, which states that elevated GCs tend to redirect resources toward survival at the expense of reproduction (Breuner et al., 2008; Patterson, Hahn, Cornelius, & Breuner, 2014). However, GC action is more complex than such a hypothesis would suggest. For example, other studies have suggested that this energetic balance depends upon individual or taxon variation in GC reactivity (the

response of the HPA axis to a stressor), with low GC reactivity encouraging reproduction and high GC reactivity encouraging survival (C. W. Breuner, S. H. Patterson, & T. P. Hahn, 2008; John C Wingfield et al., 1998; J. C. Wingfield & Sapolsky, 2003).

Sexual behavior in vertebrates has long been thought to be influenced by circulating levels of steroid hormones and sex steroids such as testosterone and progesterone have taken center stage in studies examining hormonal impacts on reproduction (citations needed). Sex steroids have both developmental and activational effects on reproduction (citations), and recent work has shown sex steroids to impact mate choice preferences (Adkins-Regan, 2005; Gordon & Gerhardt, 2009; Lynch, Crews, Ryan, & Wilczynski, 2006; McGlothlin, Neudorf, Casto, Val Nolan, & Ketterson, 2004). However, sex steroids may not account for the full variability in mate choice, especially during times of environmental stress during which sex steroids production is dampened and female mate choice variability increases (Cotton, Small, & Pomiankowski, 2006; J. C. Wingfield & Sapolsky, 2003). Leary and Baugh (2020) review studies in male vertebrates showing that GCs can impact both sexual ornaments and sexual behavior, though the direction of effect is variable by taxa and context; they note, further, that changes to male behavior may not impact reproductive success unless female mate choice responds to these behavioral changes.

Female mating behavior is also subject to substantial variation, both within and among individuals (Jennions & Petrie, 1997). Studies on mating behavior distinguish between 'proceptivity,' female willingness to select a mate, 'preference functions,' the order with which an individual ranks prospective mates, and 'choosiness,' the effort an individual is prepared to invest in mate assessment (Jennions & Petrie, 1997). Regardless of the source—genetic, environmental or social—variation in both female preferences and choosiness may have substantial impact on sexual selection and the evolution of male and female traits. Ecological challenges and stressors are known to modify female mate choice in ways that minimize costs (Cotton et al., 2006; Hunt, Brooks, & Jennions, 2005; Jennions & Petrie, 1997), although these challenges may or may not be associated with changes to GC levels. More generally than mate choice, elements of female mating behavior such as mate sampling/searching and conspicuousness to predators are linked to both GC increases and decreased female preferences (Baugh & Ryan, 2010a, 2010d; Breuner, Greenberg, & Wingfield, 1998; Forsgren, 1992). There is some evidence in correlative studies that high circulating GC levels dampen motivation (Baugh, Bee, & Gall, 2019; Gall, Bee, & Baugh, 2019) and decrease preference strength for intraspecifics (Vitousek & Romero, 2013), though proceptivity—willingness to copulate—appears to be unaffected aside from supraphysiological levels (O'Connor, Gilmour, Arlinghaus, Van Der Kraak, & Cooke, 2009) and inhibitory effects on motivation (Davis & Leary, 2015) and preferences (Bastien et al., 2018; Baugh et al., 2019; Gall et al., 2019) are not found in multiple other studies.

Leary and Baugh (2020) found only three studies that assessed female mate choice in terms of proceptivity and preferences by experimentally manipulating GC levels. Kavaliers and Ossenkopp (2001) tested odor preferences in oestrous mice following acute administration of CORT at four levels: 1.0, 2.5, 5.0 and 10.0 mg/kg. Preference strength decreased in a dose-dependent manner, with the medium CORT group (5.0 mg/kg) exhibiting no preference for male odors, and all other groups expressing significantly weaker preferences than control (no injection) and vehicle-injected mice. By simultaneous injection of CORT with one of two antagonists for two neuronal receptors, NMDA and GABA, the researchers determined that CORT-modulated preference weakening occurs at least partially through NMDA- and GABA-

mediated pathways. These results suggest an inhibitory function for CORT on female preference. Reduced locomotor activity in mice with absent or reduced preference also points to a dampening effect of CORT on sexual interest and proceptivity. In a second study, Davis and Leary (2015) administered exogenous CORT in three doses to sexually receptive green treefrogs (*Hyla cinerea*) and tested female preference for male call rate using a dual speaker phonotaxis assay. Proceptivity (latency to choice) was unaffected by treatment, but females in the two highest CORT dose groups had significantly weaker preferences for high call rate than control and low CORT dose females. This study again points to an inhibitory CORT role on expressed female preferences. The third study examined the effect of a single dose of exogenous CORT, administered twice daily for four days, on the mating behavior of female common lizards (*Zootoca vivipara*) (Romero-Diaz, Gonzalez-Jimena, & Fitze, 2019). CORT-treated females were significantly more aggressive, more reluctant to engage in mating behaviors such as approaches and tongue extrusions and copulated less frequently. There was also a significant treatment × body size interaction; larger body size correlated with increased sexual interest among CORT-treated females only. These results together indicate a decline in proceptivity for females with higher GC levels, combined with decreased mating success, that principally affects smaller females in a condition-dependent manner (Cotton et al., 2006). However, preference for familiar males over unfamiliar males was not impacted by CORT treatment or body size. These three studies collectively indicate significant taxon-, context- and condition-dependent effects of corticosterone treatment on female proceptivity and preferences, and the potential impacts on sexual selection, suggesting the need for additional studies that investigate the direct link between mate choice behaviors and GCs.

The current study seeks to build upon the understanding of GC effects on female mate choice by experimentally linking GC manipulation to female proceptivity and preferences in gray treefrogs (*H. chrysoscelis*). Furthermore, I intend to strengthen the connection between female mate choice, GCs and tradeoffs by examining female choosiness in response to temporally updated acoustic playback. Previously, HPA axis reactivity and maximum CORT levels were correlated with behavioral tradeoffs in a non-sexual context (exploratory behavior) where GCs shape individual exploratory personalities (Baugh, van Oers, Naguib, & Hau, 2013). However, GC impact on mate choice has not been studied in the context of tradeoffs, especially those in which reproduction and survival may be at a crossroads. When faced with additional barriers to a preferential mate choice, will elevated CORT levels cause females to decrease investment in reproductive effort and be less choosy? I hypothesize that elevated glucocorticoid levels beyond baseline breeding season levels will degrade aspects of female mate choice as energetic resources are shifted away from current reproductive efforts and toward survival. Specifically, I predicted that (1) elevated GC levels would attenuate proceptivity by decreasing the frequency of choices (responsiveness) and increasing latency (time) to choice, (2) elevated GC levels would modulate species-typical preference by decreasing the strength of this preference, and (3) females with elevated GC levels would be less choosy due to redirected energetic resources.

Methods

System

I studied mate choice behavior in the western genetic lineage of Cope's gray treefrogs, *Hyla chrysoscelis* (Ptacek, Gerhardt, & Sage, 1994). Females respond to male advertisement calls during the breeding season and select a mate based on the acoustic properties of this call (Wells, 2010). These advertisement calls are pulsatile, and females prefer calls of higher pulse number and higher call rate (Gerhardt, Dyson, & Tanner, 1996). As with many behavioral phenomena that are energetically taxing, male gray treefrogs face a trade-off between pulse number and call rate, despite females' preference for the upper bounds of each feature (Ward et al., 2013). Females listen to multiple male calls simultaneously and discriminate based on the above features; they then approach a preferential male and touch him to initiate amplexus (mating). Laboratory studies generally reproduce this paradigm using two-choice phonotaxis trials wherein speakers antiphonally broadcast male calls and females approach the speaker broadcasting the preferred call. Recent studies have highlighted the importance of dynamic playback, which allows temporal updating of stimuli and prompts females to modulate their behavior in real time (Baugh & Ryan, 2009, 2010a, 2010b, 2010c; Gerhardt et al., 1996).

Animals

I collected frogs as amplectant pairs from breeding aggregations at Tamarack Nature Center (Ramsey County, MN) and Carver Park Preserve (Carver County, MN) during the breeding season (May to June of 2018 and 2019) between the hours of 2130 and 2330. Frogs were left in amplexus and stored in dry plastic containers on ice at 4°C for up to three days before testing. Amplectant pairs were stored in dry containers to prevent oviposition before experimentation. Previous work has shown that storage on ice for 0-3 days has no effect on female responses in phonotaxis trials (Bee M, unpublished data). 30 min prior to phonotaxis tests, we placed amplectant pairs in 20°C water in an incubation chamber to warm them. Frogs collected from Tamarack may have been H. chrysoscelis or H. versicolor; therefore, males were allowed to call before female testing to confirm species identity (Ptacek et al., 1994). Immediately following behavioral testing of females, we took a blood sample by cardiac puncture and sacrificed each female to obtain brain tissue samples. Males were returned to their collection sites the day after female testing. All frogs were tested at the St. Paul campus of the University of Minnesota in accordance with the Institutional Animal Care and Use Committee guidelines of that institution.

Stimuli and experimental design

Male advertisement calls used in phonotaxis tests were synthesized from custom scripts (MATLAB). I used population average call parameters to design a single pulse which was concatenated (Adobe Audition) to produce three stimuli of 22, 30, and 38 pulses, respectively. The 30-pulse number (PN) call mimics a population-average male advertisement call, while the 22 PN and 38 PN calls represent two SDs below and above the population mean pulse number, respectively. Acoustic playback was controlled on a PC laptop (Dell 5520; Windows 10

Professional; MaxxAudio two-channel sound card) with SIGNAL software (version 5; Engineering Design), which was connected to two single-channel potentiometers (SPL Electronics GmbH), a power amplifier (Crown XLS 1000), and two satellite speakers (Mod1, Orb Audio). These speakers were placed opposite each other along the long axis of the phonotaxis chamber, 2m apart (Fig. 2). Before testing and between subjects, both speakers were calibrated to 85 dB SPL (re 20 μPa) at the center ("origin") of the chamber using a Larson Davis 831 SLM. The origin was located 1 m from each speaker, as 85 dB at 1 m represents the natural call volume of this species (Gerhardt, 1975). Calls were broadcast at 11 calls/min, the average natural call rate in this species (Ward et al., 2013).

