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Research paper

Hypothalamic-pituitary-adrenal axis regulation and organization in urban and rural song sparrows

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ABSTRACT

Urban habitats present animals with persistent disturbances and acute stressors not present in rural habitats or present at significantly lower levels. Differences in the glucocorticoid stress response could underlie colonization of these novel habitats. Despite urban habitats characterization as more stressful, previous comparisons of urban and rural birds have failed to find consistent differences in baseline and stress induced glucocorticoid levels. Another aspect of glucocorticoid regulation that could underlie an animal's ability to inhabit novel habitats, but has yet to be well examined, is more efficient termination of the glucocorticoid stress response which would allow birds in urban habitats to recover more quickly after a disturbance. The glucocorticoid stress response is terminated by negative feedback achieved primarily through their binding of receptors in the hippocampus and hypothalamus and subsequent decreased synthesis and release from the adrenals. We investigated if male song sparrows (*Melospiza melodia*) in urban habitats show more efficient termination of the glucocorticoid stress response than their rural counterparts using two approaches. First, we measured glucocorticoid receptor, mineralocorticoid receptor and 11β-HSD2 (an enzyme that inactivates corticosterone) mRNA expression in negative feedback targets of the brain (the hippocampus and hypothalamus) as a proxy measure of sensitivity to negative feedback. Second, we measured plasma corticosterone levels after standardized restraint and again following a challenge with the synthetic glucocorticoid, dexamethasone, as a means of assessing how quickly birds decreased glucocorticoid synthesis and release. Though there were no differences in the hypothalamus of urban and rural song sparrows, urban birds had lower glucocorticoid receptor and 11β-HSD2 mRNA expression in the hippocampus. Further, urban and rural birds had similar reductions in corticosterone following the dexamethasone challenge, suggesting that they do not differ in how quickly they decrease glucocorticoid synthesis and release. Thus, urban and rural song sparrows display similar termination of the glucocorticoid stress response even though urban birds have decreased hippocampal glucocorticoid receptor and 11β-HSD2 abundance.

1. Introduction

Urban habitats present animals with novel and unpredictable stimuli, which are inferred to be stressors, that are either not present or far less frequent in their native habitats (Lowry et al., 2013). These challenges include human disturbance, artificial light at night, increased ambient noise, habitat fragmentation and degradation, and altered anthropogenic predator communities (Injaian et al., 2018; Kleist et al., 2018; Loss et al., 2013; McKinney, 2006; Navara and Nelson, 2007; Rosenberg et al., 2019). Species differ in their responses to such urban stressors, with some species, termed urban avoiders, remaining in their native rural habitats and other species, termed urban exploiters, maintaining peak population densities in urban areas (Blair, 1996; Bonier, 2012; McKinney, 2002; Shochat et al., 2006). Species between these two

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extremes are termed urban adapters and show among them individual variation in their response to urbanization, such that some individuals within a species colonize urban habitats while others remain in native habitats. Making comparisons across species that differ in their response to urbanization, and also among individuals within urban adapter species, can identify the characteristics that allow some animals to live in urban areas despite the additional stressors present. Within species comparisons of urban adapters are of particular importance because they control for life history traits such as sociality, mating system, and migration that may themselves impact an individual's ability to respond to the challenges of urban life (Bonier, 2012).

