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In your CORT: Corticosterone and its receptors in the brain underlie mate choosiness in female Cope's gray treefrogs (*Hyla chrysoscelis*)☆

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ABSTRACT

Selecting an attractive mate can involve trade-offs related to investment in sampling effort. Glucocorticoids like corticosterone (CORT) are involved in resolving energetic trade-offs. However, CORT is rarely studied in the context of mate choice, despite its elevated levels during reproductive readiness and the energetic transitions that characterize reproduction. Few systems are as well suited as anuran amphibians to evaluate how females resolve energetic trade-offs during mate choice. Phonotaxis tests provide a robust bioassay of mate choice that permit the precise measurement of inter-individual variation in traits such as choosiness–the willingness to pursue the most attractive mate despite costs. In Cope's gray treefrogs (*Hyla chrysoscelis*), females exhibit remarkable variation in circulating CORT as well as choosiness during mate choice, and a moderate dose of exogenous CORT rapidly (<1 h) and reliably induce large increases in choosiness. Here we measured the expression of glucocorticoid (GR) and mineralocorticoid (MR) receptors in the brains of females previously treated with exogenous CORT and tested for mate choosiness. We report a large decrease in GR expression in the hindbrain and midbrain of females that were treated with the moderate dosage of CORT–the same treatment group that exhibited a dramatic increase in choosiness following CORT treatment. This association, however, does not appear to be causal, as only forebrain GR levels, which are not affected by CORT injection, are positively associated with variation in choosiness. No strong effects were found for MR. We discuss these findings and suggest future studies to test the influence of glucocorticoids on mate choice.

1. Introduction

The regulation of female reproductive behavior by catecholamines, neuropeptides, and the hypothalamic-pituitary-gonadal axis (HPG) has received considerable study (reviewed in Adkins-Regan, 1998; Burmeister, 2017). In contrast, the HPA/I (adrenal/interrenal) axis is rarely investigated in this context, though it is likely to be important and to have a complex relationship with behaviors like mate choice (reviewed in Leary and Baugh, 2020). In general, chronically elevated glucocorticoids like cortisol and corticosterone (CORT) are thought to suppress reproduction (reviewed in: Sapolsky et al., 2000; Toufexis et al., 2014), whereas acute increases are associated with reproductive facilitation (Wingfield and Kitaysky, 2002; Moore et al., 2016). As a metabolic hormone, CORT is involved in the prioritization of energetic resources (e.g., glucose) across tissue types (reviewed in Hau et al., 2016; MacDougall-Shackleton et al., 2019), and this homeostatic regulation is known to play a role in fecundity, reproductive investment and mating decisions (Wingfield and Sapolsky, 2003; Cotton et al., 2006; Breuner et al., 2008; Tokarz et al., 2011). Sexual reproduction often involves extreme energetic transitions, especially in females, which may have metabolic demands during the breeding season that are an order of magnitude higher than their reproductive male counterparts (Ryan et al., 1983). This basic observation suggests that metabolic hormones like CORT are implicated in modulating reproductive decision making during this consequential life history chapter.

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The potential for an acute facilitating role of CORT during mating is supported by the observation that it is naturally elevated during peak reproductive readiness and declines rapidly and precipitously immediately following mating (Dauphin-Villemant et al., 1990; Romero, 2002; Bastien et al., 2018; Gall et al., 2019; Amruta et al., 2020). This facilitation, however, must take into account the fact that these naturally elevated levels of CORT are also themselves hypervariable (ca. two orders of magnitude) among females within a population, and even within a single day (Bastien et al., 2018; Gall et al., 2019). Further, complexity might arise via non-linear dose-response (hormone-behavior) relationships (reviewed in: Moore and Jessop, 2003; Hau and Goymann, 2015). For instance, acute and moderately elevated CORT might facilitate reproductive efforts by mobilizing energy stores (e.g. locomotion; Breuner et al., 1998), while extremely elevated levels might suppress behavior (reviewed in: Toufexis et al., 2014), though the mechanisms underlying HPA-HPG interactions remain unclear (reviewed in: Chand and Lovejoy, 2011). Experimental approaches are necessary for understanding the potential consequences of such large amounts of standing variation in HPI activity, including the nature of dose-dependent hormone-behavior relationships.

The strength of preferences during mate choice, and the fidelity with which those preferences are expressed, determines the benefits accrued by the female as well as the strength and direction of sexual selection for male courtship traits (reviewed in Andersson, 1994; Kirkpatrick and Ryan, 1991). The process of female mate choice (reviewed in Phelps et al., 2006), however, involves at least three subcomponents, all of which introduce variation that can be explored at proximate levels. First, females can vary in sexual motivation or *proceptivity*–this can be measured as a female's responsiveness and speed with which she expresses attraction toward male courtship signals (Ward et al., 2013; Romero-Diaz et al., 2019). Second, females can express certain mate *preferences*–biases in their intraspecific mate choices according to an internal preference function that weights certain male traits as more attractive than others, which can lead to consistent patterns of male reproductive skew (Ryan, 1985; Baugh and Ryan, 2011; Ryan et al., 2019; Rosenthal, 2017). Third, there are often intrinsic (e.g., condition-dependent) and extrinsic factors (e.g. environmental circumstances) that introduce trade-offs that have the potential to modulate the fidelity with which even strong 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, and when that dynamic behavior comes at a cost, we refer to this component as *choosiness*. Studying the role of the HPA/I axis on female mate choice would benefit from experimental approaches that separate these three components.

Previous research that has examined how CORT impacts female mating behavior has largely focused on proceptivity in seasonally breeding species–organisms with brief reproductive windows. These studies, primarily in frogs and lizards, suggest that elevated CORT does not suppress proceptivity, although there may be some subtle effects (Davis and Leary, 2015; Bastien et al., 2018; Gall et al., 2019; Romero-Diaz et al., 2019). The lack of reproductive suppression may indicate that seasonal breeders are buffered against naturally elevated CORT at this critical juncture (Gall et al., 2019). While some previous work has explored how plasma CORT may relate to female mating preferences (Davis and Leary, 2015; reviewed in Leary and Baugh, 2020), the study of the endocrine basis of female mate choosiness is a nascent field. 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 decisions (Keinan, 1987; Lenow et al., 2017). Interestingly, males and females can differ in how decision making

trade-offs 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). Moreover, environmental challenges, such as diminished food availability and predation risk, which are known to stimulate the HPA/I axis and suppress the HPG axis (Wingfield and Sapolsky, 2003), have been associated with variation in female mate choice (Johnson and Basolo, 2003; Cotton et al., 2006; Willis et al., 2012). This association might suggest that glucocorticoids participate in mediating female mate choice, but animal studies that experimentally manipulate CORT are rare (Davis and Leary, 2015; Baugh et al., 2021) yet necessary to establish causation and further elucidate which elements of this endocrine system are implicated. While concentrations of the ligand (circulating hormones) have often been the target of study in behavioral endocrinology, other elements (e.g., enzymes, receptors) deserve attention. For example, a multilevel study in birds demonstrated that the abundance and distribution of glucocorticoid receptors (glucocorticoid and mineralocorticoid receptors, GR and MR, respectively) explains more of the behavioral variation (exploration) than CORT concentrations (Baugh et al., 2017). This makes sense given the large fluctuation in ligand levels as a function of an animal's current state–the sensitivity on the receiving side, rather than the hypervariable signal, might govern the endocrine system's capacity to modulate outputs such as behavior.

