Moderately Elevated Glucocorticoids Increase Mate Choosiness But Do Not Affect Sexual Proceptivity Or Preferences In Female Gray Treefrogs

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Moderately elevated glucocorticoids increase mate choosiness but do not affect sexual proceptivity or preferences in female gray treefrogs

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Glucocorticoids (GCs) are rarely studied in the context of female mate choice, despite the expression of receptors for these products in sexual, sensory and decision-making brain areas. Here we investigated the effects of GC concentrations on three aspects of female sexual behavior in breeding Cope’s gray treefrogs (Hyla chrysoscelis): proceptivity—a measure of sexual motivation, intraspecific mate preferences, and mate choosiness. To our knowledge this is the first experimental study on the endocrine basis of mate choosiness. We predicted that mate choosiness—forfeiting an initial mate preference to pursue a suddenly more attractive mate—would be particularly impacted by elevated GCs with moderate GC levels associated with greater choosiness. We found support for this predicted inverted-U relationship. Females in the control group (no injection) showed no change in choosiness across timepoints. In contrast, females in the vehicle, Low (20 ng g\(^{-1}\)) and High (180 ng g\(^{-1}\)) corticosterone groups exhibited a nominal decline in choosiness after injection, suggesting that the experience of injection has little or perhaps slightly suppressive effects on female choosiness. Females in the moderate dose group (60 ng g\(^{-1}\)), however, exhibited a significant increase (>100%) in choosiness. Further, we found no effect of elevated GCs on sexual proceptivity or the species-typical preference for longer calls. These findings may reflect a buffering of primary sensory areas in the brain against elevated GCs. The recruitment of other cognitive processes during active decision-making, however, may facilitate GC modulation of mate choosiness, thereby promoting tactical plasticity at this critical life history juncture.

KEYWORDS: choosiness; corticosterone; dynamic mate choice; mate preferences; phonotaxis; stress.
The relationship between glucocorticoids (GCs) such as cortisol and corticosterone (CORT) and female reproductive behavior is understudied and likely to be complex (reviewed in Leary and Baugh, 2020).

Chronically elevated CORT is generally thought to suppress reproduction (Sapolsky et al., 2000; Toufexis et al., 2014), while acute elevations have been hypothesized to facilitate reproduction (Wingfield and Kitaysky, 2002; Moore et al., 2016). For example, CORT is thought to be involved in the acquisition and prioritization of metabolic resources that play a role in aspects of fecundity, reproductive investment and mating decisions (Wingfield and Sapolsky, 2003; Cotton et al., 2006; Breuner et al., 2008; Tokarz and Summers, 2011). Further, reproduction is among the most demanding chapters in the life histories of female vertebrates—energy expenditures during the breeding season can be an order of magnitude higher in females compared to males (Ryan et al., 1983). Thus, an acute facilitating role of CORT is consistent with the observation that this hormone is naturally elevated immediately prior to and during peak reproductive readiness and declines precipitously after mating (Dauphin-Villemant et al., 1990; Romero, 2002; Bastien et al., 2018; Gall et al., 2019; Amruta et al., 2020). This facilitating role, however, may itself be complicated due to non-linearities in dose-response relationships. For example, moderately elevated GCs may facilitate reproductive efforts by mobilizing energy stores, while extremely elevated levels may inhibit reproduction (reviewed in Moore and Jessop, 2003). These temporal and dose-dependent effects between the HPA axis and reproduction require experimental study.

One critical aspect of female reproductive behavior is how the process of mate choice is executed because this dictates the strength and direction of sexual selection for male traits and often has implications for female fitness (Welch et al., 1998). But this process of mate choice is itself multifaceted, with at least three key components. First, females must be sexually motivated to be attracted to male signals. Tests of proceptivity—female behaviors directed toward males or male stimuli that typically result in mating—can be used to evaluate this component (Ward et al., 2015). Second, females often exhibit strong intraspecific preferences, biasing their mate choices towards males with certain attractive traits over others (Baugh and Ryan, 2011). Lastly, mating preferences are not executed in a vacuum. There are often intrinsic factors (e.g. condition-dependent) or extrinsic factors (e.g. environmental...
circumstances) that introduce tradeoffs. These tradeoffs have the potential to modulate how mate preferences are expressed (Jennions and Petrie, 1997). When females are capable of modulating the expression of those preferences on a moment-to-moment basis as a function of the dynamic male signaling environment, we refer to this component as choosiness (i.e. fickleness). We think an understanding of how CORT impacts female mate choice will benefit from an experimental decomposition of these three components in a single study system.

Most empirical studies to date focus on how CORT influences female proceptivity in seasonal breeders—i.e., species with brief windows of reproductive readiness. These studies, primarily of frogs and lizards, suggest that elevated CORT does not suppress sexual motivation, though subtle inhibitory effects may be present (Bastien et al., 2018; Gall et al., 2019; Romero-Diaz et al., 2019; but see Davis and Leary, 2015). This lack of reproductive suppression may indicate that seasonal breeders are buffered against naturally elevated CORT at this fitness-determining juncture (Gall et al., 2019).

To our knowledge only two published studies have experimentally tested the effect of GCs on intraspecific preferences in females. In common lizards (Zootoca vivipara), Romero-Diaz et al. (2019) found that exogenous CORT did not impact the female preference for familiar versus unfamiliar males. In contrast, Davis and Leary (2015) found that exogenous CORT reduced the otherwise strong species-typical preference for higher call rates in green treefrogs (Hyla cinerea). Reduced preferences and mate sampling (i.e. lower time investment) under conditions of elevated GCs could be adaptive, for example, if GCs accelerate passage through a window of reproductive readiness (e.g., gamete viability) (Bastien et al., 2018). Two additional studies report the effects of GCs on female mating behavior, with the authors’ interpreting their results in the context of female mating preferences. However, mate preferences were not explicitly evaluated. For example, Kavaliers and Ossenkopp (2001) quantified female locomotor responses to male versus blank odorants in mice and found that exogenous CORT ablated the normal male odorant association, thereby indicating an effect of CORT on proceptivity, not preferences. In marine iguanas, Vitousek and Romero (2013) found a negative correlation between circulating CORT (following an experimental stressor) and the number of male territories visited by reproductive females.
This negative correlation was interpreted as evidence of CORT’s suppressive role in mate selectivity, though mate selection was not measured and there was no stimulus control of male traits.