Phonotaxis trials were performed under infrared lighting in an anechoic acoustic chamber with sound-attenuating tiles on the walls and ceiling and low pile carpet (295×275×195 cm, L×W×H internal dimensions; Industrial Acoustics Company). Subjects' behavior was observed with a ceiling-mounted IR video camera (Basler GigE; Ahrensburg, Germany) and each trial was recorded on a second PC laptop (Dell 5520; Windows 10 Professional; NVIDIA graphics card) for later analysis using Ethovision XT software (Version 9, Noldus, Wageningen, NL). Lab tape, visible under IR light, marked the midline and other boundaries. The approach boundary was designated as a 65cm-radius arc surrounding each speaker, while the choice boundary was a 10cm-radius circle around each speaker (Fig. 2). A female was judged to have crossed a boundary if 50% or more of her body crossed a boundary line.

All females were tested in the same series of five phonotaxis trials each at two timepoints: before treatment ("pre-injection") and after treatment ("post-injection") (Fig 1). After a 30-min warming period, females were tested in five pre-injection phonotaxis trials. Immediately following these trials, females were treated in one of five groups according to random prior assignment: (a) no injection control; (b) vehicle injection (sesame oil); (c) low CORT (20 ng g^{-1}; 2 ng uL^{-1}); (d) medium CORT (60 ng g^{-1}; 6 ng uL^{-1}); and (e) high CORT (180 ng g^{-1}; 18 ng uL^{-1}) (N = 21 or 22 for each treatment group). Following treatment, females were allowed to rest in a 20°C incubator for 30 min in order for the exogenous CORT to enter circulation. Females were then again tested with the five phonotaxis trials before blood draw and brain harvest. The no-injection treatment group provided a control for the handling stress and 30-min rest period between phonotaxis testing bouts. The vehicle injection group provided a further control for the stress of injection *per se*.

Behavioral Testing

The five phonotaxis trials were designed generally as static or dynamic. In static trials, the 22 and 38 PN calls were presented with no changes made to acoustic conditions. Each female was tested in this trial first, both pre-injection and post-injection, to assay responsiveness and provide a baseline measure of behavior. Dynamic trials were divided into control and commit conditions and two acoustic conditions, for a total of four trial conditions. Both control and commit trials tested two acoustic conditions, 22 vs. 30 PN stimuli and 30 vs. 38 PN stimuli (abbreviated as Control 22/30, Control 30/38, Commit 22/30, and Commit 30/38). The order of the four trials was randomized both before and after treatment. The three acoustic conditions (22/38, 22/30, 30/38) were selected due to the asymmetry of female preference; females prefer higher pulse numbers, but discriminate more strongly against below-average calls (Ward et al., 2013).

The four dynamic trials were so named because the acoustic environment changed before females had made a mate choice. When female subjects crossed the approach boundary (see Fig. 2) toward the higher PN call, an experimenter pressed the spacebar on the computer controlling the acoustic playback, which began a custom script in SIGNAL that introduced a 500-ms pause and, depending on the trial, modified the playback. In control trials, the male stimuli continued from their original speakers following the 500-ms delay. In commit trials, male calls switched speakers (i.e. speaker with lower PN call now broadcasts higher PN call, and vice versa). The experimenter pressed the spacebar only between stimuli reproduction to ensure that no calls were clipped, and that the acoustic environment maintained seamless presentation to subjects. To control for the potential temporal and spatial confounds of side bias in the chamber and first caller preference (Bosch & Márquez, 2002) in both static and dynamic trials, I randomized stimuli location and call order (before and after manipulation) for every test.

To begin phonotaxis trials, females were placed under an acoustically transparent mesh cage at the origin. A damp paper towel on the origin floor kept the female hydrated between trials. Following 10 sec of habituation to the acoustic playback, the mesh cage top was lifted remotely, and the female was free to move. Trials outcomes were recorded as follows. Each static trial had two possible outcomes: (1) standard foul (F-S), in which a female failed to move from the origin for 5 min, did not leave the wall for 2 min, or did not make a choice within 10 min; or (2) a choice for either the 22 or 38 PN call. Each dynamic trial had 5 potential outcomes: (1) standard foul (F-S), see definition above; (2) low pulse number foul (F-PN), in which the female chose the lower PN male call without first crossing the approach boundary toward the higher PN call (required for our dynamic trial design); (3) foul after switch (F-AS), in which the female failed to make a choice within 10 min after the playback pause was introduced; (4) non-reversal choice (NR), in which the female crossed the approach boundary toward the higher PN call and then chose the call at that speaker; (5) or reversal choice (R), in which the female crossed the approach boundary toward the higher PN call and reversed direction toward the opposite speaker and crossed that choice boundary. Dynamic trials in which a female fouled were repeated until a choice was made.

In addition to reversals, I measured and recorded the following latencies: (1) latency to exit the origin, (2) latency to cross any approach boundary (higher or lower PN), (3) latency to cross the approach boundary toward the higher PN call, (4) latency to cross the choice boundary.

Hormone Sampling and Quantification

CORT injections

Crystalline corticosterone (HPLC grade, Sigma Cat. No. 27840) was dissolved in a small volume of 95% EtOH, vortexed until dissolved and diluted in sesame oil (Sigma, Cat. No. S3547), again vortexed and heated in an incubator to evaporate the EtOH. Injections of 10 uL per gram frog were prepared in the following five groups: (a) no injection control; (b) vehicle injection (sesame oil); (c) low CORT (20 ng g^{-1}; 2 ng uL^{-1}); (d) medium CORT (60 ng g^{-1}; 6 ng uL^{-1}); and (e) high CORT (180 ng g^{-1}; 18 ng uL^{-1}). Frogs were injected i.p. using a 27-gauge insulin syringe (BD 0.5 mL Tuberculin with attached needle (BD & Co, Franklin Lakes, NJ, USA). These CORT injections resulted in consistently elevated circulating CORT levels within physiological range (1-124 ng mL^{-1}; Gall et al. 2019), as previously validated (see Supplemental Materials SX1).

Blood sampling

Whole blood (ca. 50 uL) was collected immediately following post-injection testing via cardiac puncture using a 30-gauge insulin syringe (BD Micro-fine U-100, 0.3 mL) pre-rinsed with heparin. Within several hours of collection, whole blood was centrifuged (7500 RPM for 10 min; Eppendorf 5418 at 8° C) and the plasma fraction stored at -20° C for up to 3 weeks; samples were then shipped on dry ice to Swarthmore College and briefly stored at -80° C until assayed.

Steroid extraction and reconstitution

All hormone methods have been previously validated (Gall et al., 2019). A liquid diethyl ether extraction method was used, with 5 uL of plasma being sufficient to accurately quantify CORT concentrations. Plasma samples were vortexed and added to borosilicate vials, with 200 uL RO water to aid in decanting. 2 mL of diethyl ether were then added to each vial, samples were vortexed, and the aqueous layer was frozen on a dry ice-methanol slurry. The organic layer was decanted to a new vial, and the extraction was repeated once the aqueous layer thawed. Ether extracts were dried for 20 min using a Speedvac centrifuge at 37° C (Thermo Fisher Savant Speedvac SPD1010), then reconstituted using assay buffer (in kit) and reconstituted overnight at 4° C.

Enzyme immunoassays

Hormone concentrations were estimated using a commercial EIA kit (DetectX® kits, Arbor Assays, Ann Arbor, MI) for plasma corticosterone (Cat. No. K014, Donkey anti-Sheep IgG). I followed the manufacturer's protocol for steroid estimation using 50 uL of reconstituted sample per well. Samples were assayed in duplicate and mean values were accepted. Optical densities for the plates were read at 450 nm using a $Versa_{max}$ microplate reader with SoftMax Pro software (Molecular Devices, Sunnyvale, CA). The assays have detection limits and sensitivities, respectively, of 16.9 pg mL^{-1} and 18.6 pg mL^{-1}. The cross-reactivity of the antiserum is 100% for corticosterone, 12.3% for desoxycorticosterone, 0.62% for aldosterone, 0.38% for cortisol.

Statistical Analysis

Statistical modeling was performed in SPSS (version 26, IBM) while exact tests of proportions and frequencies were conducted using VassarStats (vassarstats.net, Richard Lowry). All models assumed statistical significance at $p < 0.05$. CORT concentrations were $\log_{10}$-transformed to improve normality; latency data were not transformed (Gall et al., 2019). I utilized a mixed within- and between-subject experimental design; female subjects were randomly assigned to one of five treatment groups and each female functioned as her own control for the effects of treatment.