One endocrine mechanism that could allow animals in urban habitats to persist in the face of novel disturbances is the glucocorticoid stress response. In response to stressors, animals secrete catecholamines from the adrenal medulla to initiate physiological and behavioral reactions, and glucocorticoids from the adrenal cortex that act more slowly and facilitate recovery (Romero et al., 2009; Sapolsky et al., 2000; Siegel, 1980). While the glucocorticoid stress response is essential to coping with disturbances, repeated exposure to novel and unpredictable stimuli and the resulting increases in glucocorticoids can cause stress related disease, oxidative stress, and neural damage (Dickens et al., 2009; Lin et al., 2004; Lynn et al., 2010; McEwen, 2006; Sapolsky et al., 2000). It has been hypothesized that animals in urban habitats characterized by more frequent disturbances could mitigate the damages of repeated glucocorticoid exposure by maintaining lower baseline levels or releasing less glucocorticoid during a stress response (Bonier, 2012). Indeed, several prior studies in songbirds have tested the hypothesis that individuals in urban habitats have lower baseline corticosterone (Cort, the predominant avian glucocorticoid) or dampened release of Cort in response to a standardized stressor (In the focal population: Foltz et al. (2015a); See Review: Bonier (2012)). Results from these comparisons of urban and rural individuals have been mixed, though, and there are no consistent patterns of difference in the endocrine profiles of urban and rural animals (see reviews in Vertebrates: Renthlei et al. (2017); Birds: Bonier (2012)). However, recent research has demonstrated that individuals also vary in the efficiency with which they terminate the glucocorticoid stress response (Liu et al., 1997; MacDougall-Shackleton et al., 2013; Romero et al., 2009; Zimmer et al., 2019). More efficient termination of the glucocorticoid stress response could underlie faster behavioral and physiological recovery and reduce the duration of exposure to glucocorticoids. This raises a new hypothesis; individuals that live in habitats with frequent stressors, such as urban habitats, could benefit from more efficiently terminating the stress response.

Termination of the glucocorticoid stress response could be mediated by greater expression of the receptors that facilitate negative feedback, or by reduced expression of enzymes that metabolize glucocorticoids in brain regions involved in negative feedback. Glucocorticoid secretion is primarily regulated by the hypothalamic-pituitary-adrenal (HPA) axis, a neuroendocrine cascade that is highly conserved across vertebrate species (McEwen, 2001). This cascade is triggered by internal or external stimuli that induce the hypothalamus to release corticotropin-releasing hormone, which signals the pituitary to release adrenocorticotropic hormone, which in turn stimulates the adrenal cortex to up-regulate the production of glucocorticoids (McEwen, 2001; Romero et al., 2009; Sapolsky et al., 2000). Glucocorticoid secretion is terminated when glucocorticoids bind low affinity glucocorticoid receptors (GRs) within the hypothalamus and hippocampus, causing negative feedback and reduced glucocorticoid synthesis (Dickens et al., 2009; Kadhim et al., 2019; McEwen, 2001; Jacobson and Sapolsky, 1991; Sapolsky et al., 2000; Vandenborne et al., 2005; Zimmer and Spencer, 2014). Greater expression of low affinity GRs relative to higher affinity mineralocorticoid receptors (MRs) is associated with stronger negative feedback on the HPA axis and lower behavioral stress reactivity (De Kloet et al., 1993; Harris et al., 2013; Liu et al., 1997; Meaney, 2001; Zimmer and Spencer, 2014). Additionally, the enzyme 11β-hydroxysteroid dehydrogenase Type 2 (11β-HSD2) (McEwen, 2001; Pérez et al., 2020; Rensel et al., 2018; Seckl, 1997) modulates the effectiveness of Cort by converting it into 11-dehydro-corticosterone and cortisone as it enters the cell, before it can bind GRs (Rensel et al., 2018). Increased expression of 11β-HSD2 could buffer tissue from the effects of glucocorticoids by decreasing their local availability (Pérez et al., 2020; Rensel et al., 2018; Seckl, 1997) but is thought to diminish the strength of negative feedback (McEwen, 2001; Pérez et al., 2020; Wada, 2015). Differences in potential for negative feedback on the glucocorticoid stress response can be estimated using mRNA expression of GR and 11β-HSD2 within brain tissue (Rensel et al., 2018; Zimmer and Spencer, 2014). Additionally, functional termination of the stress response can be evaluated by administering a synthetic glucocorticoid, such as dexamethasone (Dex), and measuring the magnitude of decrease in circulating Cort (Carroll et al., 1981; Lattin and Kelly, 2020). Dex, acting primarily at the pituitary and periphery, selectively binds GR's, so the magnitude of decrease in endogenous Cort reflects how quickly and dramatically an individual terminates glucocorticoid synthesis (De Kloet and Ronald, 1997; MacDougall-Shackleton et al., 2013; McEwen, 2001). Very few studies have considered how urbanization affects the termination of the glucocorticoid stress response (though see Fokidis et al., 2009). Examining the efficiency of the mechanisms that return animals to their physiological baseline could provide new insights into how variation in HPA axis function supports persistence in novel habitats including urban environments.