Seasonally breeding frogs provide a potentially powerful non-model system for answering questions about the role of GCs in regulating metabolically demanding sexual behaviors. Frogs have long served as model organisms for elucidating the mechanisms, function, and evolution of mate choice (Gerhardt, 1994, 2001; Ryan, 2001; Gerhardt and Huber, 2002; Wells, 2007). In many species, males aggregate in suitable breeding habitat and form dense choruses, where they produce loud and energetically expensive advertisement calls to attract females for the purpose of mating (Gerhardt, 1994, 2001; Gerhardt and Huber, 2002; Taigen and Wells, 1985; Wells and Taigen, 1986). While advertisement calls are species specific, there is considerable within-individual and among-individual variation in calls within a species (Gerhardt, 1991), and this variation is frequently tied to dynamic signaling interactions between males in the chorus (Wells and Schwartz, 2007; Schwartz and Bee, 2013). Females typically respond only to species-specific calls and choose their mate by exhibiting phonotaxis, a locomotor behavior that can involve hopping, walking, swimming, and climbing toward a calling male within the chorus, often over some distance, in the dark, and in structurally complex habitats (e.g., in trees or vegetated wetlands). Moreover, like many other animals (Ryan and Keddy-Hector, 1992), female frogs are often selective for males that produce calls with preferred traits, such as longer durations (Gerhardt et al., 1996). Female preferences and phonotaxis behavior can be reliably studied in the laboratory using robust and repeated behavioral assays that involve presenting acoustic stimuli to gravid females (Gerhardt, 1994). Previous work in multiple anurans demonstrates that females are sensitive to dynamic alterations in male calling behavior in real time and adjust their mate choice dynamically during the execution of phonotaxis (Gerhardt et al., 1996; Bastien et al., 2018; Baugh and Ryan, 2009, 2010a, 2010b, 2010c). This dynamic mate choice, or 'temporal updating,' however, comes at a cost. Females exhibiting it incur a time and locomotor energy cost. These costs can be substantial. In a recent study of Cope's gray treefrog (*Hyla chrysoscelis*), for example, females exhibiting temporal updating experienced on average a > 100 % increase in distance traveled and a 77 % increase in time spent approaching males (see Baugh et al., 2021). Hence, dynamic mate choice assays provide an opportunity to examine a female's energetic investment in selecting the (currently) most attractive male as a mate.

In Baugh et al. (2021) we examined this tradeoff in Cope's gray treefrog by experimentally manipulating plasma CORT in wild caught females and testing them before and after this manipulation. That study revealed an inverted U-shaped dose-response relationship wherein females that experienced a moderate increase in circulating CORT exhibited a > 100 % increase in choosiness, whereas the other four treat-

ment groups experienced no change in choosiness. Moreover, these experimental CORT effects were specific to choosiness (probability of mate choice reversal), whereas sexual proceptivity and mate preferences (for higher pulse number call alternatives) were unimpacted by treatment. This is interesting because proceptivity and preference, unlike choosiness, likely do not involve energetic trade-offs. Here, we extend that earlier work by examining the effects of this CORT treatment on the expression of glucocorticoid (GR) and mineralocorticoid (MR) receptors in subdivisions of the brain (using qPCR), and what these receptor expression results may tell us about HPI axis activity and female mate choice behavior. Specifically, we examine whether CORT treatment impacts the expression of GR and MR across the brain and subsequently, whether expression within and across treatment groups correlates with female choosiness behavior. The anuran torus semicircularis (TS, homologous to the mammalian inferior colliculus) is a midbrain region that serves as a sensorimotor interface important for the integration of acoustic mating signals and subsequent behavioral responses (reviewed in Bass et al., 2005; Hoke et al., 2004; Wilczynski and Endepols, 2007). Given its role in guiding acoustically-mediated phonotaxis behavior, we predict that GR and MR expression in the midbrain might be correlated with female behavior.

2. Materials and methods

2.1. Animals and experimental design

A full description of the animal testing components of this study can be found in Baugh et al. (2021). Briefly, in June of 2018 and 2019, we collected mating pairs of the western genetic lineage of Cope's gray treefrog (Booker et al., 2022) from wetlands located in the Carver Park Reserve (Carver County, MN), the Crow-Hassan Park Reserve (Hennepin County, MN), and the Hyland Lake Park Reserve (Hennepin County, MN). Amplexed pairs caught in the field were transported in small plastic containers to the lab at the University of Minnesota, where all behavioral testing and CORT manipulations took place. All pairs were maintained at approximately 4 °C until the following day when they were tested.

On the day of testing each mated pair was placed in an incubator set to 20 °C for 30 min (see Fig. 1). Females were then separated from male mates and subjected to a battery of phonotaxis tests (pre-treatment) before receiving one of five hormone manipulations [no injection, vehicle injection (vehicle = sesame oil), low CORT (20 ng g^{-1}), medium CORT (60 ng g^{-1}), or high CORT injection (180 ng g^{-1})]. Animals were then held in the incubator for 30 min before undergoing a second round of phonotaxis testing (post-treatment). These dosages, route of administration, and timeline were chosen following a validation study (see Baugh et al., 2021). Following the second round of behavioral testing, a blood sample was collected for CORT measurement. Animals (N = 107) were then quickly euthanized 60 min post injection and brains were collected for receptor expression analysis (see Fig. 1).

2.2. Behavioral testing

For a full description of behavioral testing methods see Baugh et al. (2021). Briefly, we tested females in two speaker playback phonotaxis assays that simulated two antiphonally calling males differing in the number of pulses in their calls. Earlier work in this system has established that females prefer males that produce calls with more pulses (Gerhardt et al., 1996; Bee, 2008; Ward et al., 2013). Our phonotaxis assays examined female preferences and choosiness for longer calls having more pulses (Fig. 2). The choosiness tests included two trials with different stimulus contrasts (22 versus 30 pulses/call; 30 versus 38 pulses/call) conducted before and after CORT treatment (see Fig. 1). In each of these trials the female was released at the midpoint between two speakers separated by 2 m that alternately broadcast two stimuli differing in pulse number (PN) (e.g. the right speaker broadcasting a 38 pulse call and the left speaker broadcasting the 30 pulse call). If the female initially approached the stimulus with a higher PN by crossing the approach boundary toward it (Fig. 2), then the positions of the two stimulus alternatives were switched between speakers, thereby resulting in the higher PN call being subsequently broadcast from the opposite side of the test arena. As a prerequisite criterion, females were required to initially cross the approach boundary toward 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 toward the higher PN call and then continued on that trajectory after the switch and chose the lower PN call; (2) *reversal* choice: the frog crossed the approach boundary toward the higher PN call and then reversed course after the switch and chose the higher PN call coming

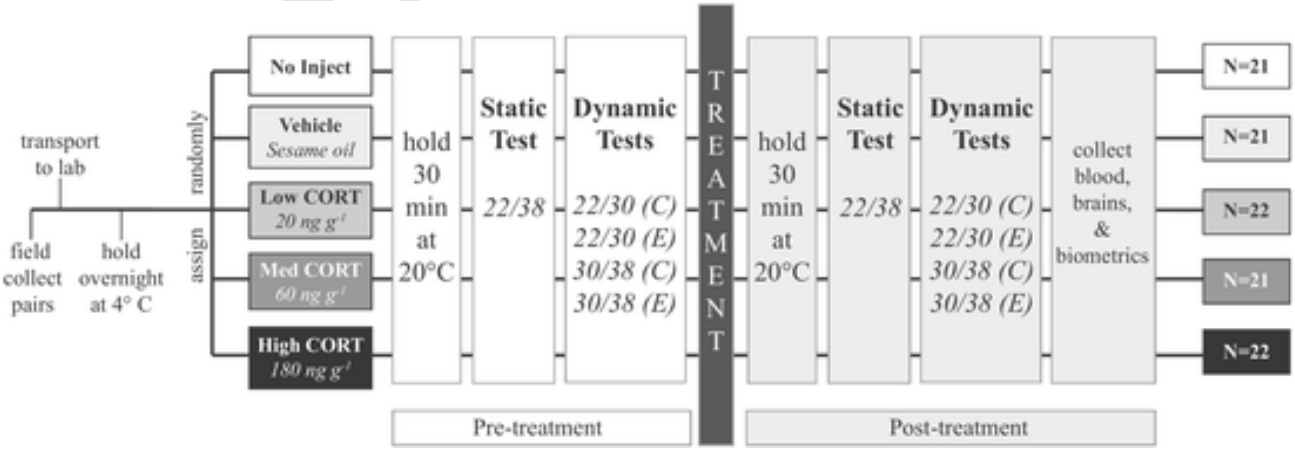

Fig. 1. Experimental design. All CORT treatment groups experienced the same handling and holding procedures, and behavioral tests (randomly ordered). For most testing days, one female was assigned randomly to each of the five CORT treatments. Females were tested in a battery of five two-alternative choice 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 posttreatment). Body measurements and blood were taken following completion of posttreatment behavioral testing. A total of 107 females were tested. Reprinted from Baugh et al. (2021) with permission.