I assessed proceptivity using only static trials because their acoustic environments were unchanging and could therefore be used to assess sexual willingness without the confounds of perception or trade-offs. I calculated proceptivity as the proportion of females that made a choice (compared to those that committed a F-S) in static trials. Binomial exact tests determined if these proportions differed from random choice. Fisher exact tests determined the significance of treatment group differences within timepoints. McNemar exact tests for correlated proportions were then used to determine significance of timepoint differences within treatment groups, as this test included repeated measures. A secondary measure of proceptivity was the latency to

various boundaries, interpreted as an indicator of sexual motivation. Because dynamic trials manipulated acoustic playback after females crossed an approach boundary, only latency to approach boundary was used from dynamic trials, while latency to both approach boundary and choice boundary was used from static trials. General linear models with repeated measures were utilized to model the effect of treatment and timepoint on latencies. Beyond treatment and timepoint, dynamic trial models tested for the effect of acoustic condition (22/30 or 30/38) and trial order within acoustic condition (i.e. regardless of control/commit designation, which trial came first chronologically). Finally, circulating CORT levels were correlated to static trial choice latencies with a regression model. Regression was performed for both timepoints, though I expected only post-injection latencies to reflect CORT impacts, as blood plasma was sampled only post-injection.

The species-typical preference of females for higher PN male calls was assayed in both static and dynamic trials; preference strength was calculated as the proportion of females moving toward the higher pulse number call for both approach boundary and choice boundary. As with proceptivity statistics, choice boundary latencies were calculated only in static trials to avoid confounds. Since females may have repeated dynamic trials due to fouls, I calculated preferences using only the first trial iteration in which females crossed an approach boundary. Control and commit trial data were collapsed, as the acoustic environment until approach boundary crossing was identical. Static trial results were easily grouped by 22 PN and 38 PN preference. Dynamic trial approach boundary preferences were grouped into three categories due to the collapsing of control and commit trials: (1) unanimous low PN preference, (2) split preference and (3) unanimous high PN preference. Females approaching the lower PN in both trials of an acoustic condition were scored as having a unanimous low PN preference, and likewise for the higher PN. Female crossing the approach boundary toward the lower PN in one trial and toward the higher PN in the other trial were scored as having a split preference for that acoustic condition. These data were then reduced to two categories: (1) unanimous low PN preference and split preference and (2) unanimous high PN preference. Generalized linear models were created for both the trinomial and binomial preference groupings, using ordinal probit and binary probit link functions, respectively. These models tested for treatment, timepoint, and treatment × timepoint interaction effects. Binomial exact tests were used as before to determine a difference from random preference, while Fisher's and McNemar's exact tests were used to calculate post hoc significance between individual treatment groups and timepoints.

Choosiness was assayed with dynamic commit trials and calculated as the proportion of females making reversal (R) choices. I introduced a trade-off challenge by switching the acoustic stimuli locations in the commit trials. The central component of this design was to test if elevated CORT levels would cause females to decrease their choosiness, thereby reversing at lower proportion after stimuli switch. Fisher's and McNemar's exact tests were used as described above to test for specific treatment and timepoint effects. A generalized linear model was then created as a more powerful test of these effects, as this model included all variance within and among treatment groups. The GLM was created using a binary response (R or NR) with a probit link function.

Results & Interpretation

Hormone dose response

As referenced in the methods, a previous dose response validation was performed to determine appropriate dosages and timelines (Supplemental Materials SX1). The dosages selected (20 ng g^{-1}, 60 ng g^{-1}, and 180 ng g^{-1}) were lower than those used by Davis & Leary (2015), whose study was a major motivator for the current study. Both studies used 3 CORT doses: Davis & Leary (2015) injections were 4, 8, or 16 ug CORT per frog; my injections were ca. 0.12, 0.25, or 1 ug CORT per frog (SX1). Despite the large difference in dosages, the realized steroid concentrations were very similar, likely due to the difference in vehicle; Davis & Leary (2015) used saline as a vehicle, while I used sesame oil. Thus, their 16 ug CORT dose resulted in mean circulating CORT levels of 80.1 ng/mL 1-2 hr. post-injection (Davis & Leary, 2015), while I achieved similar CORT levels with my highest dose (ca. 1 ug CORT, 87.6 ng/mL at 1 hr. post-injection) (SX1). The difference in drug delivery, the difference in testing timeline (Davis & Leary tested females within 2 hours of injection on the same night, I tested females 30 min after injection following an overnight hold), and the species difference (Davis & Leary (2015): *H. cinerea*; my study: *H. chrysoscelis*) may help account for any differences in results between the two studies.

Proceptivity

Females' response rate to male advertisement calls in static trials, both pre- and post-injection, was significantly greater than random in all treatment groups (binomial exact test, all $p < 0.00002$, Fig. 3). In fact, only two females failed to respond and make a mate choice at the first acoustic presentation pre-injection (one each in LO and MED CORT groups). There were no differences between pre- and post-injection response rates within any treatment group (McNemar exact tests, all $p = 1$). Nor were there any differences in response rates between treatment groups (Fisher exact tests, all $p = 1$); no-injection control was compared to vehicle control, and vehicle control was compared to each CORT injection group. As a broad measure of proceptivity, female response to male advertisement calls indicated that neither handling, injection nor CORT treatment had any impact on proceptivity.

Choice and approach boundary latencies were used as a secondary measure of proceptivity. Longer latencies would indicate a dampening of proceptivity as predicted for females with CORT injection. Choice boundary latencies in static trials did not show a main effect of timepoint (GLM: $F_{1,102} = 1.54$, $p = 0.22$, Fig. 4) or treatment group (GLM: $F_{4,102} = 0.46$, $p = 0.77$). There was no interaction of timepoint and treatment group (GLM: $F_{4,102} = 0.35$, $p = 0.85$) nor were any pairwise comparisons significant (GLM, estimated marginal means: all $p > 0.1$). Approach boundary latencies in static trials did show a nominally significant main effect of timepoint (GLM: $F_{1,102} = 4.03$, $p = 0.047$), although no main effect of treatment group was noted (GLM: $F_{4,102} = 0.90$, $p = 0.47$). There was no interaction of timepoint and treatment group (GLM: $F_{4,102} = 0.49$, $p = 0.74$) and despite the significant main effect of timepoint, no pairwise comparisons were significant (GLM, estimated marginal means: all $p > 0.05$). Approach boundary latencies in dynamic trials revealed no main effects of timepoint (GLM: $F_{1,102} = 0.00$, p

= .98), treatment (GLM: $F_{4,102} = 0.83$, $p = 0.51$), acoustic condition (GLM: $F_{1,102} = 0.65$, $p = 0.42$) or trial order (GLM: $F_{1,102} = 1.36$, $p = 0.25$). All interactions (2-way, 3-way and 4-way) were not significant (GLM: all F between 0 and 1.5, all $p > 0.05$).

Circulating CORT did not predict latency to choice in static trials. Correlations between $\log_{10}$ CORT concentrations and choice latencies were performed for pre- and post-injection timepoints. The pre-injection result was qualitatively identical whether or not statistical outliers were included (all data: $R^2 = 0.00$, $p = 0.93$; outliers excluded: $R^2 = 0.01$, $p = 0.33$; Fig. 5). The post-injection was also qualitatively identical regardless of outlier inclusion (all data: $R^2 = 0.00$, $p = 0.93$; outliers excluded: $R^2 = 0.00$, $p = 0.79$).

The only significant result of this section is the main effect of timepoint in static trial approach boundary latencies, with females crossing the approach boundary more quickly post-injection compared to pre-injection. Females may be responding to the 30-min time difference between pre- and post-injection, a time window that shortens the time horizon to oviposition. As females approach oviposition, the urgency to oviposit can shorten latencies, as shown in the closely related species Hyla versicolor (Bastien et al., 2018). Bastien et al. additionally showed that CORT correlated negatively with latency to choice, though this result was not found in the present study. Despite the tendency for females to shorten choice latencies with closeness to oviposition, CORT did not impact any level of proceptivity (response frequency or latencies) and I conclusively state that CORT does not dampen female proceptivity in the seasonal breeding Cope's gray treefrogs.

Preferences

Species-typical preference for higher pulse number advertisement calls was assayed in both static and dynamic trials using approach boundary and choice boundary crossings. Within static trials, both approach boundary and choice boundary results show females with a high species-typical preference for the higher pulse number call (38 PN) over the lower pulse number call (22 PN). Preference for the higher PN call was significantly greater than random selection for all treatment groups, both pre- and post-injection (binomial exact tests; approach boundary: all $p < 0.02$; choice boundary: all $p < 0.001$; Fig. 6,7). Approach boundary preferences showed nominal variation among treatment groups and across timepoints, but these differences were not significant (Fisher exact tests, between groups: all $p > 0.1$; McNemar exact tests, within groups: all $p > 0.4$). Final choice preferences in static trials were even more stable and consistently greater than 90% for the 38 PN call. Within treatment groups, all post-injection choice frequencies for the 38 PN call were higher than or equal to pre-injection frequencies, although this was a nominal difference (McNemar exact tests: all $p >= 0.5$). There were no differences between treatment groups (Fisher exact tests: all $p > 0.6$). Static trial results therefore show a strong, species-typical preference for calls with high pulse numbers with no effect of treatment on this preference.

Approach boundary preferences in dynamic trials did not have significant main effects in any model. Starting with the 22/30 acoustic condition, I did not find any significant main effects of treatment or timepoint using either a trinomial (Wald chi-square; treatment: $\chi_4^2 = 4.37$, $p = 0.36$; timepoint: $\chi_1^2 = 0.53$, $p = 0.47$; Fig. 8) or binomial (treatment: $\chi_4^2 = 3.79$, $p = 0.44$; timepoint: $\chi_1^2 = 1.08$, $p = 0.3$; Fig. 9) response. There was no significant overall interaction (trinomial: $\chi_4^2 = 5.09$, $p = 0.28$; binomial: $\chi_4^2 = 4.52$, $p = 0.34$). However, there was a significant

interaction between timepoint and the no-inject treatment group in both models (trinomial: $\chi_1^2 = 3.92$, $p = 0.048$; binomial: $\chi_1^2 = 4.28$, $p = 0.039$). This result indicates that the absence of injection (no-inject group) causes a significant increase between pre- and post-injection timepoints. That is, with only a rest period of 30 min, females increase their preference for higher pulse number calls, while injection per se appears to slightly diminish that effect. Although unanimous 30 PN preference remains the largest proportion of response for all injection treatments (sesame and low, med, high CORT), there is no significant interaction of treatment and timepoint for any of these groups, signaling that injection per se removes that increased post-injection preference for 30 PN calls that the no-inject group demonstrates.