Song sparrows (*Melospiza melodia*) are a common North American songbird that establish seasonal breeding territories in both rural and urban habitats. As urban adapters, song sparrows have been the focus of urbanization research for years and express both behavioral and physiological adjustments to urban environments, making them an excellent model for the current study. Past research on the focal populations have had mixed results when investigating the glucocorticoid stress response, with urban and rural birds expressing yearly variation in baseline and stress induced levels. (Beck et al., 2018; Davies et al., 2018; Foltz et al., 2015a). Exploring the endocrine mechanisms of negative feedback in urban and rural song sparrows will add insight into the physiological acclimations that may underlie the consistent behavioral differences observed between urban and rural dwelling animals.

In the current study we tested the hypothesis that individuals in urban habitats terminate the glucocorticoid stress response more efficiently than their rural counterparts, using song sparrows as a study system. We asked if urban and rural birds differed in potential for negative feedback by quantifying the expression of MR, GR, and 11β-HSD2 and we asked if the functional termination of glucocorticoid synthesis differed using a dexamethasone challenge. We predicted that urban and rural birds would have similar relative mRNA expression of MR. We also predicted that urban birds would have greater expression of GR, which would facilitate negative feedback on the HPA axis, and reduced expression of 11β-HSD2, which would increase local availability of glucocorticoids to enhance negative feedback. Additionally, in a second experiment, we predicted that urban birds would show a greater reduction in circulating Cort in response to Dex injection than rural birds.

2. Methods

2.1. Study population

Male song sparrows in breeding condition were captured in the wild from 3 urban and 3 rural sites near Blacksburg, VA. These sites have been previously characterized for their levels of urbanization using a technique validated by Seress and colleagues (See Davies and Sewall (2016) and Davies et al. (2018) for details on site selection and characteristic; Seress et al., 2014). All birds were captured via mist nets using 1 of 16 conspecific songs played at the center of previously mapped territories. All captures occurred between 0500 and 1115 h. All

procedures were preapproved by Virginia Tech's Institutional Animal Care and Use Committee and were conducted under current scientific collecting permits.

2.2. Experiment 1: qPCR quantification of targets for negative feedback

In 2016 we collected territory holding males (urban n = 16, rural n = 10) from the 6 sites during the breeding season (May 4th–May 25th). We captured males within an average of 10.2 min of playback (range: 1.2–22.22 min). We immediately anesthetized each male with isoflurane, euthanized them, and removed their brains which were flash frozen on dry ice and stored at −80 °C until qPCR was performed. We sectioned half of the brain in 40 μm sections in a cryostat set to −20 °C. Using a scalpel, we micro-dissected out the hypothalamus and hippocampus with the central fissure and anterior commissure as anatomical landmarks, found by referencing a passerine bird brain atlas (Nixdorf-Bergweiler and Bischof, 2007). We extracted total RNA from each brain region using a commercially available kit following the manufacturer's instructions (Total RNA Purification Micro Kit, Norgen Biotek, Canada, Cat. No. 35300) and we included the DNase-1 digestion step (RNase-Free DNase-1 Kit, Norgen Biotek, Canada, Cat. No. 25710). To assess RNA quantity and purity we used a nano-spectrometer (Nanophotometer Pearl, Implen, USA) and used a Bio-Analyzer 2100 (Agilent Technologies, USA, Cat. No. G2939BA) to assess RNA integrity. We reverse transcribed 100 ng of total RNA to cDNA using a commercially available kit (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Cat. No. 4368813) and diluted with RNase-free water to a final concentration of 1 ng/μl. Following the manufacturer's instructions, the thermocycler conditions were 25 °C for 10 min to start, then 37 °C for 120 min, 85 °C for 5 min, and then the thermocycler held the product at 4 °C until it was taken out. A song sparrow genome has not been published, so we used the zebra finch (*Taeniopygia guttata*) genome for primer design. We designed primers for GR, MR, 11β-HSD2, and the reference gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, which did not differ between treatment groups) using Primer Express v.3 (Applied Biosystems, USA, Cat. No. 4363991), and the oligonucleotides were synthesized by Integrated DNA Technologies, USA (see Table 1 for Accession Numbers and primer sequences). The Ct values for GAPDH were cross-calibrated against values of other reference genes including beta-actin and 18S rRNA and determined to be the most stable across all sample types.