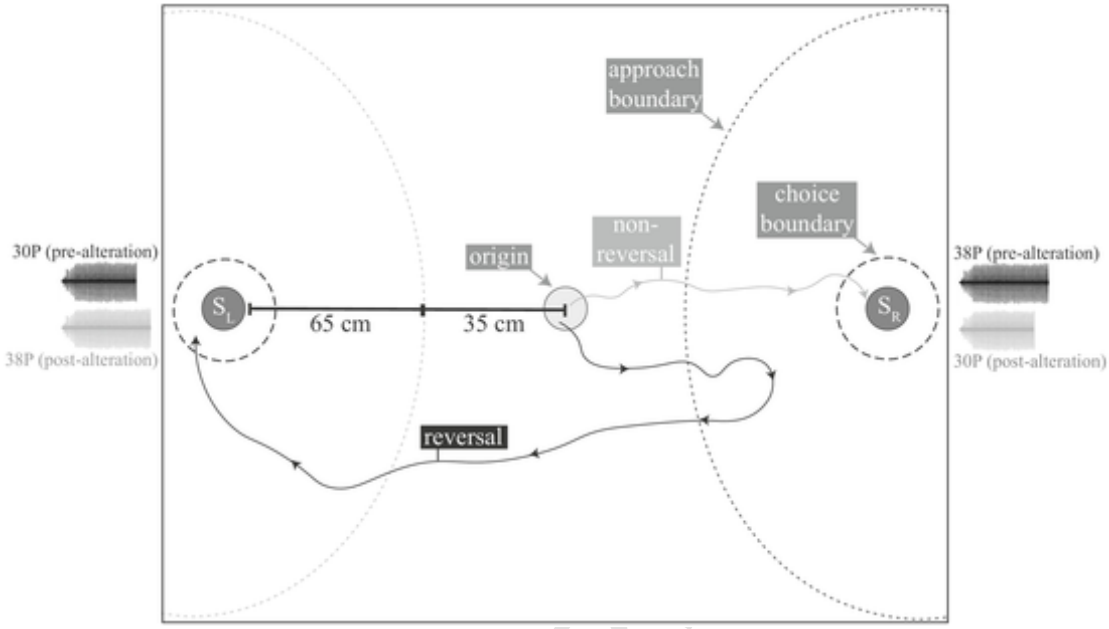

Fig. 2. The testing arena was located inside an anechoic chamber. Each trial began with the female located in the origin under an acoustically transparent cage. Speakers on the left (SL) and right (SR) were surrounded by a choice boundary with a radius of 10 cm. Approach boundaries were located symmetrically 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. Reprinted from Baugh et al. (2021) with permission.

from the opposite speaker. Reversal frequencies are used as an operationalized measure of choosiness (higher proportion of trials with reversals indicates a choosier female). 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'). Thus, both of these latencies measures precede playback manipulation and therefore are not confounded by whether the female reversed or not. Latencies are used as a measure of sexual proceptivity, and variation in latencies largely reflects the wait time prior to initiating phonotaxis, rather than the speed of movement during phonotaxis.

2.3. Tissue collection and storage

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.

Animals were then euthanized with an application of benzocaine to the ventral surface of the body followed by rapid decapitation. Brains were rapidly extracted on ice and placed in 1.5 mL centrifuge tubes containing RNAlater (Thermo Fisher Scientific). Brains were then stored at −20 °C at the University of Minnesota for up to two weeks and then shipped to Vassar College on dry ice and stored at −80 °C until further processing. Prior to RNA extraction, brains were trisected with sterile, RNaseZap (Thermo Fisher Scientific) treated double-edged razor blades. We trisected the brain into forebrain (telencephalon + diencephalon), midbrain (mesencephalon including the optic tectum and tegmentum), and hindbrain (brainstem and cerebellum) divisions. A coronal cut was made immediately posterior to the optic tectum and anterior to the cerebellum to separate the hindbrain division. A second dorsal-ventral cut was made from just anterior to the optic tectum (dorsal) to just post posterior to the hypothalamus (ventral). Trisected brains were placed into individual centrifuge tubes containing RNAlater and shipped overnight on ice to Colorado State University for RNA extraction, cDNA synthesis and qPCR.

2.4. Enzyme immunoassays for plasma CORT

For a full description of laboratory methods see Baugh et al. (2021). Briefly we used a commercial EIA kit (DetectX® kits, Arbor Assays) with all samples processed in duplicate wells using a validated protocol that we have developed for this species.

2.5. RNA extraction, cDNA synthesis and qPCR validation

Total RNA was isolated using Trizol reagent (Invitrogen) following the manufacturer's protocol. Following extraction and purification, samples were assayed on a nanodrop and only samples with 260/280 nm ratios >1.8 were used in further steps. cDNA libraries were generated from 1 μg of total RNA using ProtoScript II First Strand cDNA synthesis kits (New England Biological). Quantitative PCR was performed with iTaq Universal SYBR Green Supermix (BioRad) for two housekeeping genes (GAPDH and β-actin), GR, and MR. Housekeeping gene primers were designed based on available gene sequences from a brain transcriptome in *Hyla cinerea*. GR and MR gene sequences were obtained from *H. chrysoscelis* transcriptome sequences aligned to the reference genome available for *Nanorana parkeri* (*gr:* XM_018562858.1, *mr:* XM_018554260.1). Primers were designed to flank exon-exon boundaries, product presence was visualized using gel electrophoresis and each primer pair was observed to only have a single melt curve peak (see SM1 for primer sequences). For each sample, gene expression was measured in triplicate and amplification efficiencies for each

primer pair were determined using standard curves made from serial cDNA dilutions. All qPCR samples were randomly coded to ensure their handling was blind to treatment and identity.

The fold expression of GR normalized to both Actin and GAPDH demonstrated significant positive linear correlations, as did MR, indicating agreement between the two housekeeping genes (SM2). Furthermore, across brain divisions and receptors, there were many significant linear correlations (SM3). This is not surprising, as receptor abundances across tissues within an individual have been shown to exhibit positive correlations in sparrows (Lattin et al., 2015). Moreover, the strength of these correlations varies in a predictable manner, with stronger correlations within a receptor type across two adjacent brain divisions and between receptors within a brain divisions. For example, the correlations between hindbrain and midbrain GR levels were strongly, positively correlated, and the correlations between GR and MR within the midbrain were also strong and positive (SM3). This pattern further suggests some consistency in the qPCR methods.