A similar pattern of approach boundary preferences was found in the 30/38 acoustic condition. I again found no main effect of either treatment (Wald chi-square; trinomial: $\chi_4^2 = 2.65$, $p = 0.62$, Fig. 8; binomial: $\chi_4^2 = 3.19$, $p = 0.53$, Fig. 9) or timepoint (trinomial: $\chi_1^2 = 1.74$, $p = 0.19$; binomial: $\chi_1^2 = 1.53$, $p = 0.22$), but there was a significant overall interaction between treatment and timepoint (trinomial: $\chi_4^2 = 11.22$, $p = 0.024$; binomial: $\chi_4^2 = 10.24$, $p = 0.037$). This interaction appeared to be driven by the no-inject treatment group, which was the only treatment group with a significant parameter effect (trinomial: $\chi_1^2 = 4.92$, $p = 0.027$; binomial: $\chi_1^2 = 3.42$, p = 0.064) and the only group to have a significant interaction with timepoint (trinomial: $\chi_1^2 = = 6.2$, $p = 0.013$; binomial: $\chi_1^2 = 6.84$, $p = 0.009$). The effect of no-inject treatment on approach boundary preferences was even stronger in the 30/38 condition compared to the 22/30 condition. Unlike all other groups and acoustic conditions, in the 30/38 condition pre-injection the no-inject group had a greater proportion of females with unanimous 30 PN preference and split preference than unanimous 38 PN preference. Following treatment (handling and rest, no injection) the no-inject group preference ratio was flipped, resulting in more females with a unanimous 38 PN preference than females with unanimous 30 PN preference and split preference.

Across all trials, females generally maintained strong species-typical preferences for male advertisement calls with higher pulse numbers. Static trials showed the strongest preference for higher pulse numbers, while dynamic trials revealed somewhat competing preferences between higher and lower PN calls. These results are not surprising and point to recognized features of H. chrysoscelis biology. Static trials presented a clear choice between male calls of vastly differing quality; a 38 PN call is 2 SD's above the population average, while a 22 PN call is 2 SD's below the average. The fact that females showed greater than 90% likelihood of selecting the higher PN call is consistent with this static preference feature. When assessing approach boundary preferences in static trials, however, the preference strength appears to be somewhat reduced (Fig. 7). This apparent disparity is likely due to the difficulty in localizing male calls at distance; I noted that females often hopped in wide circles to sample both male calls before making a final mate choice. Quantifying preferences at the approach boundary may sample greater error in female localization than quantifying preferences at the choice boundary. Furthermore, the PN difference between calls in dynamic trials was half that of static trials (22 vs. 30 or 30 vs. 38 PN, compared with 22 vs. 38 PN) and therefore the female preference function had less pulse number disparity on which to operate. Weaker expressed preferences in dynamic trials might therefore be expected.

The effect of CORT on female preference for higher PN calls reflects these underlying biological principles. In the most general sense, CORT does not seem to have a large effect on female preference, in that no treatment or treatment/timepoint interaction effects are seen in static trials, either for final choice or approach boundary preferences. There are additionally no

main effects of treatment on the dynamic trial approach boundary preferences. However, the interaction effects between treatment and timepoint in dynamic trials reveal that both injection per se and potentially CORT treatment are modulating preferences. This influence is most visible where underlying preferences are weakest; approach boundaries with a lesser PN difference between calls, and in the acoustic condition to which females are the most ambivalent (30/38; female H. chrysoscelis discriminate more strongly against below-average calls than in favor of above-average calls (Ward et al., 2013)).

Choosiness

There were no significant differences in total reversals between timepoints in either the 22/30 (p = .47, Fig. 10) or 30/38 (p = 0.29, Fig. 12) acoustic condition. Comparing treatment groups in the 22/30 condition, the post-injection reversal rate of the med (60 ng g^{-1}) CORT group was significantly greater than that of the low (20 ng g^{-1}) CORT group (p = 0.027); all other comparisons between groups within timepoints were not significant (all $p > 0.05$). No treatment groups differed in pre- vs. post-injection reversal rates (all $p > 0.1$). The 30/38 condition did not yield any significant differences in exact comparisons between reversal frequencies, either between (all $p > 0.05$) or within (all $p > 0.1$) groups.

The generalized linear model, a more comprehensive analysis of choosiness, did not find significant main effects of treatment or timepoint for either the 22/30 (treatment: $\chi_4^2 = 5.67$, p = 0.23; timepoint: $\chi_1^2 = 0.81$, p = 0.37) or 30/38 (treatment: $\chi_4^2 = 1.44$, p = 0.84; timepoint: $\chi_1^2 = 1.04$, p = 0.31) condition. These GLM analyses further failed to find a main interaction between treatment and timepoint (22/30 condition: $\chi_4^2 = 5.09$, p = 0.28; 30/38 condition: $\chi_4^2 = 4.52$, p = 0.34). However, consistent with qualitative interpretations of the reversal data, the med CORT treatment group emerged with significant parameter estimates in both acoustic conditions. In the 22/30 condition, both the med CORT treatment ($\chi_1^2 = 4.59$, p = 0.032) and treatment/timepoint interaction ($\chi_1^2 = 4.01$, p = 0.045) parameters were significant. In the 30/38 condition only the med CORT treatment parameter was significant ($\chi_1^2 = 4.29$, p = 0.038), though the treatment/timepoint interaction parameter was close to significant ($\chi_1^2 = 3.1$, p = 0.079).

Injection *per se* had a tendency to decrease choosiness, and CORT treatment generally did not differ from the vehicle control. This effect was not significant but can be seen visually in Figures 8 and 9. The medium CORT treatment group, however, reversed the overall trend of injection treatment groups; females in the med CORT treatment and only in this treatment reversed more post-injection than pre-injection in both 22/30 and 30/38 acoustic conditions. This effect was significantly different than other treatments and provides strong evidence for a non-linear dose-response curve. Specifically, choosiness in the three CORT groups defines an inverted U function; reversal likelihood nominally decreases in both low and high CORT groups, while reversal likelihood significantly increases in the medium CORT group. This inverted U shape is one of several hormone-behavior response curves observed in animal populations (Hau & Goymann, 2015). Hau and Goymann (2015) discuss how behaviors may be especially prone to plastic, context-dependent hormone responses (compared with more static morphological traits). Gambel's White-Crowned Sparrows (*Zonotrichia leucophrys gambelii*) that orally ingested CORT show dose-dependent activity levels by an inverted U-function; only the medium CORT dose increased perch hopping, a behavior associated with response to stressful natural perturbations (Breuner et al., 1998). Similarly, in my study, regardless of underlying (pre-

injection) choosiness within treatment groups, only the medium CORT treatment was significant and caused an increase in reversal choices.

Synthesis & Conclusions

My results suggest that proceptivity in female gray treefrogs is unaffected by elevated levels of circulating CORT. Nor do handling and injection, potent stressors themselves, have any impacts on responsiveness or latencies in this population. These results are consistent with prior studies showing that responsiveness in anurans is not affected by elevated CORT (Davis & Leary, 2015; Gall et al., 2019). My results also show that CORT did not increase latencies, contrary to my prediction; this result differs from two previous studies that show a correlation between elevated CORT and longer latencies, suggesting a slight dampening effect of CORT on proceptivity (Bastien et al., 2018; Gall et al., 2019). However, my result concurs with Davis and Leary (2015) who found no impact of CORT on latencies. As noted in Bastien et al. (2018), the Davis and Leary (2015) study manipulated CORT levels (as did I), while Bastien et al. (2018) and Gall et al. (2019) considered correlational effect of CORT on latency. That proceptivity in anurans seems largely immune to manipulated CORT effects may be a result of the seasonal breeding nature of these animals. Seasonal breeders that have limited time and opportunity to fertilize an egg clutch may be buffered against elevated levels of CORT in order to ensure successful reproduction (J. C. Wingfield & Sapolsky, 2003).

The impact of CORT treatment on preferences largely corresponded with previous studies. While past studies in a variety of species have linked environmental stressors independently to both elevated GCs and decreased preferences (reviewed in Leary & Baugh, 2020) and previous GC manipulation studies found decreased preference as a result of elevated GCs (Davis & Leary, 2015; Kavaliers & Ossenkopp, 2001), correlational studies in anurans have suggested that preferences are not correlated with GC levels and that anurans with high GCs maintain strong species-typical preferences (Bastien et al.. 2018; Baugh et al., 2019; Gall et al., 2019). I found that elevated CORT levels had little to no effect on female preferences for higher pulse number calls. Choice boundary preferences in static trials were consistently high regardless of treatment; approach boundary preferences in static trials had greater variation but no significant changes in preferences due to CORT. Dynamic trial approach boundary preferences showed that injection *per se* appears to weaken approach boundary, while CORT-treated females actually showed a nominal strengthening of preferences compared with vehicle-injected females (Fig. 8, 9). It appears that GC effects on female preferences are highly dependent on species and context, as past studies in green treefrogs and mice found an attenuation of preference with elevated GCs (Davis & Leary, 2015; Kavaliers & Ossenkopp, 2001), while a study in lizards (Romero-Diaz et al., 2019) and the current study of gray treefrogs found that preferences were unaffected by elevated GCs. Additional studies with experimentally manipulated GCs in other species will be necessary to elucidate consistent trends, if these exist.