Primer specificity was confirmed before experiments via Primer-BLAST (NCBI; default search parameters and taxid: 59729) of each primer pair against reference genomic sequences for the zebra finch. No additional target templates were identified. Specificity of starting template (i.e., lack of genomic DNA carryover) was confirmed via lack of PCR amplification for negative control samples that were generated from reverse transcription reactions that did not contain reverse transcriptase. We performed the qPCR reactions in MicroAmp Fast Optical 96-Well Reaction Plates (Applied Biosystems, USA, Cat. No. 4346906) with MicroAmp Optical Adhesive Film (Applied Buosystems, USA, Cat. No. 4311971) using Fast SYBR Green Master Mix (Applied Biosystems, USA, Cat. No. 4385612). We ran all samples in duplicate, with all samples for a specific gene of interest within a specific brain region run on a single plate (six plates total). For instance, plate 1 being GR in the hippocampus, plate 2 being GR in the hypothalamus etc. Each well contained 3 μl cDNA, 0.25 μl of 5 μM forward primer, 0.25 μl of 5 μM reverse primer, 5 μl Fast SYBR green, and 1.5 μl RNase-free water. We calculated standard curves to assess amplification efficiency (90–110%). These were validated to be similar between target genes and the reference gene via standard curves generated from serial dilutions of cDNAs from multiple samples (90–110%; 100% for reference gene). We ran all plates on an Applied Biosystems 7500 Fast Real-Time PCR System (SeqGen Inc., USA) with the following cycling parameters: 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Post-PCR, amplicon specificity was confirmed via melting curve analysis where a single peak representing the amplified product for each reaction was observed at the correct melting temperature (95 °C for 15 s 60 °C for 1 min, 95 °C for 15 s and 60 °C for 15 s).

2.3. Experiment 2: Dexamethasone challenge

In 2019, using a separate cohort of birds, we compared the efficiency with which urban and rural males terminated the glucocorticoid stress response using a Dex challenge in the pre-breeding season (March 23rd–April 13th). We targeted territories that had been established in the previous breeding season (2017 and 2018). To confirm a male was on the territory, we played 1 of 16 recordings of conspecific songs for 5–20 s within the boundaries of a known territory. When we confirmed that a male was present, we placed a mist net in an observed flight path and played pre-recorded conspecific song for up to 68 min. We captured males within 24.4 min on average (range of 1–68 min) and immediately removed them from the net and placed them in a breathable cloth bag to induce a glucocorticoid stress response. To determine stressor-induced Cort levels, we collected a 70 μl blood sample 30 min after capture via puncture of the brachial veins using a 26-gauge needle. We randomly assigned every other bird captured to receive an injection of either Dex or vehicle (phosphate buffered saline, PBS) except for two days of the experiment, when all birds received injections of Dex (Dex: urban n = 13, rural n = 14; saline: urban n = 7, rural n = 8). Birds assigned to the Dex condition were given a 100 μl intramuscular injection of 1 mg/kg Dex (crystalline Dex, Sigma Aldrich, Cat. No. D1756) dissolved in ethanol, brought to volume with autoclaved PBS (ethanol was <5% of the final volume; Holberton et al., 2006), while birds assigned to the saline condition were given an intramuscular injection of saline (autoclaved PBS diluted with ethanol; ethanol was <5% of the final volume). This Dex dose has been shown to effectively induce termination of the glucocorticoid stress response in several songbirds, including song sparrows (Bauer et al., 2016; MacDougall-Shackleton et al., 2013; Schmidt et al., 2012). After injection, we placed all birds in covered cages with seed and water for 1 h (Schmidt et al., 2012) and then collected a second 70 μl blood sample to assess the effect of Dex or saline injection on glucocorticoid levels (Lattin and Kelly, 2020; MacDougall-Shackleton et al., 2013). We stored all blood samples on ice immediately after collection and centrifuged them later the same day. We stored plasma at −20 °C until assayed for Cort.

2.3.1. Cort assay

We quantified Cort levels using a commercially available enzyme-linked immunosorbent assay (ELISA; Lot No.: 1201810, Enzo Life Sciences, Inc, Farmingdale, NY) following the manufacturer's instructions. Previous research has validated the assay for song sparrows, and we assessed cross-reactivity with Dex (see below; Davies and Sewall, 2016; Davies et al., 2018). Briefly, we diluted samples 1:40 and added, 1%

Table 1
Accession numbers and primer sequences.