To our knowledge, the endocrine basis of female choosiness is unexplored. Studies of decision-making performance in humans, however, suggest that GCs could play a role in attentional-cognitive aspects (reviewed in Starcke and Brand, 2012). In general, it appears that decision-making under experiential stressors may lead to more haphazard and impulsive decision-making (Keinan, 1987; Lenow et al., 2017). Interestingly, males and females can differ in how decision-making tradeoffs are resolved (van den Bos et al., 2009), with some evidence that stress can induce disassortative mating preferences in women (Lass-Hennemann et al., 2010). Animal studies that experimentally manipulate CORT are needed.

In the present study, we used female Cope’s gray treefrogs (*Hyla chrysoscelis*) to evaluate how these three components of mate choice (proceptivity, preferences and choosiness) are influenced by experimentally elevated CORT (3 doses) and control treatments. First, we tested the conventional hypothesis that female sexual proceptivity is dampened by elevated CORT (Toufexis et al., 2014). We predicted, however, that the effect of CORT on this essential aspect of reproductive behavior would be relatively minor owing to the fact that seasonal breeders are likely buffered against the deleterious effects of potential stressors because of the fitness-determining consequences and tightly constrained time horizon for breeding—a female frog that foregoes reproduction, might foreclose her lifetime fitness (Lynch et al., 2005; Bastien et al., 2018). Again, this prediction is supported by the observation that plasma CORT is near peak values during reproductive readiness in seasonally breeding anurans (reviewed in Leary and Baugh, 2020).

The second aim was to evaluate whether elevated CORT influenced intraspecific preferences—how females discriminate among multiple conspecific males varying in some trait. In virtually all animal species studied, females have strong and predictable preferences for parameters of male advertisement signals, irrespective of signaling modality (Kirkpatrick and Ryan, 1991; Andersson, 1994). In *H. chrysoscelis*, females have strong preferences for a variety of male vocalization parameters, including call duration which is represented by the number of pulses in the call (Pulse Number, PN; Gerhardt 1994;
Gerhardt et al., 1996; Bee, 2008; Ward et al., 2013; LaBarbera et al., 2020). Here we used experimental manipulations of PN to test the hypothesis that elevated CORT impacts the species-typical preference for higher PN calls. Given the conflicting results from the two empirical studies conducted on this topic to date (Davis and Leary, 2015; Romero-Diaz et al., 2019), and the observation that species-typical preferences can be highly stable over adulthood (Ryan et al., 2019), present during early development (Baugh et al., 2012a) and intact despite naturally elevated CORT during breeding in anurans (Bastien et al., 2018; Gall et al., 2019; Amruta et al., 2020), we predicted the effects of elevated GCs on species-typical preferences would be relatively minor compared to a more cognitively demanding aspect of mate choice—how females update their decision under uncertainty.

Temporal updating behavior, also known as mate choosiness, is evaluated by dynamically altering the signaling environment during phonotaxis—in an ecologically relevant manner—thereby enabling us to evaluate how females sample, execute and reconcile tradeoffs during mate choice (see Baugh and Ryan, 2009, 2010a,b,c; Bastien et al., 2018). Following Baugh and Ryan (2009, 2010a,b,c) choosy behavior was operationally defined as the likelihood that a female will forego her initially preferred signaler when he suddenly becomes less vocally attractive compared to an alternative signaler. We achieved this using dynamic playbacks that seamlessly reduce PN in the initially approached male and symmetrically and simultaneously increase PN in the unapproached male. Dynamically varying male sexual advertisements are a natural phenomenon in many taxa including birds (Hill et al., 1999), fish (Rosenthal et al., 1996) and frogs (Bernal et al., 2009), and is also the case specifically for PN in gray treefrogs (Gerhardt et al., 1996). A choosy female is thus defined as one that changes her approach following stimulus manipulation, reversing course and eventually selecting the currently more attractive (higher PN) caller. This procedure allows the experimenter to introduce a tradeoff into the decision-making process, as the female frog has invested search time, locomotor effort and conspicuous movement that becomes protracted if she reverses and pursues the suddenly more attractive male. When tradeoffs are incorporated into mate choice assays (e.g. increased predation risk associated with preferred mates), otherwise strong species-typical biases can be altered (e.g. choosing heterospecific mates; Willis et al.,
2012). Such findings suggest that female decision-making is plastic—yet the physiological basis for this plasticity is largely unknown. Because many of these tradeoff circumstances introduce stressors or energetic demands or both, it is possible that GCs are involved. Indeed, recent work in songbirds indicates that locomotor behavior in the context of uncertainty is predicted by circulating CORT (reviewed in Hau et al., 2016). Further, though rarely tested (Hau and Goymann, 2015), elevated CORT has been shown to modulate behavior with an inverted-U dose-response function in three species. In a songbird, Breuener et al. (1998) demonstrated this relationship with general locomotor activity. In mice, Kovács et al. (1977) demonstrated this inverted-U relationship for passive avoidance behavior and linked this with an inverted-U relationship with 5-HT metabolism in the brain. In anesthetized rats, Diamond et al. (1992) showed an inverted-U relationship between experimentally elevated CORT and the bursting of hippocampal neurons. Given these previous empirical findings, and the idea that moderate elevations might maximally stimulate locomotor investment and appetitive behavior, we predicted that moderate CORT elevations would yield the choosiest females.

MATERIALS AND METHODS

Animals

This study was carried out on the St. Paul campus of the University of Minnesota, and the protocols used for collecting, handling, and testing frogs closely followed those described by Gerhardt (1995) and reported in previous studies (Baugh et al., 2019; Gall et al., 2019; Tanner and Bee, 2019, 2020a, 2020b; LaBarbera et al., 2020). We collected mating pairs of the western genetic lineage of Cope’s gray treefrog (Ptacek et al., 1994) from wetlands located in the Carver Park Reserve (Carver County, MN, USA, 44°53′49.08″ N, 93°43′03.11″ W) and the Tamarack Nature Center (Ramsey County, MN, USA, 45°06′08.50″ N, 93°02′28.89″ W) in June of 2018 and 2019. Pairs found in amplexus were collected and placed in small plastic containers in the field, returned to the lab, and then maintained at approximately 4° C until the following day, when they were tested (Fig. 1). Gravid female treefrogs captured in amplexus...
are as discriminating as females captured just prior to making a mating decision (Murphy and Gerhardt, 1996).