I found that female gray treefrogs administered a medium dose of CORT increased their reversal likelihood more than females in any other condition, while females given a low or high CORT dose did not differ from control females in choosiness. This non-linear dose response in behavior is remarkable, especially since CORT did not strongly affect either female proceptivity or preference strength in my study. An overly simple explanation of this effect might suggest that the medium CORT dose balanced energetic output in such a way as to provide females with

sufficient energy resources to be more choosy; that is, the increased effort (time, distance) required to select the higher pulse number call—following stimuli translocation—was a lower barrier at to this medium CORT dose. Another interpretation deals with GC receptors; mineralocorticoid (MR) and glucocorticoid (GR) receptors both bind glucocorticoids and are omnipresent in vertebrate brains (Senft, Meddle, & Baugh, 2016). Choosiness may depend upon an activational balance between these two receptors, as MR type receptors have a much greater affinity for glucocorticoids than GR type receptors. The MR may be predominately binding CORT at lower concentrations that include the low CORT dose in this study, while the medium CORT dose may be sufficient to bind to the GR at meaningful levels. A nearly supraphysiological CORT level (high CORT dose) may then cause saturation of both receptors. If this is the case, moderate activation of the GR might increase choosiness by elevating locomotor activity (c.f. Breuner, Greenberg, & Wingfield, 1998). Choosiness might also be elevated at a moderate CORT dose via a membrane receptor in a non-genomic manner (see Breuner, Greenberg, & Wingfield, 1998 for further discussion). Future studies should investigate these potential mechanisms and assess choosiness at additional CORT concentrations to improve granularity of the dose-dependent behavioral response. Regardless of the mechanism, increased mate choosiness at a moderately elevated GC level provides compelling evidence of a role for GCs in modulating tradeoffs in a mate choice context.

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Figures

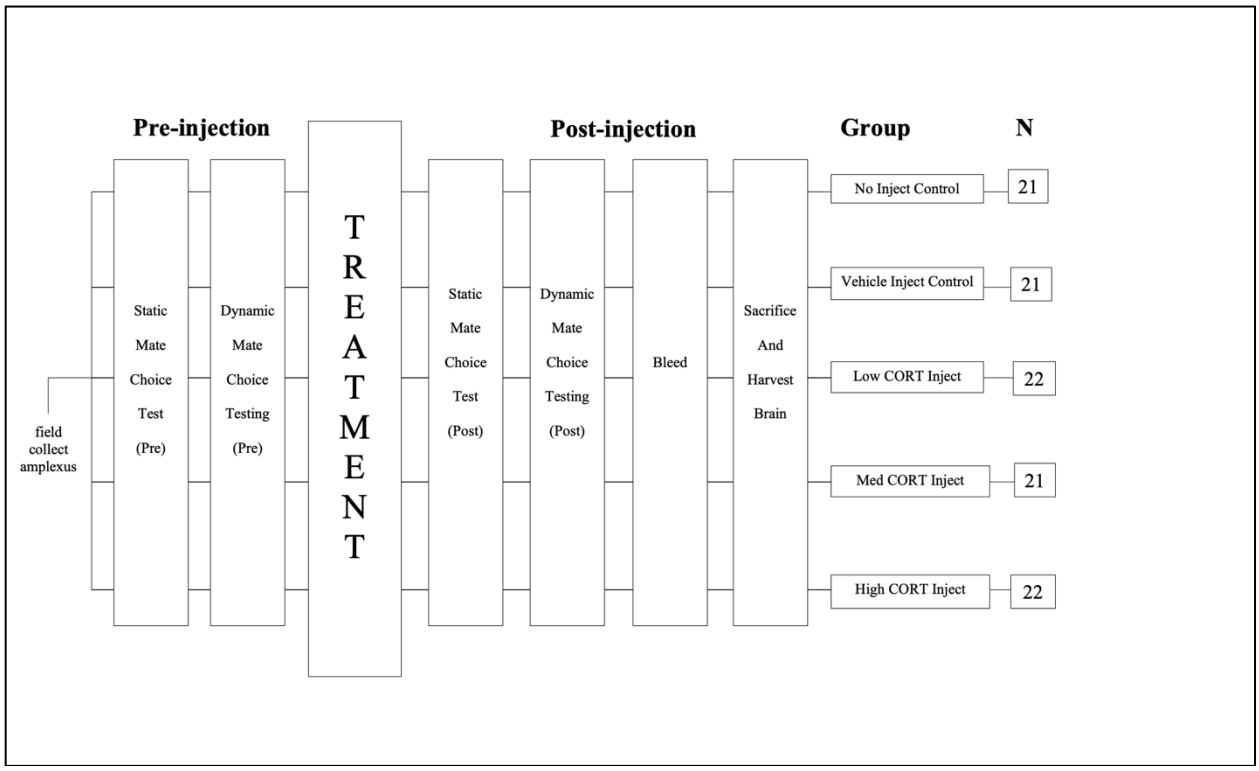

Fig 1. Experimental design. All treatment groups experienced the same handling and behavioral tests on the same time track. Thus, the following comparisons were permitted: (i) pre-injection no-inject control versus post-injection no-inject control: the effect of handling and a 30 min hold between behavioral tests on behavior; (ii) pre-injection vehicle inject control versus post-injection vehicle inject control: the effect of injection *per se* on behavior; (iii) pre-injection CORT groups versus post-injection CORT groups: the effect of exogenous low, medium and high CORT doses on behavior. A total of 107 females were used. Each female served as her own control due to the repeated measures design.

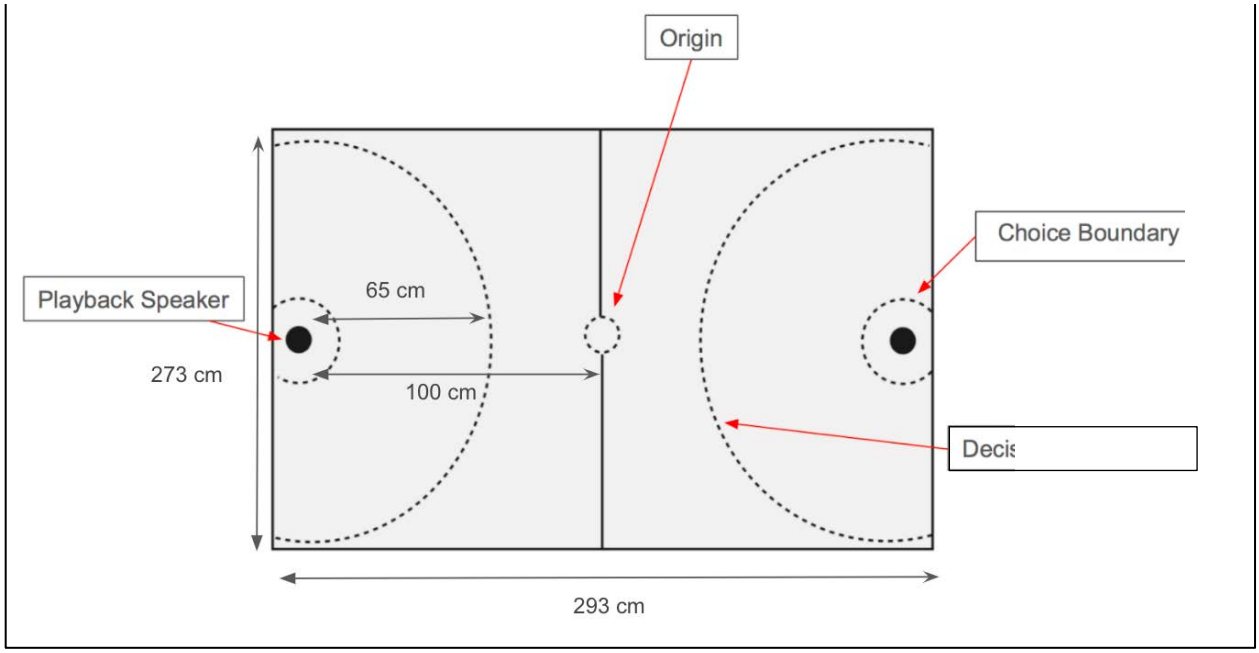

Fig 2. Phonotaxis arena. Females were placed in an acoustically transparent mesh cage at the origin to begin trials. After 10 sec, the cage top was lifted, and females were free to move. Trials continued until the female selected a speaker broadcasting a male call. Trials were repeated if the female fouled. Dimensions and boundary names are designated in the figure.