2.6. Statistical analysis

We used the $2^{-\Delta\Delta Ct}$ method to process Cq values to calculate relative expression of GR and MR in each brain division (Livak and Schmittgen, 2001). This method yields relative abundances (hereafter 'fold') that quantify receptor levels against that of each of the two housekeeping genes (β-actin, GAPDH). All fold values were $\log_{10}$-transformed and plasma CORT concentrations were square root-transformed to yield Gaussian residuals. A small number of housekeeping gene Cq values were very high (i.e. very low RNA concentrations). When the Cq of the housekeeping gene is more than the target gene (MR or GR), this results in extreme outliers for the calculated fold values, which strongly impacts fold distribution and model leverage. We therefore omitted these high housekeeping gene Cq values (11 GAPDH samples, 16 β-actin samples), which resulted in the loss of six MR and six GR estimates (1.8 % of receptor estimates; two frogs' hindbrain MR/GR, four frogs' midbrain MR/GR) due to these high Cq values being observed for *both* housekeeping genes for these samples. A small number of samples exhibited variable Cq values among replicate wells, with coefficients of variation (CV) among triplicate wells that exceeded 3 %. In most cases a single well was a clear outlier and could be omitted, thus accepting the average of duplicate wells (with CV $<$ 3 %). In a small number of samples (23; 1.9 % of all samples) duplicate wells continued to have CV values that exceed 3 % (mean CV: 7.1 %; range CV: 3.0–48.9 %). Given the small number of such samples, we elected to retain these samples in all final analyses and examined if their inclusion/exclusion qualitatively influenced the results.

We used four linear mixed effects models (LMM) to test for CORT treatment effects on MR and GR expression—one for each receptor-housekeeping gene combination. Each model had two fixed factors (treatment group; brain division) and one random factor (subject ID). An additional four models were constructed with the addition of a random effect to designate samples as either exceeding 3 % CV among wells in the qPCR or not. Those models were qualitatively identical to the first models and are thus omitted.

To confirm these qPCR results, we analyzed the differences in expression of both housekeeping genes, β-actin and GAPDH, as well as GR and MR across all treatment groups and brain divisions by fitting a generalized mixed effect model with Poisson-lognormal distribution and a Bayesian Markov Chain Monte Carlo sampling approach. These analyses were carried out using RStudio 2022.07.1 and the MCMC.qpcr (Matz et al., 2013) package. This statistical approach accounts for random variation between triplicate samples, increased power by analyzing data for all target genes in one model, and it does not require housekeeping genes. The package first converts all raw Cq values into molecule count data with consideration of the amplification efficiency of each gene. We then fit three models - (1) a one-way model looking at CORT treatment effects on gene expression across all brain divisions (SM10), (2) a two-way generalized linear mixed model (GLMM) comparing the effect of CORT treatment on gene expression across brain divisions with no housekeeping gene specified (SM11B) and (3) a two-way GLMM with housekeeping genes specified (SM11A).

To model the relationship of receptor levels and choosiness (number of reversals), we used generalized linear mixed effects models (GLMM) with the number of reversals (events) as the response variable. We collapsed across the two acoustic conditions within each timepoint. Hence each female could have 0, 1 or 2 reversals (out of a denominator of 2) at pre- and post-treatment timepoints. We used a binary logistic regression for the distribution and link function to the linear model, and fitted a random effect for subjects with the intercept included in the model. Given the large number of fixed effects and interaction terms, we used a backwards elimination model selection approach with an initial full factorial model with all terms (treatment group, pre/post-treatment time-point, and each of the brain division specific receptor fold values for a single housekeeping gene) and all two-way interactions. The term with the largest non-significant *p*-value was eliminated sequentially and re-run until only significant *p*-values remained. We performed this GLMM model selection process for each housekeeping gene. Lastly, we used linear regression analysis to evaluate the relationship between the change in choosiness (post-treatment minus pre-treatment) versus plasma CORT ($\log_{10}$) in the medium CORT group.

For latency behavior, we constructed LMMs (origin latency and decision boundary latency as dependent variables in separate LMMs) with fixed effects for pre/post and treatment group with each model having a single covariate for a brain division-receptor-housekeeping gene combination (e.g. Model 1: forebrain GR_{actin}). This yielded 12 models total, each one of them with a full factorial design. Similarly, we constructed four models with three covariates (all three brain divisions for a given receptor (e.g. Model 1: forebrain GR_{Actin}, midbrain GR_{Actin}, and hindbrain GR_{Actin}).

To examine the relationships between plasma CORT levels and GR/MR expression, we used bivariate Pearson's correlations (with 95 % CI bounds) on fold receptor levels (for each of the two housekeeping genes) against plasma CORT (square root-transformed). These are within-group correlations. All statistical analysis was performed using SPSS (version 28; IBM) and all residual errors were checked visually for normality.

3. Results

3.1. Behavior

We have described the behavioral effects in detail in Baugh et al. (2021). Briefly, the medium CORT treatment doubled the number of reversals after treatment compared to before. All other treatment groups exhibited no change or a nominal decline in reversals. This behavioral effect was replicated across two separate years and two acoustic stimulus contrasts.

3.2. Validations

3.2.1. Plasma and behavior

We describe the validations of treatment dosages, timelines and behavioral methods in detail in Baugh et al. (2021). Briefly, the two control treatments (no injection and vehicle) resulted in similarly low concentrations of plasma CORT approximating the population average for unmanipulated amplexed females sampled in the field. In contrast, low, medium and high CORT dosages caused a stepwise increase in plasma CORT within the physiological range (Fig. 3).

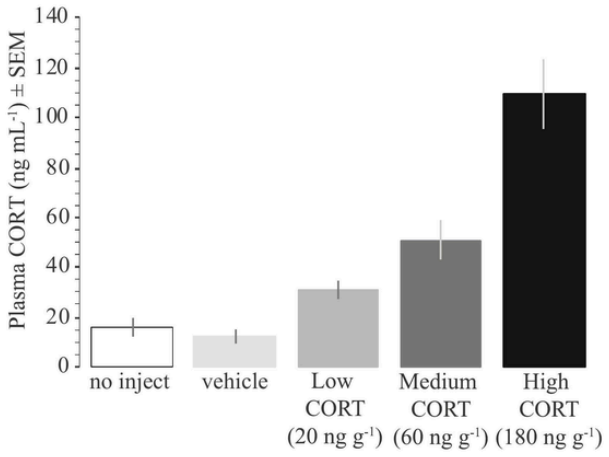

Fig. 3. Mean (±SE) plasma CORT concentrations per treatment group. Reprinted from Baugh et al. (2021) with permission. Our dosages and experimental timeline generated consistent and predictable variation in plasma CORT concentrations among CORT treatment groups (omnibus model: F4,100 = 33.3, p < 0.0001, η2 = 0.57, N = 21 females/treatment; N = 21/treatment; note that one sample from both the Low CORT and the High CORT treatment groups had inadequate plasma volumes). 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 effect between the Low CORT and Medium CORT treatments (p = 0.06, Cohen's d = 0.70).

3.2.2. Housekeeping genes

Expression of both housekeeping genes (Cq) were influenced by treatment (SM4, SM5). The LMM for Actin (Cq) demonstrated significant effects of treatment group ($F_{4,286}$ = 9.06, p < 0.001) and for brain division ($F_{2,286}$ = 46.5, p < 0.001), though no interaction effect ($F_{8,286}$ = 1.64, p < 0.11). Similarly, the LMM for GAPDH (Cq) demonstrated a significant interaction between treatment group and brain division ($F_{8,259}$ = 3.31, p = 0.001; SM5). Hence, though the effect of treatment varied in quantitative ways between the two housekeeping genes, the primary effect was that the CORT treatments generally had higher Cq values (lower mRNA) relative to the two controls. Among CORT treatments the medium CORT group tended to have the highest Cq (i.e. lowest mRNA levels). These results were corroborated with Bayesian models of housekeeping gene expression across CORT treatment (SM11).