**Acoustic stimuli and apparatus**

In a series of two-alternative choice tests (Gerhardt, 1995), we used phonotaxis in response to synthetic advertisement calls differing in PN to investigate the influence of CORT on proceptivity, preference, and choosiness. Synthetic *H. chrysoscelis* calls were generated using custom scripts in MATLAB to have acoustic properties close to the average values of calls recorded at 20°C in local populations (Ward et al., 2013). Each 10-ms pulse in these calls was created by adding two phase-locked sinusoids with frequencies (and relative amplitudes) of 1250 Hz (-11 dB) and 2500 Hz (0 dB) and then shaping the amplitude envelope with species typical onsets (3.1-ms pulse rise time) and offsets (5.4-ms fall time). We concatenated pulses together (Adobe Audition) to create sequences of pulses that simulated natural calls having a pulse rate of 50 pulses/s (20-ms pulse period, 50% pulse duty cycle). Three stimulus calls were made that differed only in PN based on the mean (±SD) PN in the population (Ward et al., 2013) of 30 ± 4 pulses/call: a low-PN call (22 pulses, $\bar{X}$-2SD), an average-PN call (30 pulses, $\bar{X}$), and a high-PN call (38 pulses, $\bar{X}$+2SD). Alternative stimuli consisted of sequences of calls created by concatenating calls and silent inter-call intervals so that calls were broadcast at the average call rate for the population (11 calls/min; Ward et al., 2013). Within a given choice test, calls in the two alternatives alternated in time such that each call was preceded and followed by an equivalent duration of silence. All calls were broadcast at a sound pressure level (re 20 µPa) of 85 dB SPL at 1 meter, which approximates a natural call amplitude in this species (Gerhardt, 1975). We used a Larson Davis 831 SLM (LCF$_{\text{max}}$) to calibrate the sound pressure levels of each experimental stimulus prior to testing each day.

Testing was carried out in an arena (Fig. 2) within a sound-attenuating chamber outfitted with acoustic foam tiles on the walls and ceiling and carpeted with low pile carpet (L×W×H, cm, internal dimensions of chamber: 295×275×195; Industrial Acoustics Company). Playbacks were performed using a laptop PC (Dell 5520; MaxxAudio two-channel sound card) running SIGNAL software (version 5;...
Engineering Design) and connected to two single-channel high fidelity gain control potentiometers (SPL Electronics GmbH), a power amplifier (Crown XLS 1000), and two satellite speakers (Mod1, Orb Audio). Speakers were centered at opposite ends of the arena along its long axis and placed away from the arena walls to permit females to walk behind each speaker. Subjects were released during tests from an acoustically transparent release cage located at the center of the arena (the ‘origin’) and 1 meter from each speaker. Two hemi-circles were outlined with infrared (IR) reflective tape on the arena floor: (1) an ‘approach boundary’ was located at a radius of 65 cm from the face of each speaker; and (2) a ‘choice boundary’ was located at a radius of 10 cm from the face of each speaker. The location of the approach boundary was determined empirically by identifying symmetrical arcs that were equidistant from speakers (i.e. average locomotor investment from origin to approach boundary was symmetrical) and approximately isomorphic in peak dB levels for the stimulus along each arc (see Fig. 2). Furthermore, the distance from the origin to the approach boundary is significantly predictive of a female’s final choice under static and control playback conditions (see Results).

A separate PC laptop (Dell 5520, NVIDIA graphics card) was connected to a ceiling mounted IR camera (Basler GigE) that permitted observers to monitor the frog’s movement in real time under IR illumination and to video record the test for later review and movement analysis using Ethovision XT (Version 9, Noldus). Three judges recorded female choices in real time and a naïve judge blinded of treatment conditions confirmed the results of each coded trial using the recorded videos. The arena dimensions were calibrated prior to testing so that path lengths reflected real world distances traveled.

**Experimental design**

Using a mixed within-subjects and between-subjects design, each female was tested in a battery of five choice tests both before and after treatment in one of five CORT treatment groups, for which they were randomly assigned (Fig. 1). Dosages and timelines were determined through a validation study conducted previously (see Supplementary materials S1), and a linear dose-response relationship was also confirmed in the present study (see Results).
We blocked each testing day by CORT treatment so that one frog per each of the five treatment groups was tested per day, in a randomized order, thereby ensuring nearly equal sample sizes among the five treatment groups and minimizing the possibility of a day-by-treatment confound. Approximately 30 min prior to pre-treatment behavioral testing, mated pairs were placed in an incubator and allowed to reach a body temperature of 20 ± 2° C inside their plastic containers with water. Pairs quickly resumed amplexus during this period. Immediately prior to testing, females were separated from their mate and tested individually. Males were housed in a separate room to prevent females from hearing spontaneous male calling. Immediately after completing the battery of five pre-treatment choice tests, females were administered their assigned treatment and maintained for 30 min at 20 ± 2° C in the incubator in their original small plastic container with water and without their mate (to avoid vocal stimulation by the male and control for variation in amplexus duration). This 30-min post-injection time course yields significantly elevated plasma CORT (Supplementary materials S1) and permitted us to interleave females throughout each day of testing. After this 30-min hold, females were then tested in the same battery of five post-treatment choice tests. After post-treatment testing, frogs were measured for body mass (in increments of 0.01 g) and two estimates of body length (snout-vent length, SVL; tibia-fibula length, TFL) using calipers (in increments of 0.01 mm) by the same experienced researcher. Body mass in this species correlates strongly with both SVL and TFL ($R^2 = 0.50$-$0.74$; Baugh et al., 2019), but the correlation is nominally higher using TFL. Thus, residual body mass (RBM) was calculated from the TFL versus body mass regression. Blood and brains were collected after body size measurements (see below).

**Behavioral testing**

In each of the five choice tests administered pre-treatment and post-treatment, the two alternatives differed only in PN. To conduct a test, the female was placed in the release cage at the origin on a damp paper towel. We broadcast the stimuli for 10 sec prior to releasing the female by remotely lifting the lid of the release cage. Females were permitted up to 5 min to cross an approach boundary and up to 10 min to
cross a choice boundary; typical response latencies in this species are ca. 70-90 s (Gall et al., 2019; Tanner and Bee, 2020b).

The first test was always a static test that paired the low-PN call (22 pulses) against the high-PN call (38 pulses) (Fig. 1). We used this static choice test as the first test before and after CORT treatment because we have noticed that rarely but occasionally females exhibit slower response latencies in their first but not subsequent tests (Bee, unpublished) and because it provided a means to test and confirm female sexual proceptivity before proceeding with the remaining four experimental tests (rarely females do not complete this static test, and in these instances are not tested further). Furthermore, we used the pre-treatment static trial as a behavioral screen to eliminate females with potentially high endogenous CORT levels. Gall et al. (2019) showed that females with naturally high plasma CORT (> 2 SD above the mean) can be predicted by long choice latencies (>200 s). Because we experimentally elevated CORT levels above their endogenous concentrations, we sought to minimize inclusion of highly elevated CORT females through this screen. Two females were excluded from further testing because they failed this initial screen.