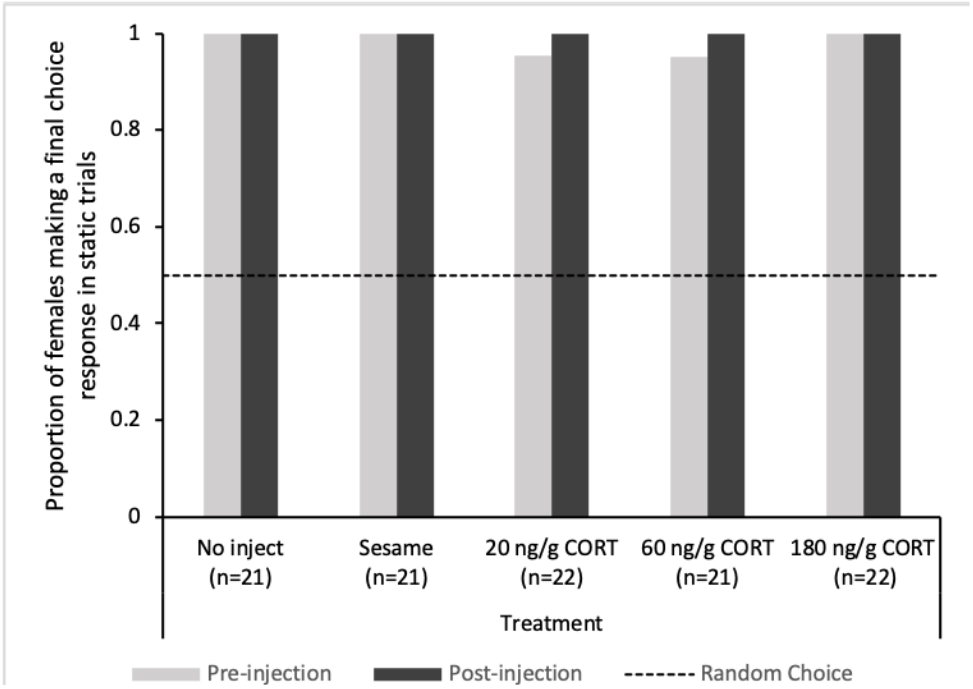

Fig 3. Proportion of females that crossed a choice boundary in the first static trial presentation. Dashed line reflects random choice. Responses were significantly greater than random.

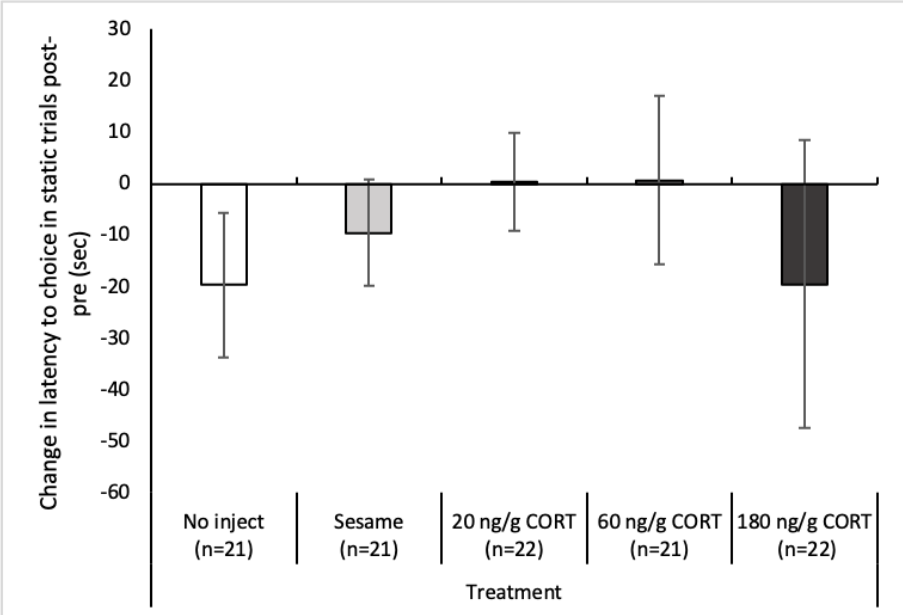

Fig 4. Latency to choice boundary delta (post-pre) by treatment group, ± SEM. Latencies to choice in static trials were significantly faster post-injection compared to pre-injection (main effect). No individual treatment was significant, nor was there a treatment × timepoint interaction.

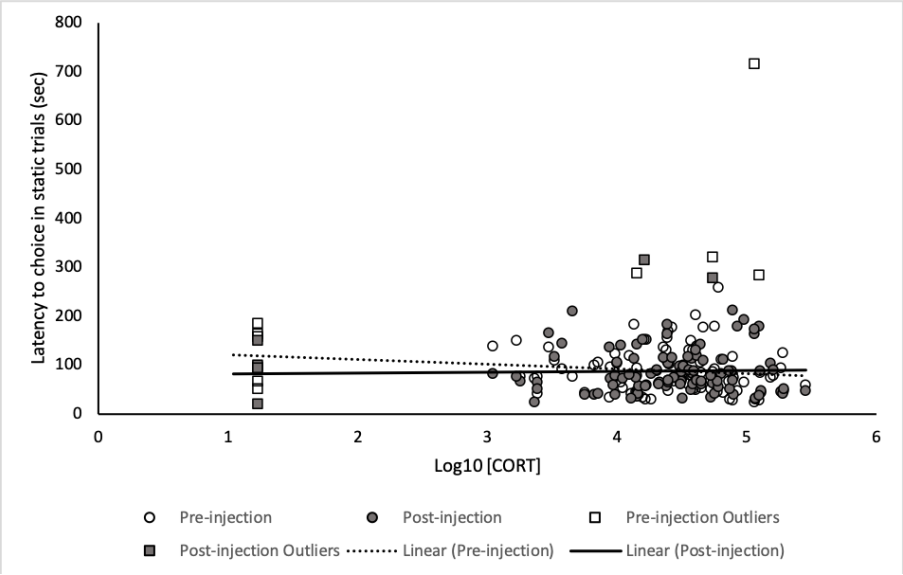

Fig 5. Regression of $\log_{10}$-transformed hormone concentrations against latency to choice boundary. Open points represent pre-injection latencies, while shaded point represent post-injection latencies. Squares represent statistical outliers. Dashed line represents a best-fit line for pre-injection data, while the solid line represents a best-fit line for post-injection data. Qualitative results of the regression were identical if statistical outliers were omitted. No effects were significant.

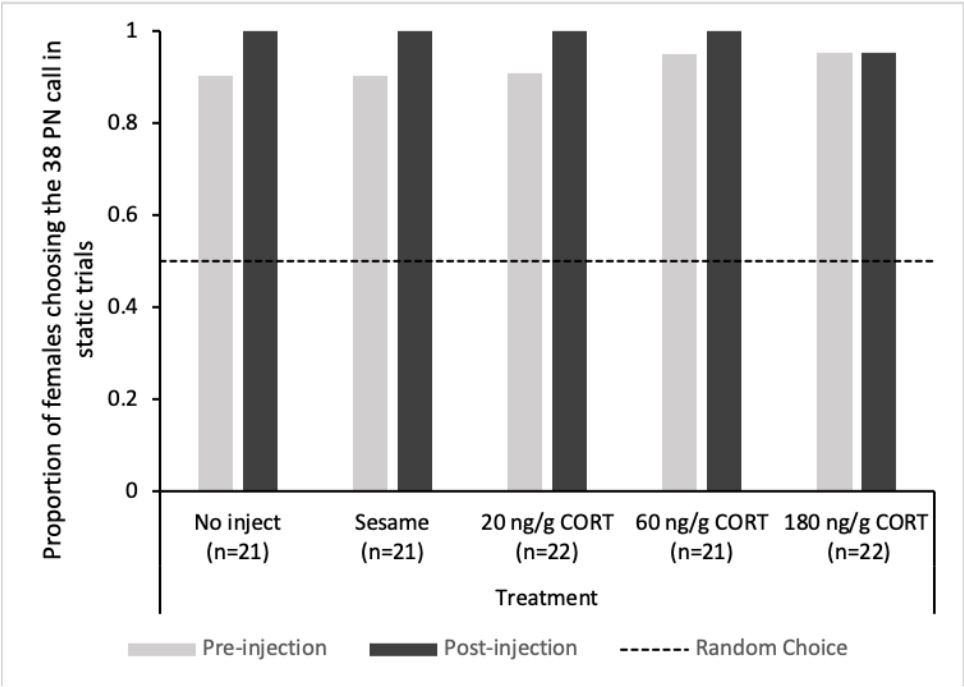

Fig 6. Proportion of females crossing the choice boundary toward the higher pulse number call in static trials. Dashed line reflects random choice by females. No effects were significant.

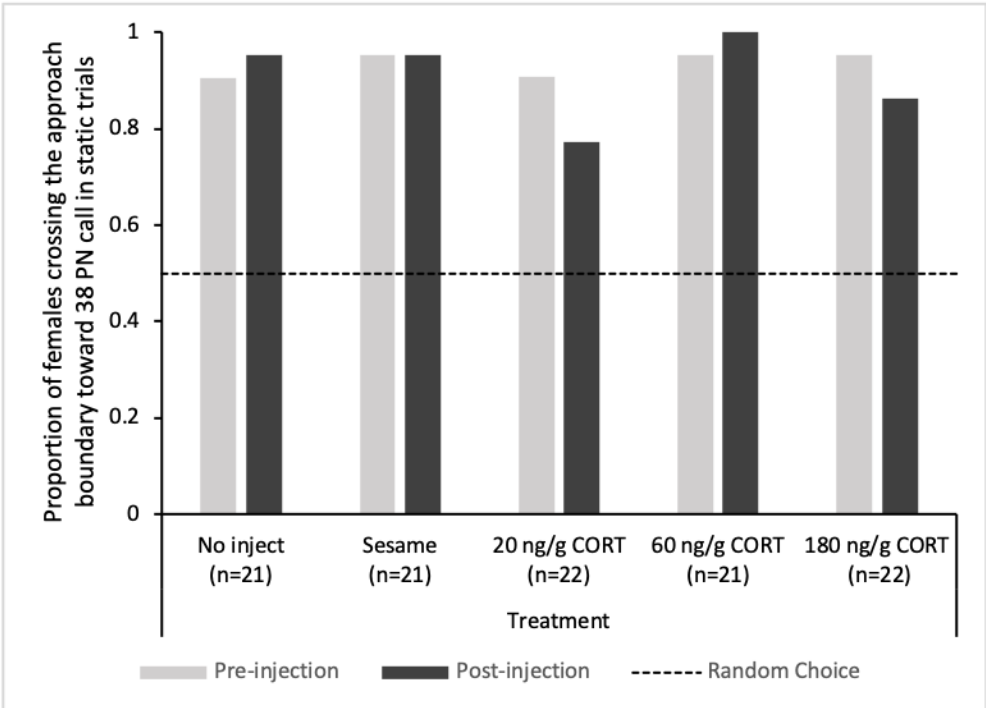

Fig 7. Proportion of females crossing the approach boundary toward the higher pulse number call in static trials. Dashed line reflects random choice by females. No effects were significant.