3.3. Effect of experimental CORT treatments on GR and MR expression

3.3.1. GR

We found a significant interaction between treatment and brain division on GR expression (*actin*: $F_{8,273}$ = 4.47, p < 0.001; *gapdh*: $F_{8,253}$ = 5.7, p < 0.001; Fig. 4, SM6). The significant interaction was because GR varied among treatment groups in the midbrain (*actin*: $F_{4,273}$ = 7.13, p < 0.001, *gapdh*: $F_{4,253}$ = 10.1, p < 0.001) and hindbrain (*actin*: $F_{4,273}$ = 4.32, p < 0.001; *gapdh*: $F_{4,253}$ = 4.67, p = 0.001) but not forebrain (*actin*: $F_{4,273}$ = 2.13, p = 0.08, *gapdh*: $F_{4,253}$ = 1.19, p = 0.32) based on the univariate F-tests for within each brain division. Hence, hindbrain and midbrain GR changed as a function of CORT treatment, with the lowest GR values observed in the medium CORT group. In the midbrain, the two GR-housekeeping gene models were similar - increasing CORT dosages causes in an inverted U-shaped GR pattern with the lowest GR levels in response to medium CORT. For GR in the hindbrain, patterns of expression normalizing against the two housekeeping genes were consistent and showed no difference in GR levels between the two control treatments along with a selective and large decrease in GR in the medium CORT treatment (Fig. 4). In the forebrain, both GR-housekeeping gene models agreed that treatment did not influence GR expression. Forebrain GR levels were stable in response to the treatment manipulation.

3.3.2. MR

We found a significant interaction between treatment group and brain division on MR expression (*actin:* $F_{8,265}$ = 5.9, p < 0.001; *gapdh*: $F_{8,272}$ = 2.5, p = 0.01; Fig. 4, SM7). The significant interaction was because MR varied among treatment groups in the midbrain (*actin*: $F_{4,265}$ = 11.9, p < 0.001; *gapdh*: $F_{4,272}$ = 3.24, p = 0.01), while it varied in the forebrain when normalized to actin only ($F_{4,265}$ = 4.32, p = 0.002; *gapdh*: $F_{4,272}$ = 1.15, p = 0.33) but not the hindbrain (*actin:* $F_{4,265}$ = 1.9, p = 0.10; *gapdh*: $F_{4,272}$ = 1.7, p = 0.15) based on the univariate F-tests for within each brain division. However, closer inspection of the data indicates that these patterns were driven largely by differential expression in the no injection and vehicle control groups relative to the CORT groups in the midbrain and forebrain (Fig. 4). In contrast, in the hindbrain, where the interaction term was non-significant, the high CORT treatment stands out with distinctly high MR levels. Hence, forebrain and midbrain MR changed as a function of treatment but this was largely due to variation from the control groups, whereas hindbrain MR exhibited a strong trend with singularly elevated levels in response to high CORT.

3.4. GR and MR expression, CORT and mate choice behavior

3.4.1. Receptor expression and choosiness

Females with higher GR levels in the forebrain exhibited more reversals and this effect was similar for both housekeeping genes. Specifically, the only variable retained during backwards elimination was forebrain GR levels which were positively associated with choosiness (Forebrain GR_{Actin}: $F_{1,192}$ = 2,8, p = 0.09, coefficient = 0.50; Forebrain GR_{gapdh}: $F_{1,188}$ = 4.01, p = 0.047, coefficient = 0.67). All other fixed effects and interactions were not significant (all p > 0.3) indicating that this forebrain GR effect was not dependent upon treatment; further examination of the patterns here suggest that this relationship was mostly present after CORT treatment (Fig. 5). Lastly, within the medium CORT treatment group alone–the only one that experienced a change in GR levels due to CORT–there was a positive trend for females with higher final plasma CORT concentrations (potentially reflecting higher endogenous baseline CORT levels) to exhibit the largest increase in choosiness after treatment (r = 0.32, $F_{1,19}$ = 2.14, p = 0.16; SM8).

3.4.2. Receptor expression and latency behavior

None of these models yielded significant effects of receptor expression and latency behavior. This indicates that both latency to exit the origin and decision boundary latency did not vary among treatment (these findings were previously demonstrated in Baugh et al. (2021) for this dataset without the receptor effects) and brain receptor expression did not explain variation in either behavior. Hence, GR and MR were not implicated in variation in sexual proceptivity in gravid *H. chrysoscelis*.

3.5. Plasma CORT and receptor expression within treatment groups

There were no significant correlations between plasma CORT and receptor expression in either of the control treatments for any of the brain division-housekeeping gene-receptor combinations (all p > 0.05; SM9). In contrast, we found divergent correlations between the medium CORT treatment and the low/high CORT treatments. In the medium CORT group there were significant positive correlations between plasma CORT versus GR (in the hindbrain and midbrain), and

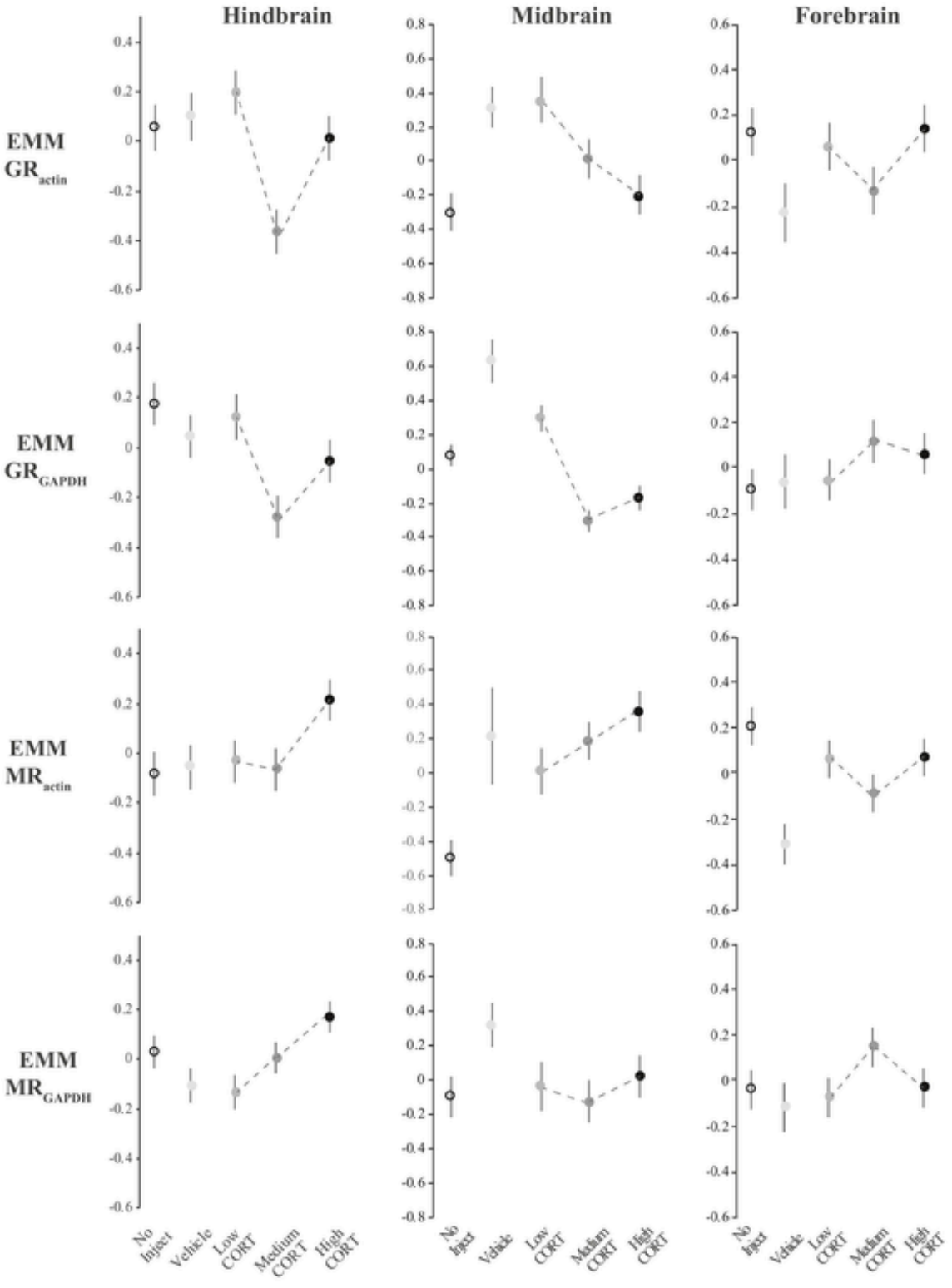

Fig. 4. Estimated marginal means (EMM) (± SE) of fold expression for GR and MR for each brain division-housekeeping gene combination across treatment groups. In the hindbrain and midbrain, GR levels were significantly lower in the medium and high CORT treatment groups when normalized for both housekeeping genes. In the forebrain, these patterns differ between housekeeping gene though there are no significant differences by treatment. MR levels in the hindbrain were significantly higher in the high CORT treatment group when normalized for both housekeeping genes. In the midbrain, there was a significant effect of vehicle injection on MR levels while in the forebrain they only varied when normalized to actin.