GOI	Accession No.	Forward Sequence	Reverse Sequence
MR	NM_001076690.1	CGAGCCCTCCGTCAACAC	GGAGTAAGTGCTGGTGAGATAGCA
GR	XM_002192952.4	TCTCCCCTCGTGCACCAT	TGTTCGTAACAGCCTCAGAGCTT
11β-HSD2	XM_030282630.1	GCGAGGACTATGTGGAGGAGAT	TCCACTGCCACCTTCATGAA
GAPDH	XM_030266469.1	GTGGTGCCAAGCGTGTGA	CACGAACATGGGAGCATCAG

steroid displacement reagent. We assayed samples in duplicate, with all samples from a given bird assigned to the same plate and all birds randomly assigned to one of two plates. Intra-assay variation was 11.14% and assay sensitivity was 27 pg/ml. Inter-assay variation was controlled for by including plate number as a random effect in the final model. To evaluate cross-reactivity with Dex for this brand of ELISA we ran samples of song sparrow serum spiked to 2, 20, 50, 100 and 200 ng/mL Dex. We found low cross-reactivity at 50 ng and below, but it increased at higher concentration (see Supplementary Materials). Because of this and recommendations from recent publications (Lattin and Kelly, 2020) we will be referring to relative amounts (relative change from stress induced Cort to 60 min post Dex injection) of Cort and not absolute values after injection.

2.4. Statistical analysis

The statistical analyses presented in the manuscript were conducted using SPSS (v.25) and R (v. 3.6.1: R Core Team, 2019). For qPCR data, we calculated relative expression of each gene of interest using the ΔΔCt method, i.e. $2^{-\Delta\Delta Ct}$ (ΔCt = target gene Ct − GAPDH Ct, ΔΔCt = ΔCt − calibrator ΔCt where the calibrator is the mean ΔCt of rural birds) (Schmittgen and Livak, 2008). We analyzed relative expression of each target gene (MR, GR, 11β-HSD2) in each brain region (hippocampus, hypothalamus) in separate generalized linear models with data fitted to a gamma error distribution and habitat type entered as a fixed factor in each model. The deviance residuals from these models were examined for normality. We explored all significant effects further using Sidak *post hoc* tests, and calculated Cohen's d effect size for all significant *post hoc* tests.

We assessed termination of the stress response by calculating the relative change in Cort concentration from stress induced levels (((30 min sample − 90 min sample)/30 min) × 100%) following recommended methods in Lattin and Kelly (2020). We entered the relative change in Cort as the dependent variable in a linear model with habitat type, injection type, and the interaction between them entered as fixed factors. We entered time of capture as a co-variate to account for circadian changes in Cort concentration and negative feedback. We assessed the effect of habitat type on stressor – induced (30 min) Cort by entering 30 min Cort as the dependent variable in a second linear model with habitat type as the fixed factor. We used time to capture (also referred to as playback exposure time) and day of year as co-variates. Plate number was entered as a co-variate in both these models to account for inter-assay variation. Residuals were normally distributed (Shapiro-Wilk, $p > 0.05$). We used an alpha value of 0.05 as the threshold for statistical significance. All figures show mean ± 1 standard error of the mean.