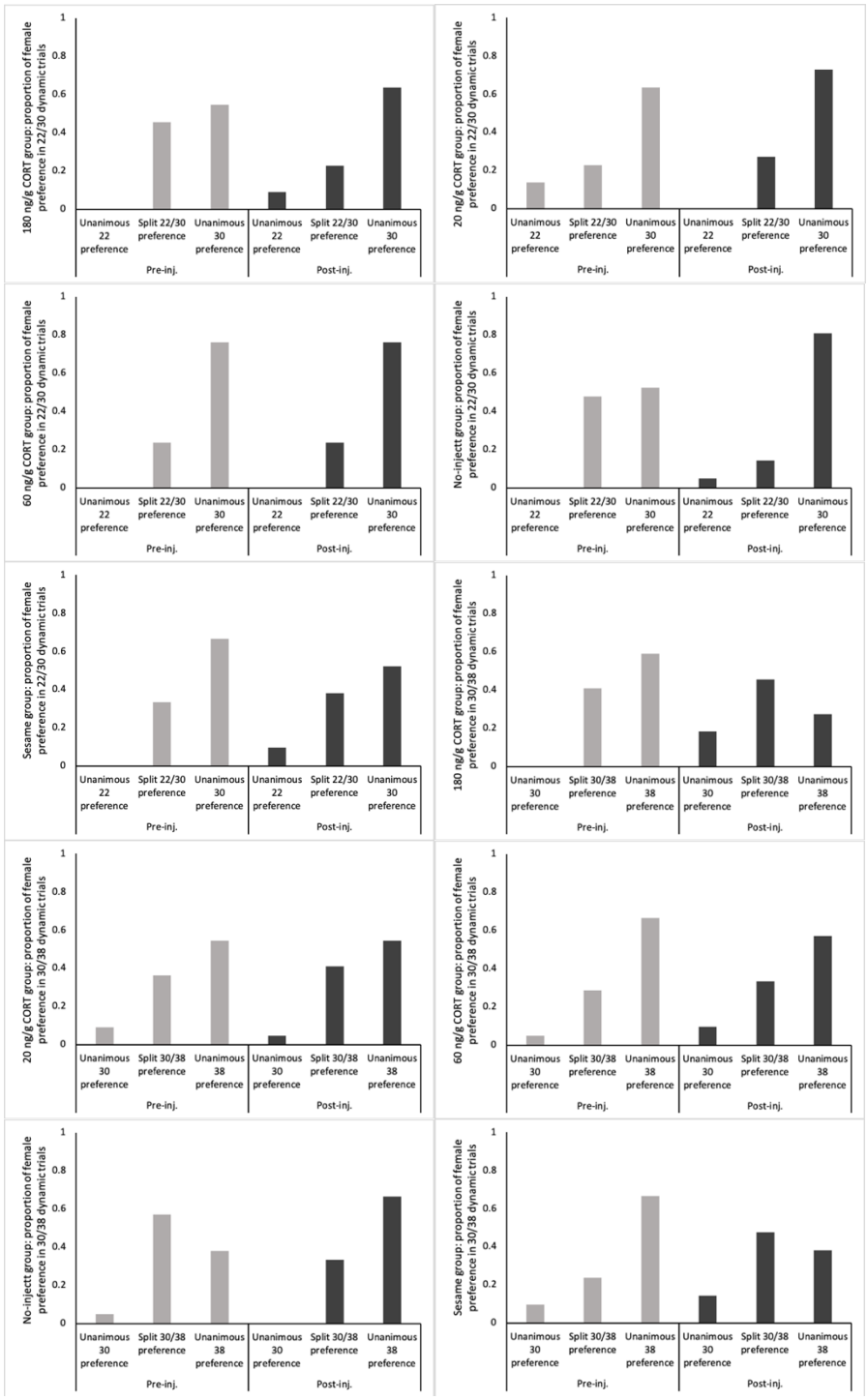

Fig 8. Proportion of females with higher pulse number preference in dynamic trials. Preferences grouped in three categories: (1) unanimous lower PN pref., (2) split pref., (3) unanimous higher PN pref. Individual graphs displayed for treatments and acoustic conditions. 22/30 graphs on the left, 30/38 graphs on the right. There was a significant interaction of no-inject treatment with timepoint: females in the no-inject group significantly increased preference for higher PN calls in both acoustic conditions.

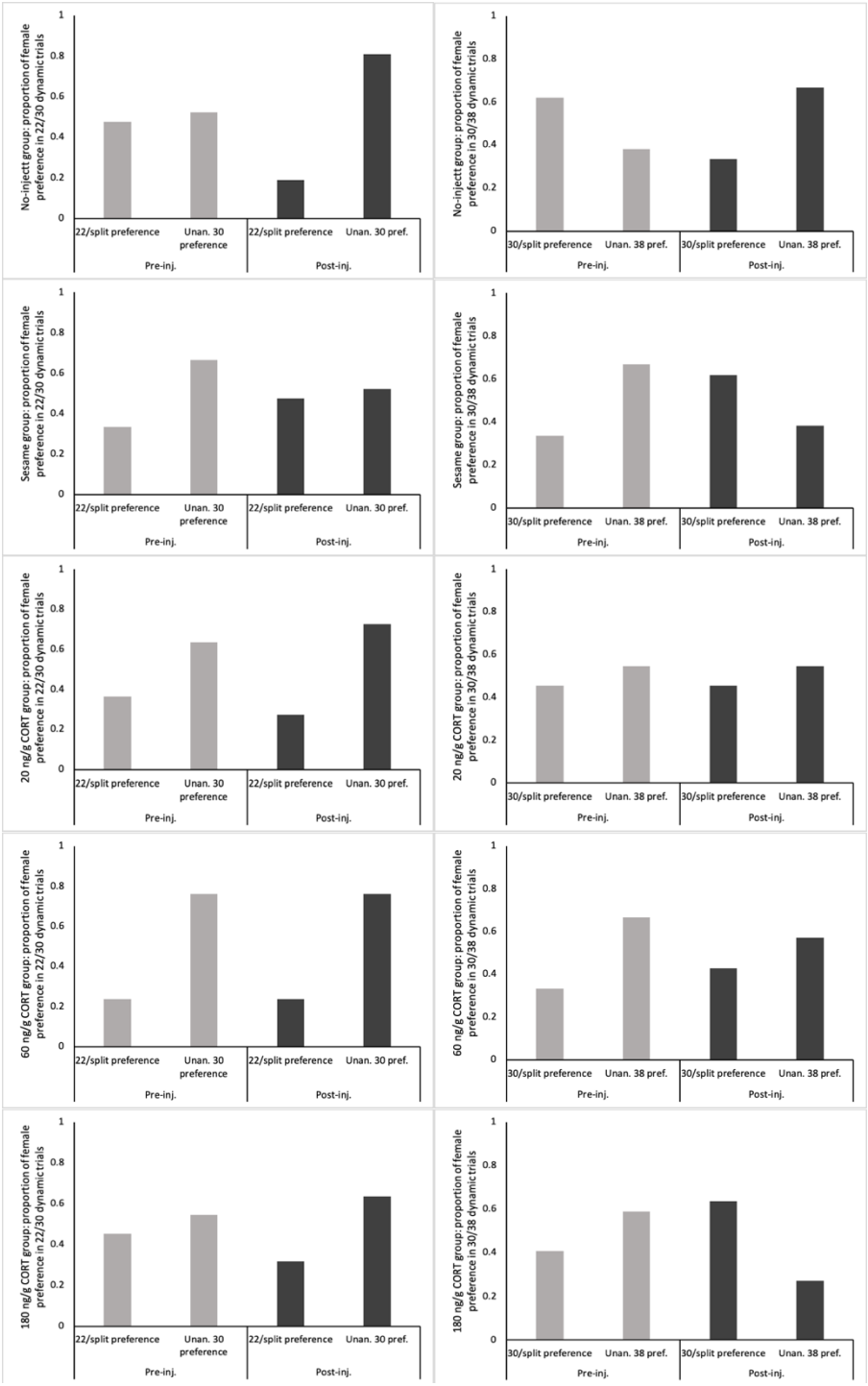

Fig 9. Proportion of females with higher pulse number preference in dynamic trials (data reduction from figure 8). Preferences grouped in two categories: (1) unanimous lower PN pref. or split pref., (2) unanimous higher PN pref. Individual graphs displayed for treatments and acoustic conditions. 22/30 graphs on the left, 30/38 graphs on the right. There was a significant interaction of no-inject treatment with timepoint: females in the no-inject group significantly increased preference for higher PN calls in both acoustic conditions.