this was consistent for both housekeeping gene (midbrain: GR_{gapdh}: r = 0.54, p = 0.016, N = 19; GR_{actin}: r = 0.49, p = 0.033, N = 19; hindbrain: GR_{gapdh}: r = 0.54, p = 0.014, N = 20; GR_{actin}: r = 0.63, p = 0.003, N = 20; Fig. 6). In contrast, the low and high CORT treatments showed significant negative correlations between plasma CORT versus forebrain MR and GR (Low CORT: forebrain: GR_{actin}: r = −0.60, p = 0.006, N = 20; MR_{actin}: r = −0.67, p = 0.001, N = 20; Hi CORT: forebrain: GR_{gapdh}: r = −0.63, p = 0.003, N = 20; MR_{gapdh}: r = −0.63,

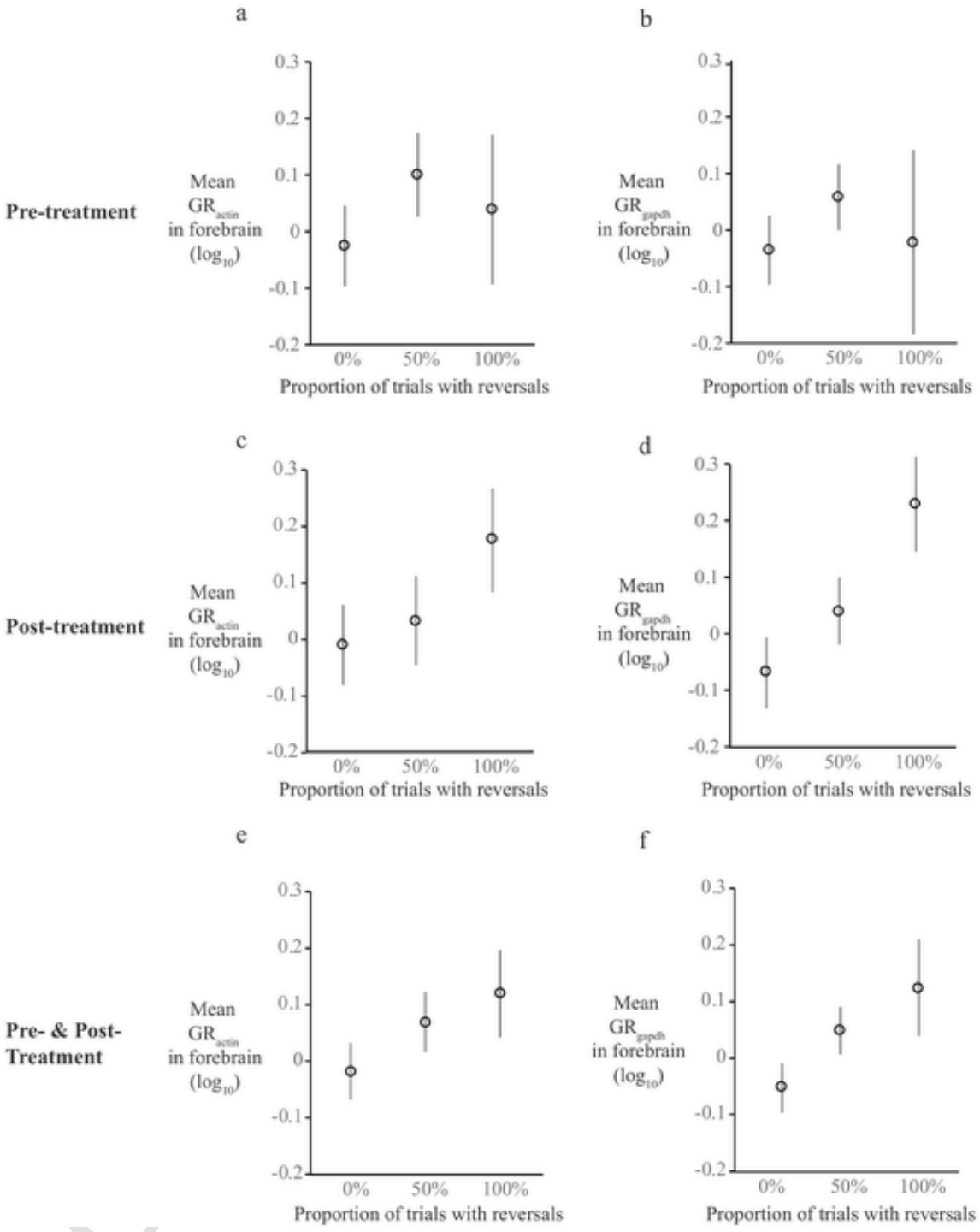

Fig. 5. Fold GR expression (mean ± SE; log_{10}-transformed) in the forebrain in relation to the proportion of trials with reversals. (a) and (b) depict Pre-treatment reversals for Actin and GAPDH normalized GR expression, respectively. (c) and (d) depict Post-treatment reversals for Actin and GAPDH normalized GR expression, respectively. (e) and (f) depict the combined (pre- and post) reversal data.

p = 0.003, N = 20; Fig. 6). Additionally, in the low CORT group there was a significant negative correlation between plasma CORT versus MR in the hindbrain (MR_{actin}: r = −0.45, p = 0.049, N = 20; Fig. 6).

4. Discussion

In Baugh et al. (2021) we demonstrated that female treefrogs that were administered a moderate dose of exogenous CORT experienced a rapid and large increase in choosiness for longer calls, compared to their pretreatment behavior an hour earlier. Here we show that this exogenous CORT also had an effect on levels of glucocorticoid receptor mRNA across the brain, with the medium CORT injection decreasing GR expression in the hindbrain and midbrain compared to no injection and vehicle controls. MR expression was typically elevated by the high CORT injection, though this effect was not consistent across brain divisions. It is important to note that evidence of changes in transcript abundance are not a demonstration of changes in receptor protein levels. Though it seems unlikely, we do not know if these differences in