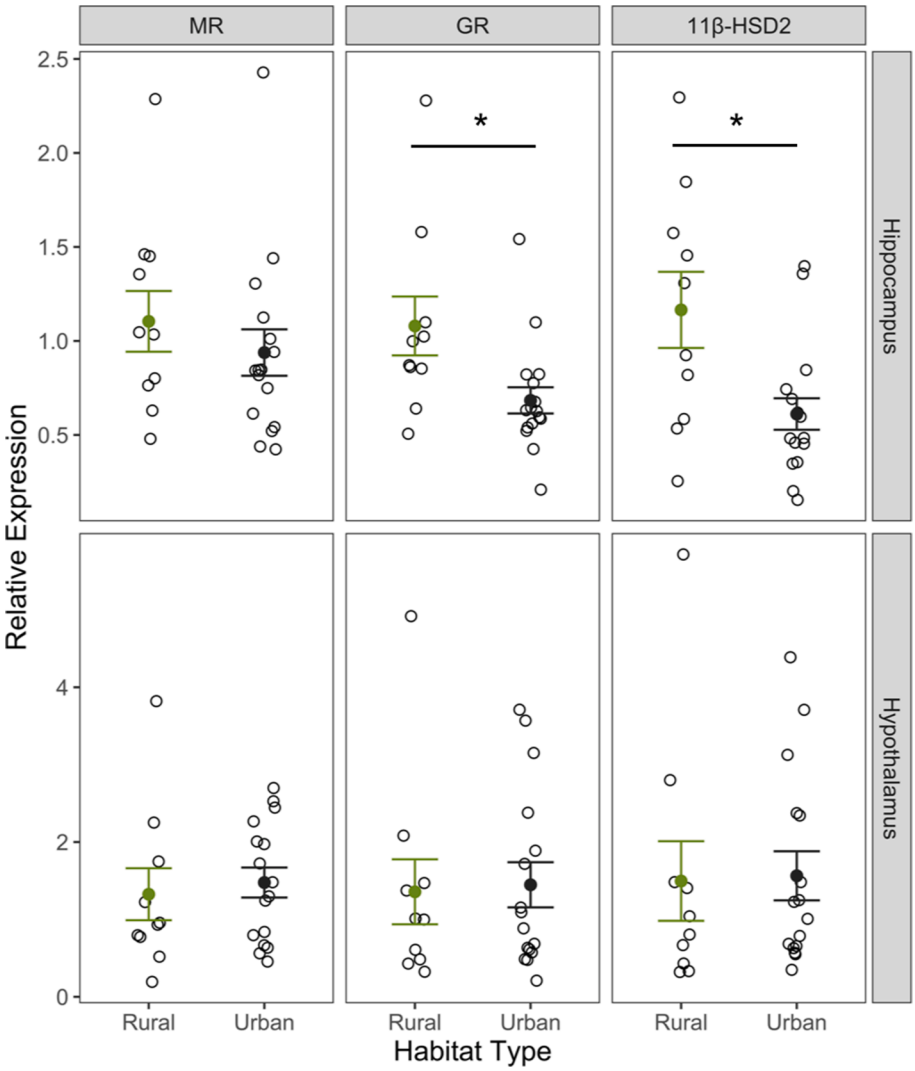

Fig. 1. Relative expression of hypothalamic and hippocampal mRNA levels of glucocorticoid receptors (GR), mineralocorticoid receptors (MR), and 11β-hydroxysteroid dehydrogenase (11β-HSD2) between urban and rural song sparrows. Open circles are individuals collected in 2016, with means ± 1 standard error of the mean and data were normalized to GAPDH. Song sparrows, regardless of habitat, have similar hypothalamic mRNA levels of GR, MR, and 11β-HSD2. Urban male song sparrows, relative to rural males, have lower hippocampal mRNA levels of GR and 11β-HSD2 (* = $p < 0.05$). Hippocampal MR mRNA expression is similar in all birds regardless of habitat.

3. Results

3.1. Experiment 1: qPCR quantification of targets of negative feedback

We found no difference between urban and rural song sparrow's hypothalamic mRNA expression of GR ($\chi^2_{1,24} = 1.473$, $p = 0.237$), MR ($\chi^2_{1,24} = 0.778$, $p = 0.386$) or 11β-HSD2 ($\chi^2_{1,24} = 0.820$, $p = 0.374$) (Fig. 1). In the hippocampus however, urban song sparrows had lower hippocampal mRNA levels of GR ($\chi^2_{1,24} = 6.953$, $p = 0.014$, $d = 1.056$) and 11β-HSD2 ($\chi^2_{1,24} = 10.474$, $p = 0.004$, $d = 1.171$) relative to rural song sparrows (Fig. 1). Hippocampal MR mRNA expression did not differ between habitats ($\chi^2_{1,24} = 0.367$, $p = 0.550$).