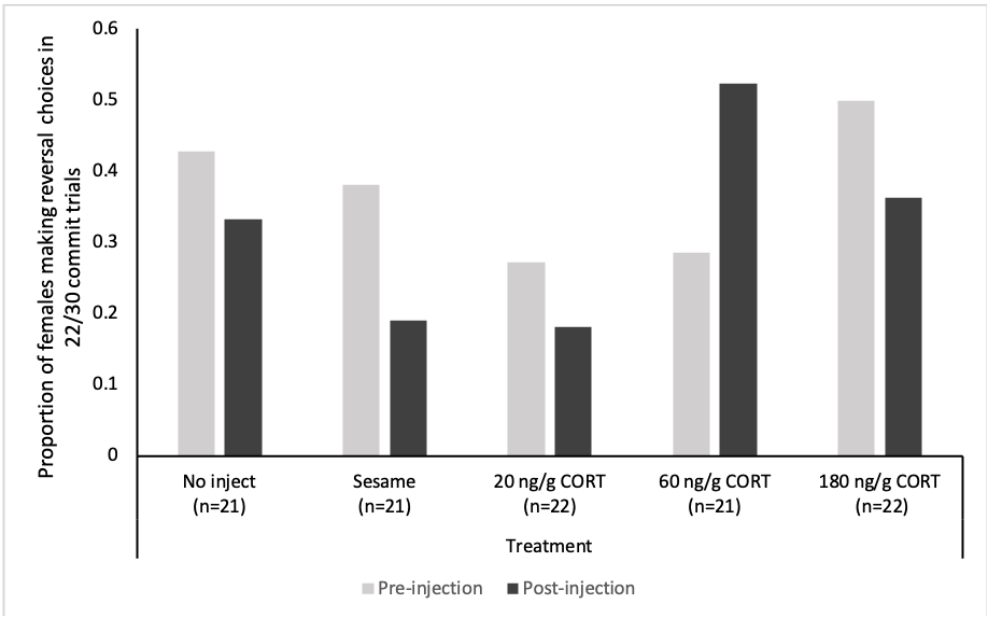

Fig 10. Proportion of females making reversal choices in the 22 vs. 30 PN acoustic condition. Gray bars represent pre-injection reversals and black bars represent post-injection reversals. Females in the medium (60 ng/g) CORT treatment reversed significantly more following treatment compared with all other treatment groups (generalized linear model).

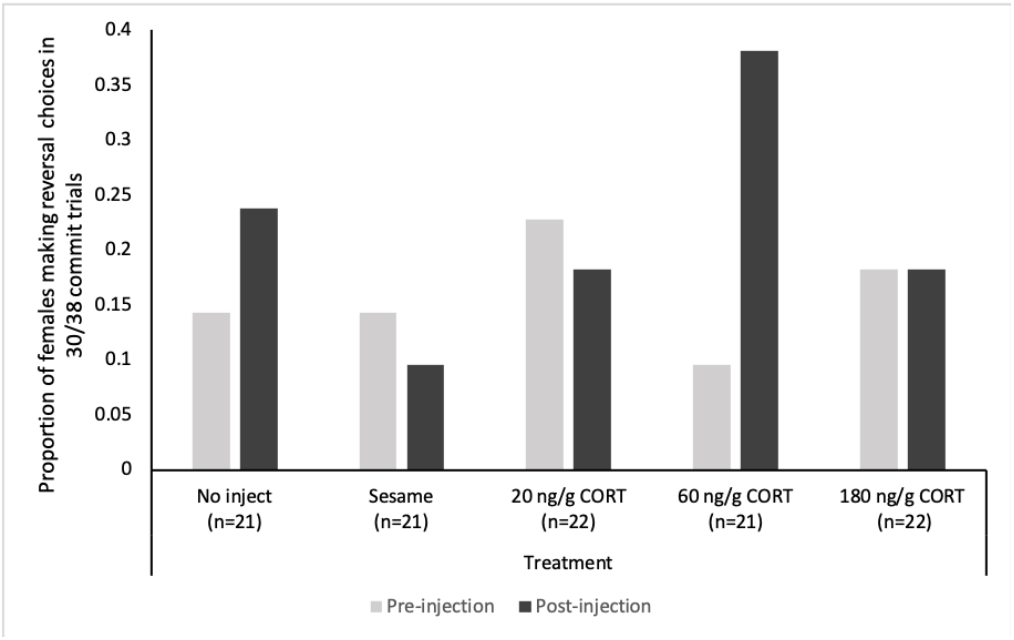

Fig 11. Proportion of females making reversal choices in the 30 vs. 38 PN acoustic condition. Gray bars represent pre-injection reversals and black bars represent post-injection reversals. Females in the medium (60 ng/g) CORT treatment reversed significantly more following treatment compared with all other treatment groups (generalized linear model).

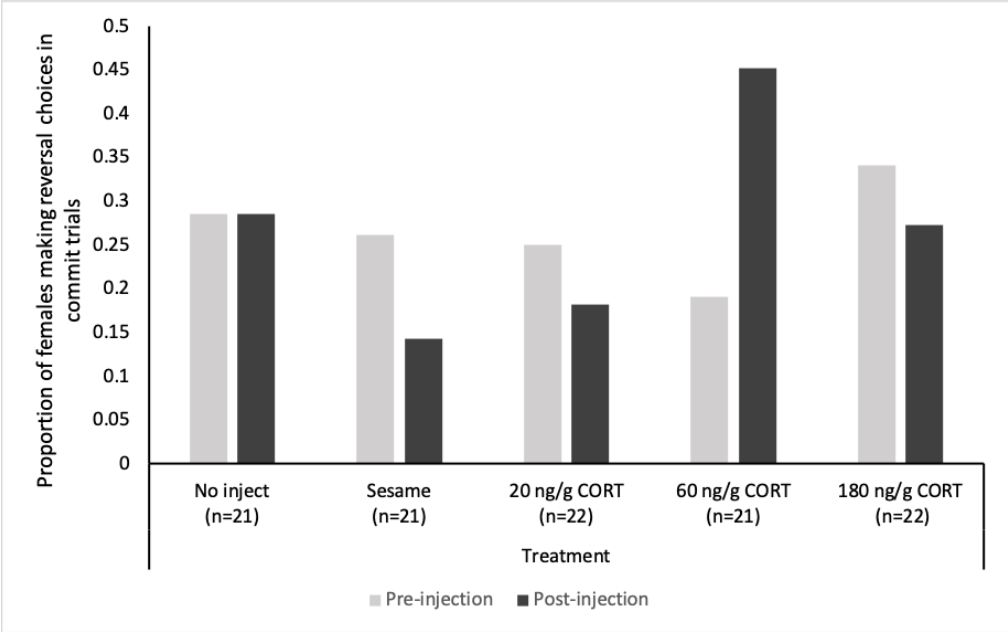

Fig 12. Proportion of females making reversal choices in all commit trials (two acoustic conditions combined). These reversal proportions were not used for statistical analysis; this graph is included for visual purposes only. Gray bars represent pre-injection reversals and black bars represent post-injection reversals.

Supplemental Materials

SX1: Hormone injection validation

A dose-response validation was performed during May 2018 prior to the current study, using captive eastern gray treefrogs (*Hyla versicolor*, N=15). CORT injections were prepared as described previously in this paper, dissolving crystalline corticosterone in 95% EtOH and then diluting in sesame oil (Sigma, Cat. No. S3547). Two control groups were used: no injection, vehicle injection (sesame oil); and 9 CORT doses were used: ranging from 25-6400 ng g^{-1} in doubling intervals. This method expands upon the work done previously by Davis and Leary (2015) by utilizing oil rather than saline as a vehicle and extending the range of CORT doses. We sampled blood at 30 min, 60 min, 120 min and 360 min intervals and quantified steroid concentrations using the methods described previously in this study. Females were each used 3 times separated by a 72-hour recovery interval. The results indicated that doses of 20 ng g^{-1} (2 ng uL^{-1}), 60 ng g^{-1} (6 ng uL^{-1}) and 180 ng g^{-1} (18 ng uL^{-1}) and a 30 min recovery period would consistently elevate CORT within natural physiological range (1-124 ng mL^{-1}; Gall et al. 2019).

Supplemental Materials SX1: CORT Injection Validation *Hyla versicolor* (May 2018, Swarthmore College)		**Mean (+/- SEM) Plasma CORT (ng/mL)**			
Treatment CORT concentration	**Sample Size (N)**	**30 min post injection**	**60 min post injection**	**120 min post injection**	**360 min post injection**
No Injection	3	14.19 (1.5)	27.42 (9.33)	n/a	23.82 (12.78)
Vehicle Injection (oil)	3	6.27 (2.38)	11.93 (1.35)	7.92 (1.15)	10.14 (4.67)
2.5 ng/uL (25 ng/g frog; ca. 125 ng CORT per injection)	3	18.51 (7.53)	25.83 (7.88)	n/a	28.76 (9.34)
5 ng/uL (50 ng/g frog; ca. 250 ng CORT per injection)	3	34.38 (2.51)	34.80 (3.79)	n/a	58.88 (21.62)
10 ng/uL (100 ng/g frog; ca. 500 ng CORT per injection)	3	31.29 (5.72)	70.38 (18.87)	49.84 (30.57)	14.61 (3.60)
20 ng/uL (200 ng/g frog; ca. 1 ug CORT per injection)	3	n/a	87.64 (19.58)	97.25 (35.45)	n/a
40 ng/uL (400 ng/g frog; ca. 2 ug CORT per injection)	3	n/a	130.49 (18.44)	106.69 (27.61)	n/a
80 ng/uL (800 ng/g frog; ca. 4 ug CORT per injection)	3	n/a	244.47 (20.59)	221.63 (34.53)	n/a
160 ng/uL (1600 ng/g frog; ca. 8 ug CORT per injection)	3	n/a	1302.87 (764.9)	302.28 (95.46)	n/a
320 ng/uL (3200 ng/g frog; ca. 16 ug CORT per injection)	3	n/a	2300 (200.0)	732.11 (28.09)	n/a
640 ng/uL (6400 ng/g frog; ca. 32 ug CORT per injection)	1	n/a	1554.5 (n/a)	668.47 (n/a)	n/a