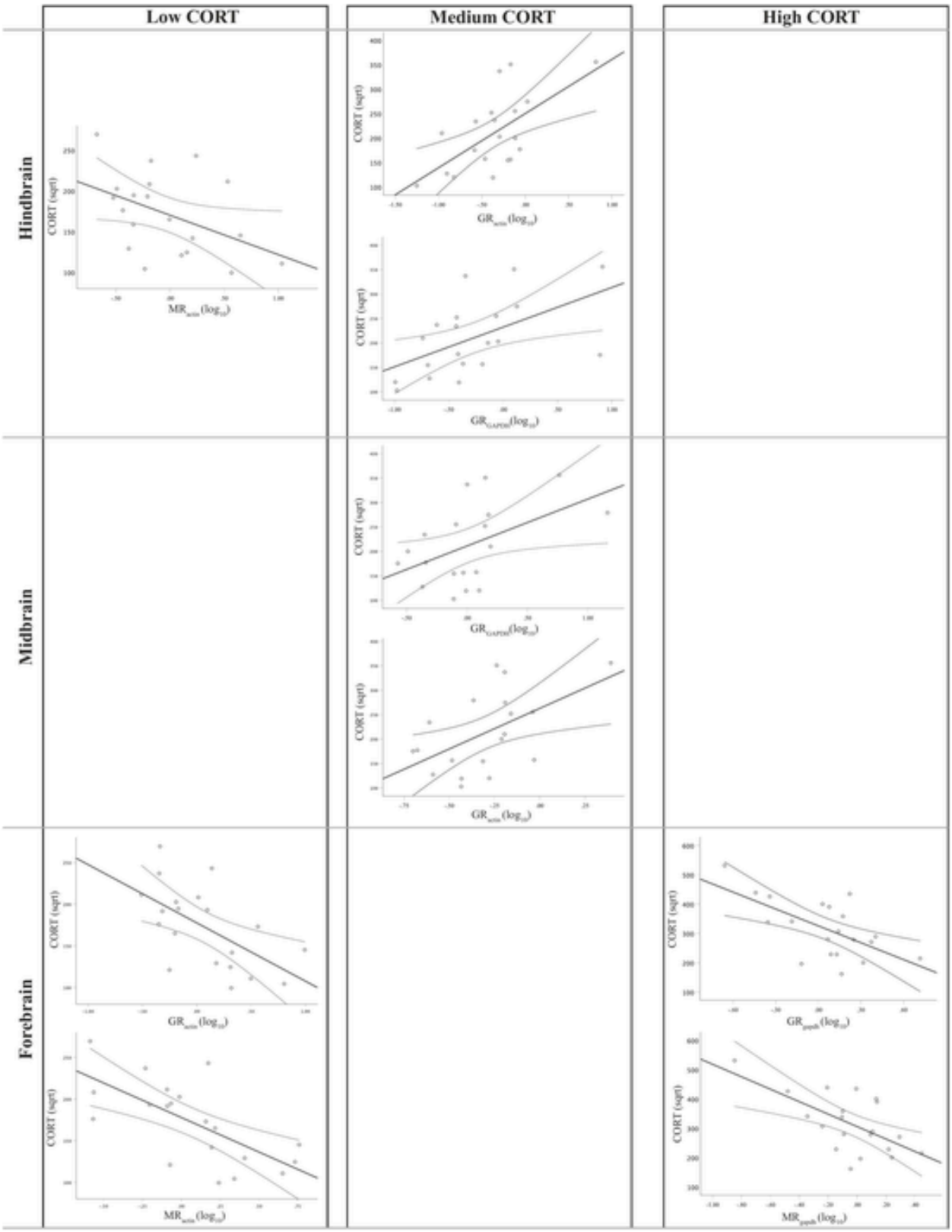

Fig. 6. Depicted are significant bivariate correlations between plasma CORT (square root-transformed) and fold receptor expression (log_{10}-transformed) for each of the three brain divisions within the three CORT treatment groups (no correlations were observed in the two control groups).

mRNA levels translate to protein changes within this ca. 1 h time window. This is important because interpreting any functional (behavioral) implications of changes in receptor transcript abundance is only possible if receptors themselves have changed. Hence, given that CORT treatment influenced GR transcript abundance in the hindbrain and midbrain (but not the forebrain), and given that it is unlikely those rapid receptor transcript changes translate to protein changes, we were not surprised to find a lack of correlation between the degree of choosi-

ness behavior and GR expression in the hindbrain and midbrain. On the other hand, we did identify that forebrain GR expression–which is variable among frogs but stable in response to CORT treatment–was positively correlated with female choosiness.

We also found that only in the medium CORT group, GR levels in the hindbrain and midbrain were consistently positively correlated with plasma CORT levels. This suggests that moderate elevations in circulating CORT can have multiple, potentially interacting effects. For one, it can selectively shape behavioral responses and gene expression independent of each other (at least at short timescales), which is reflected by the fact that GR changes in the hindbrain and midbrain in response to CORT treatment are not correlated with choosiness while stable (presumably endogenous) GR levels in the forebrain are positively correlated with choosiness. These results suggest a potentially interesting underlying mechanism by which endogenous GR levels relate to female choosiness behavior irrespective of the short-term effects of CORT treatment on receptor mRNA abundance.

4.1. CORT treatment effects on the expression of housekeeping genes across brain divisions

The finding that CORT injections drive changes in two conventional housekeeping genes (namely, actin and GAPDH) is relevant for future qPCR studies that involve HPA/I activation. Previous work found that acute and chronic stress changes the expression of both actin and GAPDH transcripts in a brain region-specific manner in multiple species (Bustin, 2002; Proudnikov et al., 2003; Maeda et al., 2005; Bonefeld et al., 2008; Derks et al., 2008; Fernandes et al., 2008). Given the extensive metabolic impacts of elevated GCs perhaps it is not surprising that there are many transcriptional consequences (Austin et al., 2021), including constitutively expressed genes. The fact that CORT treatment drove changes in housekeeping gene abundance makes interpretation of the results complicated, though not impossible. We want to highlight that for actin, CORT treatment significantly decreased expression levels across brain divisions, whereas for GAPDH, expression levels were significantly changed in a brain division-specific manner (SM5). However, in relation to the behavioral results, we found that the forebrain expression of GR correlates with choosiness and that this effect is similar for both housekeeping genes and is not dependent on CORT treatment (Fig. 5).

4.2. CORT treatment influences brain division-specific GR and MR expression

Both medium and high CORT doses significantly decreased GR levels, while for MR, the high CORT treatment significantly increased receptor levels only when normalized against actin housekeeping gene. Closer inspection of the results reveals a consistent trend wherein both housekeeping genes models agree that the high CORT treatment elevated MR levels in the hindbrain (Fig. 4). In the midbrain, both housekeeping gene models agreed that CORT treatment significantly impacted MR levels. That result, however, was driven in part by low MR levels in the no injection treatment and high MR in response to vehicle treatment. A conservative interpretation of this effect is that the stress of injection drives elevated MR in the midbrain. These patterns of expression are further supported by Bayesian models of gene expression (SM11) which show that medium CORT impacted all gene expression, including the abundance of housekeeping genes, and that these effects are brain region specific. In the forebrain, the two MR-housekeeping gene models demonstrated disparate effects–this lack of agreement between models leads to the conservative interpretation that CORT treatment does not impact MR in the forebrain.

Given that, compared to controls, CORT treatments decreased housekeeping gene transcript abundance as shown by a higher Cq value (SM4, SM5), the observed decrease in fold GR levels in the hindbrain and midbrain in response to the medium CORT treatment should reflect a conservative estimate. We interpret this finding as indicating a substantial GR diminishment in response to medium CORT. In contrast, that same decrease in the housekeeping gene presents the opposite problem for interpreting fold MR, which exhibited an apparent increase in response to high CORT in the hindbrain and midbrain; thus, the decrease in the housekeeping gene (reflected by an increase in Cq) alone should increase fold MR. This makes interpreting the MR patterns more challenging. Nevertheless, because the housekeeping genes saw their highest Cq values (lowest mRNA abundance) in response to the medium CORT treatment, whereas it was the high CORT treatment that resulted in the highest fold MR, this suggests that increased MR expression in response to high CORT likely represents a biological effect, though the effect size should be interpreted cautiously.