The remaining four tests comprised two pairs of dynamic tests in which one alternative was the average-PN call (30 pulses) and the other alternative was either the low-PN call (22 pulses) or the high-PN call (38 pulses). Hence, in each test, the alternative stimuli differed by the same absolute number of pulses (8) yet the two test types differ in the proportional difference between stimuli. Our design thus incorporates a type of non-linearity described by Weber’s Law and known to be relevant for mating call preference functions in this species (Ward et al., 2013; LaBarbara et al., 2020) and others (Akre et al., 2011). One test within each pair was designated as the experimental test and the other was the control test (Fig. 1). The protocol for both experimental and control tests depended on the behavior of the subject. If the frog initially approached the stimulus with a higher PN by crossing the approach boundary towards it (Fig. 2), then the observer pressed the spacebar on the computer controlling the playback, leading to the activation of a custom program in SIGNAL that introduced a 500-ms delay, and, depending on the type of test, alterations to stimulus presentation. In experimental tests, when a female crossed the approach
boundary toward the higher PN call, a 500-ms delay was followed by a switch of the two stimulus
alternatives between speakers, thereby resulting in the higher PN call being subsequently broadcast from
the opposite side of the arena. In control tests, the same operation was conducted except that the two
stimulus alternatives were simply rebroadcast (following the 500-ms delay) from their original speakers.
This process of call alteration was perceptually smooth for the frog: observers only pressed the spacebar
during the inter-call interval between calls in order to prevent artificially truncating a call, because
interrupting a call can decrease its attractiveness (Henderson and Gerhardt, 2013). In order to minimize
any potential side bias in the chamber or first caller preference (Bosch and Márquez, 2002), we randomly
assigned the order of the four dynamic tests, the order of stimulus onset (leading versus lagging speaker,
both before and after manipulation), and the location of the stimuli (left, right speaker).

As a prerequisite criterion, females were required to initially cross the approach boundary
towards the higher PN call, which happened in almost all cases. In a minority of cases, females initially
approached and chose the lower PN speaker; here we retested the female and she invariably approached
the higher PN call on the second attempt. Thus, each test had one of two outcomes: (1) Non-reversal
choice: the frog crossed the approach boundary towards the higher PN call and then continued on that
trajectory crossing the choice boundary at the near speaker; (2) Reversal choice: the frog crossed the
approach boundary towards the higher PN call and then reversed and crossed the choice boundary of the
far speaker (see Supplementary materials S2 and S3 for representative non-reversal and reversal trials,
respectively). During each dynamic test we additionally measured the following latencies using digital
stopwatches: (1) latency to exit the origin (‘origin latency’); (2) latency to cross an approach boundary
(‘approach latency’); and (3) latency to cross the choice boundary (‘choice latency’). Latencies were used
as one measure of proceptivity.

CORT injections

We prepared CORT injection solutions by dissolving crystalline corticosterone (HPLC grade, Sigma Cat.
No. 27840) in 1 mL of 95% EtOH and vortexing until dissolved. We then diluted this solution in 10 mL.
of sesame oil (Sigma, Cat. No. S3547), vortexed and heated it in an incubator to evaporate the EtOH. This stock solution was prepared fresh every 3 days and aliquots of each fresh solution were frozen and processed by ELISA to ensure injected concentrations were accurate and precise. The stock solution was serially diluted each day at three concentrations. Frogs were injected i.p. using a 30-gauge insulin syringe (BD Micro-fine U-100, 0.3 mL). We used five CORT treatment groups. These included two control groups: (a) no injection control (N = 21); (b) vehicle injection control (sesame oil; N = 21); and three experimental groups: (c) Low CORT (20 ng g\(^{-1}\); 2 ng uL\(^{-1}\); N = 22); (d) Medium CORT (60 ng g\(^{-1}\); 6 ng uL\(^{-1}\); N = 21); and (e) High CORT (180 ng g\(^{-1}\); 18 ng uL\(^{-1}\); N = 22). All injections were 10 uL per gram frog, rounded to nearest gram (e.g. a 6.49 g female received a 60 uL injection). This resulted in an average female receiving a 50 uL injection that contained the following amounts of exogenous corticosterone: 100 ng (Low CORT), 300 ng (Medium CORT), and 900 ng (High CORT). These dosages and the experimental timeline resulted in consistent elevations in circulating CORT in a dose-dependent manner that was typically within the natural physiological range (1-124 ng mL\(^{-1}\); Gall et al., 2019). Prior to the experimental component of this study we validated these and alternative dosages and timelines (see Supplementary materials S1).

Blood sampling

Immediately following the completion of post-treatment behavioral testing, we collected whole blood via cardiac puncture—a technique that we have used successfully in gray treefrogs without adverse health effects (Baugh et al., 2019; Gall et al., 2019; Bastien et al., 2018). Briefly, we rapidly (< 3 min) collected blood (ca. 50 µL) using a 30-gauge insulin syringe (BD Micro-fine U-100, 0.3 mL) pre-rinsed with heparin. We then centrifuged whole blood (7500 RPM for 10 min; Eppendorf 5418 at 8° C) and stored the plasma fraction at -20° C for 3 weeks and then shipped the samples on dry ice to Swarthmore College where they were stored at -80° C for 7 days until assayed. Gall et al. (2019) demonstrated that the transport and holding procedures described here do not impact plasma CORT concentration in these populations.
Steroid extraction and reconstitution

We have previously validated all the hormone methods for this species, including recovery determination, parallelism, and optimal dilution (see Gall et al., 2019). We used a liquid diethyl ether extraction method that has proven effective for small volumes of plasma and results in high recoveries (see Baugh et al., 2012b), including in frogs (Baugh et al., 2018; Bastien et al., 2018; Gall et al., 2019). Our validations indicated that 5 µL of plasma is a sufficient volume to accurately and precisely quantify CORT in this species. Plasma samples were vortexed prior to subsampling and then added to borosilicate vials. Next, 200 µL of RO water was added to each vial in order to increase the aqueous volume for ease of decanting. We then added 2 mL of diethyl-ether to each vial and thoroughly vortexed and then froze the aqueous layer on a slurry of dry ice and methanol. The organic layer was decanted to a new borosilicate vial and the aqueous layer was allowed to thaw; this extraction process was repeated a second time. The ether extracts were then dried for 20 min using a Speedvac centrifuge at 37°C (Thermo Fisher Savant Speedvac SPD1010) and resuspended in assay buffer (supplied by kit) at a 1:40 dilution and allowed to reconstitute overnight at 4°C.