3.2. Experiment 2: Dexamethasone challenge

Injection with Dex significantly decreased Cort from stress induced levels compared to saline injection ($t_{1,35} = -2.40$, $p = 0.022$, $d = 1.25$). Thus, our Dex injection successfully induced negative feedback. However, the interaction between habitat type and injection type was not significant ($t_{1,35} = 0.29$, $p = 0.77$; Fig. 2), indicating that there was no difference in how urban and rural birds responded to Dex. Independent of treatment group, rural birds had significantly higher stressor-induced Cort (pre-injection) compared to urban ($t_{37,1} = -2.14$, $p = 0.04$, $d = 0.78$; Fig. 3).

4. Discussion

Urban habitats are often considered more stressful than native habitats because they present individuals with unpredictable and novel stimuli (Birnie-Gauvin et al., 2016; McKinney, 2002) and the focus of many urbanization studies has been understanding how animals cope with these potential stressors. In the current study, we hypothesized that more efficient termination of the glucocorticoid stress response might underlie the ability of some individuals that colonize and persist in novel urban habitats. To test this hypothesis we compared (1) the expression of mRNA for receptors and enzymes (MR, GR, and 11β -HSD2) that mediate negative feedback within the hypothalamus and hippocampus and (2) the magnitude of relative decrease in circulating glucocorticoids after injection with the synthetic glucocorticoid Dex (aka a Dex challenge), between urban and rural male song sparrows. We predicted that urban birds will have greater mRNA expression of GR, and lower expression of 11β-HSD2, which would facilitate negative feedback of the HPA axis. Additionally, we predicted that urban birds will have a stronger response to the Dex challenge than rural birds and reduce circulating Cort to a greater degree following injection.

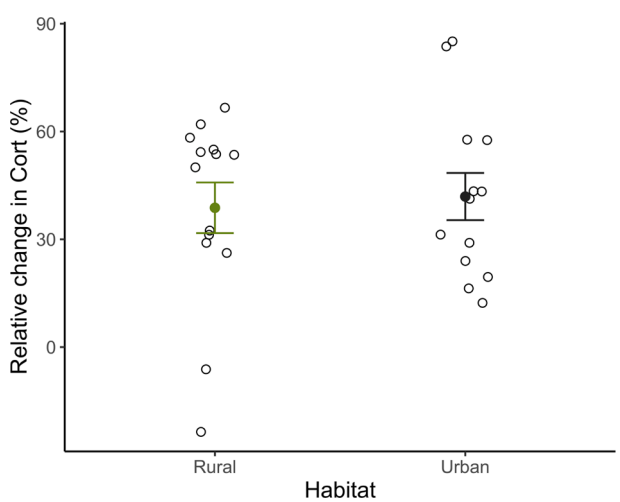

Fig. 2. Relative reduction of circulating Cort (ng/ml) following Dex injection in 2019 urban and rural male song sparrows. Open circles are individuals, with means ± 1 standard error of the mean. Birds had comparable relative reductions in Cort in response to Dex regardless of habitat.

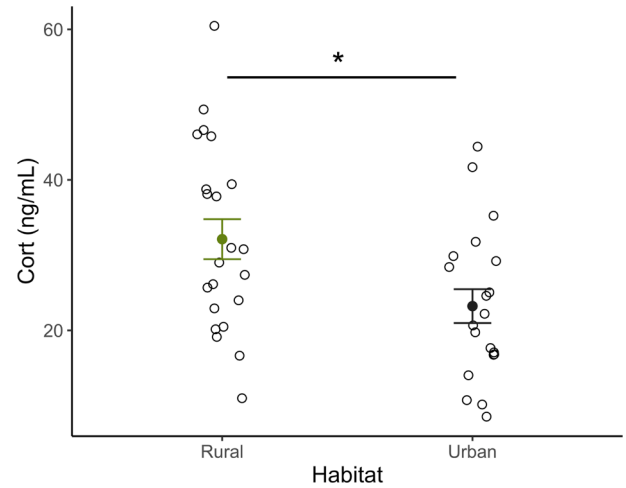

Fig. 3. Stress induced Cort levels following 30 min of restraint in urban and rural male song sparrows. Open circles are individuals, with means ± 1 standard error of the mean. In 2019 rural song sparrows (n = 22) had higher stress induced Cort compared to urban (n = 20) pre-injection and independent of treatment group.