4.3. Decoupling the effect of medium CORT on choosiness and receptor expression

Rapid effects of hormones, which generally occur on the timescale of seconds to minutes (<1 h), are independent of protein synthesis and act instead upon membrane bound receptors and can modulate processes such as excitatory amino acid release (Gray et al. 2017). Such rapid effects of CORT have been shown to impact sexual behavior (amplexus) in urodeles via membrane bound receptor actions (reviewed in Moore et al., 2005; Reedy et al., 2014). Here we found that an exogenous medium CORT dose rapidly (<1 h) enhanced choosiness during mate choice. This same medium CORT treatment had no effect on GR expression in the forebrain, yet stable or existing GR expression in the forebrain is positively associated with choosiness. Our interpretation of these results is twofold. First, the medium CORT dose could be binding to GR in the forebrain of females that have naturally high forebrain GR protein levels. Thus, these treatment effects could be modulating circuits known to play a role in female mate choice behavior (Walkowiak et al., 1999) through longer term, genomic changes in nuclear GRs. Second, a moderate CORT dose is simultaneously binding to receptors (GR or MR or both) in hindbrain and midbrain cells and downregulating GR expression there. Given the non-lability of forebrain GR expression in response to treatment, the association with choosiness suggests that individual differences in forebrain GR (standing variation) influence female choosiness, with higher forebrain GR potentially increasing choosiness when CORT levels are elevated. Of course, causality in this relationship requires experimental tests.

In the amphibian brain, GR is highly expressed in forebrain regions such as the medial pallium and preoptic area (Yao et al., 2008), which are important for female reproductive behavior. Though less densely expressed, GR and MR are still found widely throughout other brain regions (Denver, 2009), particularly in auditory midbrain regions that are important for regulating the audio-motor integration required for selective phonotaxis in gray treefrogs (Endepols et al., 2003). Here, we found that a medium CORT dose was associated with low GR in the hindbrain and midbrain yet it was females with higher GR expression in the forebrain, not hindbrain or midbrain, who exhibited more choosy behavior. The distinction, therefore, of the among-group patterns of covariation between receptors and behavior (medium CORT group has low GR in the hindbrain and midbrain and high choosiness) compared to the within-individual covariance patterns (females with high forebrain GR express high choosiness) is important.

4.4. Within group variation in plasma CORT levels and its relationship to receptor expression

Within the medium CORT treatment group, there was a positive trend for females with higher final plasma CORT concentrations (potentially reflecting higher endogenous baseline CORT levels) to exhibit the largest increase in choosiness (SM8). Moreover, in this treatment group

alone, there were strong positive correlations between plasma CORT and GR levels in both the hindbrain and midbrain (Fig. 6). Therefore, females that were administered a medium CORT dose, and as a consequence experienced a moderate CORT elevation, still vary in their final plasma CORT–because of either initial differences in endogenous plasma CORT levels or variation in the *Kd* for exogenous uptake, or both–and this variation appears to matter. Females in this treatment group with relatively high final CORT levels tend to have relatively high GR in the hindbrain and midbrain and also tend to be the most choosy females after CORT treatment. At first glance this seems counterintuitive because, among treatment groups, the medium CORT females experienced a large decline in GR in these brain divisions along with increased choosiness. But within this key treatment group, a somewhat opposite pattern is observed: females with the highest CORT have relatively high GR and high choosiness.

The metabolic demands of reproduction in gravid females combined with the important consequences of mate choice on a female's fitness (Welch et al., 1998) provide an opportunity to examine how metabolic endocrine systems like the HPI axis influence energetically demanding and ecologically relevant behavior (choosy mate choice). This is interesting for a few reasons. First, receptor abundances seem to have considerable power in explaining endocrine-behavior relationships; and receptor levels in the brain are changing rapidly. It remains unknown if the changes in receptor mRNA levels seen here, which occurred in <1 h, contributed to the observed behavioral effects; and if they did, any effects would likely be non-causal and suggest alternate mechanistic circuits may be mediating such a fast behavioral response. Alternatively, moderate CORT elevations alone might have modulated behavior unrelated to changes in receptor abundance changes. There is some evidence to support this latter interpretation because it was higher forebrain GR levels that were associated with elevated choosiness, and forebrain GR levels were not changed in response to CORT treatment. The transcriptional effects of moderately elevated plasma CORT binding to higher endogenous expression of GR in the forebrain presents a possible mechanism for the observed behavioral effects.

These patterns underscore the complexity of hormone-behavior relationships. In principle, this is unsurprising, given the diversity and scope of transcriptional targets of glucocorticoids (Austin et al., 2021), the non-linear dose-response patterns (reviewed in Hau and Goymann, 2015), and the multiple timelines of biological action. And it presents challenges for elucidating the detailed mechanisms involved in these patterns.

On the surface there is what may appear to be a paradox, namely that moderately elevated plasma CORT reduces GR (which is especially intriguing in the midbrain, where the audio-motor integration areas that underlie phontaxis lie) and increases choosiness. It is tempting to consider that midbrain expression effect at the among-group level to be a neural correlate of the midbrain-controlled behavioral effect. However, we found that it is higher forebrain GR levels that are associated with choosy females and forebrain GR levels are not impacted by CORT treatment. Of course, it is possible that the behavioral effects are unrelated to standing variation in receptor levels entirely–that this association and this correlation are spurious. It should also be noted that this study measured mRNA levels, not protein. It is unknown whether changes in GR mRNA expression in such a short duration of time (1 h) translates into protein level changes. In fact, that seems unlikely. Hence, we currently favor the hypothesis that the moderate dose of exogenous CORT induced rapid transcriptional declines in GR, especially in the midbrain, and the behavioral consequences of that transcriptional effect are unlikely to be manifested in such a short period of time. Hence, if there is an influence of corticosterone receptor expression in the brain on choosiness during mate choice, we favor the hypothesis that elevated binding of CORT to stable (in response to CORT treatment) but variable (among females) GR levels in the forebrain is a potential mechanism of action. Future studies using central administration of selective GR agonists and antagonists (e.g. RU486) would permit an experimental test of this hypothesis.

5. Conclusions

With the exception of *Xenopus*, anuran amphibians are not traditional model systems in behavioral endocrinology, yet they offer some important advantages. As seasonal breeders, their endocrine profiles and sensory systems are rapidly remodeled during reproduction (Baugh et al., 2019; Gall et al., 2019), during which time they are highly motivated and exhibit robust mate choice behavior under controlled laboratory conditions. These rapid changes in the HPI and HPG axes offer windows into the mechanisms that underlie variation in female sexual behavior, including mate choice. For example, the elevated and highly variable concentrations of plasma CORT on the night of mating, along with its role in regulating energetic priorities, indicates a potential role for this steroid in modulating fitness relevant behavior, including female phonotaxis. To our knowledge this is the first experimental study examining the neuroendocrine basis of female mate choosiness. We show that exogenous corticosterone rapidly influences the abundance of GR, and to a lesser extent, MR transcripts in the brain. In particular, GR expression was diminished significantly in the hindbrain and midbrain in response to medium doses of exogenous CORT. The medium CORT dose was also responsible for a large increase in mate choosiness. However, those two findings do not appear to be causally linked, as forebrain GR levels–which were not impacted by CORT treatment–were positively correlated with choosiness. Hence, we propose that exogenous CORT elevations rapidly adjust GR mRNA abundance in the hindbrain and midbrain as well as promote mate choosiness, potentially through actions of stable individual differences in GR expression in the forebrain centers that may underlie cognitive elements involved in complex decision making.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2024.105477.

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 #2001-37746A).

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CRediT authorship contribution statement

Mariana Rodriguez-Santiago: Formal analysis, Investigation, Resources, Writing – original draft. **Annika Ruppert:** Investigation. **Megan D. Gall:** Investigation, Resources, Writing – review & editing. **Kim Hoke:** Resources, Writing – review & editing. **Mark A. Bee:** Resources, Writing – review & editing. **Alexander T. Baugh:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

Uncited references

Declaration of competing interest

We have no competing interests to declare.

Data availability

Data will be made available on request.

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