Enzyme immunoassays

We estimated steroid concentrations using commercial EIA kits (DetectX® kits, Arbor Assays) for plasma corticosterone (Cat. No. K014, Donkey anti-Sheep IgG). Reconstituted samples and kit reagents were allowed to reach room temperature prior to use and samples were vortexed prior to plating. Following methods described in Gall et al. (2019), samples were randomly assigned to wells and assayed in duplicate along with blanks, standards, stripped samples (see Delehanty et al., 2015), and stripped/spiked samples. Samples were assayed following manufacturer instructions. Briefly, 50 µL of sample or standard were plated into wells along with conjugate and antibody. Plates were then placed on an orbital shaker (500 RPM) at room temperature for 1 hour and then washed four times with wash buffer (supplied by kit). Substrate was then added and the plate was incubated at room temperature for 30 min
without shaking. The reaction was stopped and optical densities were read at 450 nm on a VersaMax microplate reader with SoftMax Pro software using a four-parameter curve fitting equation (Molecular Devices). Intra- and inter-assay coefficients of variation (CV) were estimated by including three stripped and spiked samples per plate, thereby incorporating cumulative technical error during extraction and assaying (see Gall et al., 2019). We accepted the average of duplicate wells. 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 as follows: 100% for corticosterone, 12.3% for desoxycorticosterone, 0.62% for aldosterone, 0.38% for cortisol.

**Statistics**

We used SPSS® (Version 21, IBM) and SAS® (Version 9, SAS Institute Inc.) for statistical analyses. Plasma CORT concentrations were square root-transformed to improve the normality of error distributions. We used general linear models (GLM) with planned post-hoc comparisons to evaluate the effect of CORT treatment group on plasma CORT concentrations. We used binomial exact tests and McNemar’s tests (both two-tailed) to evaluate the frequencies of preferences (choices and approach boundaries) at among-individual and within-individual levels, respectively. We estimated effect sizes for effects in GLM and GLMM as eta-squared ($\eta^2$) or partial eta-squared ($\eta_p^2$) and for pairwise comparisons for continuous response variables as Cohen’s $d$.

For the analysis of choosiness in dynamic tests, we used the GLIMMIX procedure in SAS to conduct generalized linear mixed models (GLMM) with a logistic link function to evaluate how reversal/non-reversal frequencies were influenced by factors at one within-individual level (timepoint: pre-treatment, post-treatment) and two among-individual factors (CORT treatment group and acoustic condition, i.e., low-PN versus average-PN and average-PN versus high-PN). We omitted the two dynamic control tests from this model because there were so few reversals (1.4%). Planned post-hoc contrasts permitted tests of three assumptions. First, testing for variation in reversal frequencies among the CORT treatment groups at the pre-treatment timepoint evaluates if there was sampling error in the random
assignment of females to CORT treatment groups. Second, evaluating the pre-treatment versus post-treatment timepoint for the no injection control group indicates if there was an effect of handling and a 30-min hold on reversal frequency. Third, evaluating the pre-treatment versus post-treatment timepoint for the vehicle treatment group indicates if there is an effect of the injection per se on reversals.

RESULTS AND DISCUSSION

Assumptions and validations

We found no differences among CORT treatment groups for extraneous variables that we randomized yet could not experimentally control, including day, time of day of testing, body mass, TFL and RBM (all p>0.4). Our dosages and experimental timeline generated consistent and predictable variation in plasma CORT concentrations among CORT treatment groups (omnibus model: $F_{4,100}=33.3$, $p<0.0001$, $\eta^2=0.57$, N=21/treatment; one sample from both the Low CORT and the High CORT treatment groups had inadequate plasma volumes), which largely fell within the physiological range (Fig. 3). Gall et al. (2019) established that the endogenous range of plasma CORT in identically treated females from these same populations was large (1-124 ng mL$^{-1}$) and nearly identical to the endogenous range of plasma CORT from amplexant females in these populations that were immediately bled in the field upon collection (1-123 ng mL$^{-1}$). Though elevated due to the exogenous injections, the range in the present study was similar (1-282 ng mL$^{-1}$). Planned post-hoc comparisons of plasma CORT levels indicated the following: (i) no difference between the two control treatments (no inject vs. vehicle; $p=0.94$, Cohen’s $d=0.27$); (ii) significant differences between each of the two controls and each of the three CORT groups (all $p<0.01$, all Cohen’s $d>0.93$); and (iii) between each of the CORT groups (all $p<0.001$, all Cohen’s $d>1.12$), with the exception of a non-significant difference between the Low CORT and Medium CORT treatments ($p=0.06$, Cohen’s $d=0.70$), likely owing to high variability in the latter (Fig. 3). Intra- and inter-assay CVs were 10.2% and 10.4%, respectively. Recovery efficiencies were high and largely invariant (mean ± SE; 85.5 ± 4.2%), and therefore we did not attempt to adjust plasma concentrations for extraction loss.
All trial choice outcomes were confirmed by a naïve and blinded judge and path lengths were estimated from Ethovision by two independent judges (inter-judge agreement: $R^2=0.99$, $p<0.0001$). The crossing of approach boundaries significantly predicted final choice when no stimulus manipulation occurred, validating that this locomotor investment is behaviorally relevant (proportion of females choosing near speaker after crossing approach boundary: range: 92-100%, all $p<0.0001$).

**Proceptivity**

There was no effect of CORT treatment on sexual proceptivity and this negative result was evident in a number of different metrics. First, nearly unanimously females responded by making mate choices in all acoustic conditions, pre-treatment and post-treatment. Because the static (i.e. conventional) trials do not involve stimulus manipulation, they permit us to simply evaluate how likely a female is to begin and complete the canonical phonotaxis response and if that probability hinges on her CORT treatment and timepoint. All but two of the 107 females responded by making a choice pre-treatment and 100% of females responded post-treatment (Fig. 4a), clearly indicating no large suppressive effects on general proceptivity. The lack of an effect of a pharmacological stressor on sexual responsiveness is consistent with an earlier report demonstrating that the application of a physical stressor (sampling blood via cardiac puncture) also has no detectable effect on female mate choice responsiveness in this species (Gall et al., 2019).

Latencies to mate choice are sometimes used as a more subtle proxy for sexual motivation in a variety of taxa (Gerhardt and Huber, 2002; Leary and Baugh, 2020). Here again we found no evidence that CORT treatment had any effect—in the static trials, latencies were short pre-treatment (mean ± SE; origin latency: 12.0 ± 2.5 s; approach boundary latency: 46.0 ± 3.6 s; choice latency: 98.9 ± 7.9 s) and post-treatment (origin latency: 7.2 ± 2.5 s; approach boundary latency: 41.4 ± 3.7 s; choice latency: 91.1 ± 5.0 s). There was a small (5 s) and significant decrease in latency to exit the origin post-treatment compared to pre-treatment (GLM: $F_{1,102}=4.5$, $p=0.036$, $\eta^2_p=0.042$), which may indicate a marginal increase in urgency as a function of remaining fecundity time. These latency measures did not differ
among CORT treatments (treatment×timepoint: origin latency: $F_{4,102}=0.27$, $p=0.9$, $\eta^2_p=0.01$; approach boundary latency: $F_{4,102}=0.15$, $p=0.96$, $\eta^2_p=0.005$; choice latency: $F_{4,102}=0.35$, $p=0.85$, $\eta^2_p=0.01$; Fig. 4b).

Lastly, the correlation between latency to choice in the static trials and the plasma CORT concentration in these females was nearly zero for both pre-treatment and post-treatment timepoints (both $R^2<0.01$, $p > 0.99$). Two previous studies reported a positive correlation between endogenous plasma CORT and choice latencies in *P. pustulosus* (Leary and Baugh, 2020) and the treefrog used in the present study, *H. chrysoscelis* (Gall et al., 2019). Both studies found a positive correlation that appears to be driven by a small number of females with very high endogenous plasma CORT concentrations. There are a number of methodological differences between these prior studies and the current one, including (1) the use of different signal discrimination assays (e.g. inter- versus intraspecific discrimination tests), which are known to reliably affect latencies even within a female across trials (Baugh et al., 2008)—indicating that variation in latencies reflects in part the nature of the acoustic choice task; (2) different arena dimensions and choice boundary distances; (3) the screening method used in the present study aimed to exclude females with high endogenous CORT levels, thereby possibly eliminating the correlation driven by high CORT females. However, this last possibility has some interesting implications because the current study did generate females with very high plasma CORT (high CORT dosage group). Therefore, the lack of a positive correlation in the current study between total plasma CORT (endogenous plus exogenous) and latencies might indicate that a longer time course of circulating CORT (>30 min) is necessary for the expression of any dampening effect. There are many other possibilities, including that an acute spike in injected corticosterone differs in its biological activity than the endogenous steroid, for example due to differences in how it interacts with binding globulins or the differential effects of activation of cytosolic versus membrane-bound receptors on sexual behavior (reviewed in Orchinik and McEwen, 1994), the latter of which can rapidly (< 8 min) modulate sexual behavior in amphibians (Moore and Orchinik, 1991). Taken together, we suggest that phonotaxis latencies in female anurans may
only narrowly reflect variation in stress physiology and sexual motivation and that future efforts would
benefit from examining an extended time course of action for experimentally manipulated GCs.
Together, this set of findings clearly establishes that experimentally elevated CORT, within and
occasionally exceeding the physiological range, has no observable impact on female sexual proceptivity.
This result is in contrast with a recent study in female lizards (Romero-Diaz et al., 2019) which found
diminished proceptive behavior in experimentally elevated CORT treatments. This discrepancy could be
caused by differences in breeding biology (e.g., fecundity time horizon), pharmacology, or behavioral
testing methods as the lizard study evaluated spontaneous (i.e. no stimulus control) female behaviors. We
encourage future studies to experimentally evaluate the effects of elevated GCs on female proceptivity
across a range of taxa varying in breeding biology (e.g., discrete versus protracted fecundity), using
stimulus-response behavioral assays and a range of time courses and pharmacological doses, and routes of
administration including non-invasive methods to manipulate (Dalm et al., 2008; Wong et al., 2008;
Sopinka et al., 2015) and measure GCs (Baugh and Gray-Gaillard, 2020).

Preferences
We evaluated whether the species-typical preference for higher PN calls was influenced by CORT
treatment and timepoint. In static trials (low-PN versus high-PN), the bias for crossing the approach and
the choice boundaries toward the preferred call (high-PN) was statistically significant and equally strong
across each CORT treatment group, both pre-treatment and post-treatment, indicating that this strong
species-typical preference was intact throughout the experiment (all p<0.05, binomial exact tests; Fig. 5).
Further, at the within-subject level (i.e. comparing pre-treatment to post-treatment) there were no
differences for any of the five CORT treatment groups in the frequencies of these initial approaches or
final choices toward the higher PN alternative in the static trials (all p>0.9, McNemar test, Fig. 5). In
dynamic trials (low-PN versus average-PN and average-PN versus high-PN), wherein we can only
evaluate the frequencies of crossing the approach boundary, the species-typical preference for the higher
PN alternative was intact in all five CORT treatment groups at both pre-treatment and post-treatment
timepoints (Supplementary materials S4). In the control tests, wherein stimuli are not modified, we can also evaluate the proportion of females demonstrating a strong preference for the call with the relatively higher PN by both initially approaching and ultimately choosing that speaker. Again, the preference for higher PN calls was intact across CORT treatment groups and both acoustic conditions, and there was no change in that preference strength between pre-treatment and post-treatment timepoints for 9 of 10 comparisons (McNemar’s test; all p>0.05; Supplementary materials S5). The single exception was the vehicle injected group in the average-PN versus high-PN condition, which exhibited a statistically significant decline in the preference for the high-PN call after injection compared to before (p=0.013).

Results from these preference tests indicate that experimentally elevated CORT, within and occasionally exceeding the physiological range, has no observable impact on species-typical female mate preferences. A small handful of studies have examined female mating preferences in relation to GCs (reviewed in Leary and Baugh, 2020), only one of which experimentally manipulated GCs and evaluated female preferences using a stimulus-response design. Davis and Leary (2015) found a dose-dependent diminution by CORT for the normally robust species-typical preference for males with higher call rates. Our results contrast with those findings. We found no evidence, robust (observed final choice behavior in the static test of low-PN versus high-PN) or subtle (initial approach preferences observed in all test conditions), for any influence of exogenous CORT on female mate preferences using stimulus alternatives known to be salient in this population (Ward et al., 2013). There are several differences between Davis and Leary (2015) and the present study that may explain these contrasting findings, including different treefrog species (\textit{H. cinerea} versus \textit{H. chrysoscelis}, respectively); different acoustic parameters tested (call rate versus call duration, respectively); different testing conditions (field versus lab, and same night versus next day testing, respectively); different injection-behavior timelines (post-treatment holds of 1-2 hours versus 30 min, respectively); and different CORT dosages—Davis and Leary (2015) injected much higher concentrations of CORT which was likely necessary to achieve physiologically relevant circulating levels given that they dissolved crystalline CORT in saline, rather than oil. More research is needed across a range of species and breeding strategies to understand if lability in species-typical mate
preferences is modulated by GCs, and we encourage expanding the range of pharmacological methods (e.g. GC receptor antagonists) to do so.

**Choosiness**

We demonstrated that the experimental tradeoff implemented in the present study was effective. Choosier females incurred a time and locomotor effort cost. The latency to choice in dynamic trials with reversals (mean ± SE; 138 ± 12 s) was 77% longer than non-reversal trials (78 ± 5 s). Likewise, the distance traveled (path length), a proxy for locomotor investment, was approximately twice as long in reversal trials (mean ± SE; 616 ± 51 cm) compared to non-reversal trials (300 ± 15 cm).

Reversals were significantly more common in the experimental (dynamic) tests (26.6%; 114 of 428 trials) compared to the control tests (1.4%; 6 of 428 trials; p<0.0001, McNemar test), consistent with the findings of a similar protocol in eastern gray treefrogs (Bastien et al., 2018). In experimental tests, the reversal rate in the low-PN versus average-PN condition (34.6%) was nearly double that of the average-PN versus high-PN condition (18.7%). This finding is consistent with the asymmetric prediction that females both discriminate (Gerhardt, 1994; Gerhardt et al., 1996; Bee, 2008; Ward et al., 2013) and resolve tradeoffs (current study) more strongly against below average PN callers than they prefer high PN callers—a type of nonlinear preference function consistent with predictions from Weber’s law (LaBarbera et al., 2020). Recent work in this species (Tanner and Bee, 2020) indicates that temporally variable signaling from males degrades sexual selection for attractive callers, which is consistent with the less than 100% reversal rate observed in the present study’s dynamic tests.

For most CORT treatments and in both acoustic conditions, reversal rates were slightly lower in the post-treatment compared to the pre-treatment timepoint, suggesting a nominally suppressive effect of the experimental procedure (Fig. 6). For example, this effect can be observed in both acoustic conditions (low-PN versus average-PN and average-PN versus high-PN) for the vehicle group and may indicate that the experience of being injected, the vehicle itself or an interaction between these variables and the mere passage of time (fecundity) slightly inhibits reversal probabilities. This potentially mild suppressive effect
was observed for most treatments and conditions, with one important exception. The Medium CORT treatment exhibited a large increase in reversals following injection, in both acoustic conditions (Fig. 6).

The GLMM revealed a significant main effect of acoustic condition ($F_{1,315} = 14.9$, $p<0.0001$, $\eta^2_p=0.13$), indicating that reversals, though elevated post-treatment compared to pre-treatment in both acoustic test conditions, were expressed at a significantly higher frequency in the low-PN versus average-PN condition compared to the average-PN versus high-PN condition. This result is similar to the earlier findings, yet in this case indicates that female choosiness is modulated by GCs in the same direction (against below average males) as static preferences are expressed. In other words, moderately elevated CORT levels appear to reinforce the preference function that underlies the bias against below average males. Likewise, consistent with our prediction, there was a significant interaction between timepoint (pre-treatment versus post-treatment) and CORT treatment group ($F_{4,315} = 2.6$, $p=0.037$, $\eta^2_p=0.08$). A post-hoc test indicated that this interaction effect was driven exclusively by the increase in reversals after injection in the Medium CORT group ($p=0.007$; Supplementary materials S6), whereas all other treatment groups did not experience a significant change between these two timepoints (all $p>0.05$).

The experimental design also permitted several additional planned contrasts within the GLMM. First, comparison of all five CORT treatment groups (pre-treatment)—these were not significant for any pairwise comparison (all $p>0.13$)—indicated no sampling error in the random assignment of females to CORT treatment groups with respect to choosiness. This is relevant because it is possible that there are significant individual differences (i.e. repeatability) in choosiness, as has been shown in another anuran (Baugh and Ryan, 2009). Second, the no-inject contrast (pre-treatment versus post-treatment) was not significant ($p=0.99$), indicating no effect of handling and a 30-min hold on reversal frequency. Third, the vehicle contrast (pre-treatment versus post-treatment) was not significant ($p=0.44$), indicating no effect of the injection per se on reversals.

Path lengths (distances traveled) were highly variable within and among CORT treatment groups across all test types (Supplementary materials S7). Descriptively, in the dynamic control tests in which
reversals were rare (1.4% of trials), successively higher CORT treatment groups had shorter path lengths, indicating a nominally suppressive effect on locomotion. In the dynamic experimental tests, females in the Medium CORT group exhibited an increase in path length after injection whereas females in the other treatment groups tended to show a decrease, but this merely reflects the higher frequencies of reversals in the Medium CORT group—the static tests (low-PN versus high-PN), which lacked any reversals, showed no pattern across groups. The GLMs indicated that path lengths did not differ significantly among treatment groups for any of these five acoustic conditions ($F_{4, 99-101}=0.4–2.2$; all $p>0.05$, $\eta^2_p=0.02–0.04$; Supplementary materials S7). Therefore, the path length data suggests that CORT does not increase locomotor effort and therefore increase reversals. Instead, the opposite directionality appears present: moderate CORT elevations increase choosiness and this behavior necessitates greater locomotor effort.

This choosiness finding along with the lack of CORT effects on proceptivity and preferences supports the prediction of an inverted-U function, wherein moderate GC elevations enhance performance in this decision-making task. Again, each of these CORT treatment groups maintained the species-typical preference for longer calls pre-treatment and post-treatment. What changed was that females in the Medium CORT group alone increased their choosiness after injection, whereas females in all the other CORT treatment groups remained unimpacted.

Together, the dynamic testing results may indicate that reproductive females occupy a spectrum of choosiness, from merely preferring higher PN males (under invariant signaling circumstances) to evincing strong choosiness towards higher PN males (under dynamic signaling circumstances). This is consistent with the range of choosiness observed in another species. Baugh and Ryan (2009) showed that amongst choosy female túngara frogs there are individual differences from nominal choosiness to highly choosy females—the latter will reverse up to 16 times to pursue the most attractive caller in a looped stimulus manipulation test (Baugh and Ryan, 2009). The current study suggests some of this behavioral variance may be explained by differences in HPA axis activity at reproductive readiness.

**CONCLUSIONS**
The current study represents a comprehensive experimental study of how GCs influence female sexual behavior. We demonstrated that exogenous elevations in CORT have no effect on a female frog’s sexual proceptivity or intraspecific mate preferences for call duration. Likewise, small and large elevations in CORT have no effect on female choosiness, yet a moderate elevation considerably increases choosiness, and this effect is not explained by merely increasing general locomotor behavior. We posit that for seasonal breeders that have tightly constrained fecundity schedules, a buffering of sex drive and species-typical preferences to elevated GCs (congruent with their peak though highly variable GC levels during reproductive readiness) ensures breeding (with conspecifics) at this critical juncture. Proximately, this buffering might be the consequence of differential responsiveness of primary sensory (e.g., auditory pathways; Endepols et al., 2003; Hoke et al., 2004) and motor (e.g., acoustic guidance motor pathways; Hoke et al., 2007) behavior network; Newman, 1999) recruited during more demanding cognitive tasks including when animals temporally update their decisions. This could include limbic and hypothalamic circuits that are implicated in anuran mate choice (Hoke et al., 2005, 2007) and are essential components of the HPA axis. The widespread distribution of receptors (MR, GR) that bind GCs, including in high abundances in the hypothalamus (reviewed in Senft et al., 2016), makes this a compelling area for future research on GC-mediated effects on sexual decision-making. By selectively modulating choosiness GCs may serve to enable condition-dependent tactical plasticity, wherein females in optimal energetic or metabolic states can pursue more costly options. Moreover, the buffering of sexual motivation and species-typical preferences may minimize the deleterious fitness consequences of lost breeding opportunities or mating with low PN males, which has indirect fitness consequences for females owing to the heritability of call duration in gray treefrogs (Welch et al., 1998).

In contrast to the HPA axis, there is more evidence that HPG axis hormones regulate female sexual behavior (Adkins-Regan, 1998). However, here too more experimental work is needed. For example, it is known that gonadal steroids can induce proceptive behavior and species-typical preferences, but often it is unknown whether and how hormone concentrations modulate these preferences.
A rare exception is a study by Lynch et al. (2006) which found that increasing doses of a luteinizing hormone analog elevated female sexual proceptivity, yet do not impact the species-typical preference for the complex call (Lynch et al., 2006). Likewise, in humans estrous hormones impact sexual motivation but intraspecific preferences appear not to be linked to the estrous cycle (Jones et al., 2018). We encourage future studies to simultaneously evaluate the roles of HPA and HPG axis products at greater breadth (e.g., taxa) and depth (e.g. pharmacological manipulations) using decision-making assays that incorporate tradeoffs.

ETHICS
Animal collections were made under Special Permits 23543 and 28347 from the State of Minnesota Department of Natural Resources. This study was approved by the Institutional Animal Care and Use Committee at the University of Minnesota (Protocol 1701-34456A, approved 03-March-2017).

COMPETING INTERESTS
We have no competing interests to declare.

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REFERENCES


FIGURE LEGENDS

**Figure 1.** Experimental design. All CORT treatment groups experienced the same handling and holding procedures, and behavioral tests (randomly ordered). For most testing days, one female was assigned randomly to each of the five CORT treatments. Females were tested in a battery of five two-alternative choice tests both pre-treatment and post-treatment. In static tests, females choose between low-PN and high-PN. Two acoustic conditions (low-PN versus average-PN and average-PN versus high-PN) were also tested using dynamic playbacks, each of which had a control (C) test (no stimulus alteration) and an experimental (E) test (stimuli altered). The mixed within- and among-subjects design allowed each female/treatment to serve as their own control (pre-treatment versus post-treatment). Body measurements and blood were taken following completion of post-treatment behavioral testing. A total of 107 females were tested.

**Figure 2.** The testing arena was located inside an anechoic chamber. Each trial began with the female located in the origin under an acoustically transparent cage. Speakers on the left (S_L) and right (S_R) were surrounded by a choice boundary with a radius of 10 cm. Approach boundaries were located symmetrically at a 65 cm radius from the speakers (35 cm from the origin). A stylized trace of a reversal
(black) and non-reversal (gray) path are depicted. Oscillograms for the average-PN call (30P) and high-PN call (38P) are depicted in their initial (pre-alteration; black) and final (post-alteration; gray) states.

**Figure 3.** Mean (± SE) plasma CORT concentrations per CORT treatment group (N = 21 females/treatment).

**Figure 4.** Proceptivity results. (A) Proportions of females responding by making a choice in the static test (low-PN versus high-PN). There were nearly unanimous responses in all CORT treatments and timepoints except the pre-treatment timepoint for the Low CORT group (21 of 22 females responded) and the Medium CORT group (20 of 21 females responded). (B) Change in latency to choice (post-treatment minus pre-treatment) in the static test by treatment group (mean ± SE). Sample sizes are indicated in parentheses.

**Figure 5.** Preferences for the call having the higher PN in the static trials (low-PN versus high-PN) was intact and not influenced by CORT treatment (Pre versus Post). This result was observed when examining the frequencies of approach boundary crossings toward the high-PN call (A) and the final choice for the high-PN call (B). Dashed lines (two-tailed) indicate cut-off values (p<0.05) for binomial exact tests. Effect sizes are simply the difference between the observed proportions and 0.5, and sample sizes are indicated in parentheses.

**Figure 6.** Choosiness results. Proportion of females exhibiting reversal choices across the five CORT treatments at pre-treatment (Pre) and post-treatment (Post) timepoints, and the low-PN versus average-PN (A) and the average-PN versus high-PN (B) acoustic conditions. The two acoustic test conditions are combined in (C) for summary illustration of the effects of timepoint and hormone treatment. Sample sizes are indicated in parentheses.
transport to lab

field collect pairs
hold overnight at 4°C

randomly assign

No Inject
Vehicle Sesame oil
Low CORT 20 ng g⁻¹
Med CORT 60 ng g⁻¹
High CORT 180 ng g⁻¹

hold 30 min at 20°C

Static Test
22/38

Dynamic Tests
22/30 (C)
30/38 (C)
30/38 (E)

TREATMENT

hold 30 min at 20°C

Static Test
22/38

Dynamic Tests
22/30 (C)
30/38 (C)
30/38 (E)

bleed and measure

N=21
N=21
N=22
N=21

Pre-treatment
Post-treatment