Counter to our predictions, urbanization is not associated with any detectable differences in the termination of the glucocorticoid stress response as measured by the Dex challenge. Treatment with Dex significantly lowered circulating levels of Cort relative to saline injection, but urban and rural song sparrows did not differ in how much their circulating Cort was reduced in response to Dex (Fig. 2). Urban birds had significantly lower stress induced Cort following a standardized stressor as compared to urban (Fig. 3) and urban birds expressed a similar negative feedback to rural birds suggesting that overall urban birds are minimizing their HPA activation in response to stressors. A muted glucocorticoid stress response could protect individuals from the negative effects of chronic exposure to glucocorticoids. Additionally, this could be a physiological acclimation to a novel habitat, where disturbances and encounters with acute stressors occur often. However, previous research on this population has shown variation across years in both stress-induced and baseline levels of Cort in adult male song sparrows (Foltz et al., 2015a). Foltz et al. found that in some years males from urban habitats would express increased baseline and/or stress induced levels of Cort compared to rural, while in other years the patterns would be reversed or nonexistent. These conflicting results over the glucocorticoid stress response of urban birds and a lack of functional differences in negative feedback mechanisms highlight the complex interactions occurring within urban habitats and the variability of the glucocorticoid stress response. It is also important to note that a Dex challenge primarily reflects negative feedback mechanisms occurring at the pituitary and the periphery and the termination of glucocorticoid synthesis and mechanisms of negative feedback are not completely synonymous (De Kloet and Ronald, 1997; Lattin and Kelly, 2020). Future studies that bring animals from urban and rural habitats into captivity could examine urban males ability to release Cort through an ACTH challenge, and would be able to further examine negative feedback by administering Dex directly to the brain (Pérez et al., 2020).

Though we found no evidence of functional differences in the termination of glucocorticoid synthesis, we found habitat differences in mechanisms implicated in mediating negative feedback of the HPA axis. We found, contrary to predictions, lower relative expression for both GR and 11β-HSD2 in the hippocampus of urban birds (Fig. 1). Lower hippocampal GR expression is generally associated with reduced negative feedback on the glucocorticoid stress response, which would suggest urban males have less capacity for negative feedback on the HPA axis

(Liu et al., 1997; McEwen, 2001; Dickens et al., 2009; Zimmer and Spencer, 2014). The finding that urban males had reduced hippocampal 11β-HSD2 is less straight forward. 11β-HSD2 metabolizes glucocorticoids as they enter the cell, inactivating them, so decreased abundance would increase available glucocorticoids in the hippocampus. Without the reduction in hippocampal GR, this could increase the strength of negative feedback (Pérez, et al., 2020), but a reduction in both could cancel out any negative feedback effects. However, another hypothesized function of 11β-HSD2 is protection against the damaging effects of elevated glucocorticoids (Oppermann et al., 1997; Pérez et al., 2020; Rensel et al., 2018). Therefore, urban males with lower 11β-HSD2 may be at risk of greater damage from glucocorticoids in the hippocampus. We found no habitat differences in the expression of receptors or enzymes in the hypothalamus (Fig. 1). Collectively, these results do not support the hypothesis that urban male song sparrows terminate the glucocorticoid stress response more efficiently and even suggest that urban males have reduced capacity for negative feedback. By evaluating both the function of the HPA axis using a Dex challenge and the key brain mechanism by which we understand negative feedback to act, we find that negative feedback on the HPA axis and termination of the glucocorticoid stress response does not facilitate the settlement of urban habitats by song sparrows. However, future studies in species that are more recent colonists of urban areas or across more dramatic habitat gradients should be pursued. Also, it is important to acknowledge that relative mRNA expression is not the same as functional protein levels, and that urban and rural birds could differentiate levels of protein production downstream of mRNA expression. Additionally, we view this as a starting point, and that further studies using immunohistochemistry to look at protein expression and receptor binding as well as enzyme activity assays are required to gain a full understanding of the changes occurring in these brain regions of urban adapters.

Though our findings do not support our hypothesis that faster termination of the glucocorticoid stress response underlies colonization and persistence in novel urban habitats, they do raise a new hypothesis. In previous work, we found no evidence of population genetic structure (Brewer et al., 2020) and though theoretically there could be specific genetic alteration between these groups, we believe they are one continuous interbreeding population. This suggests individuals in our urban populations are experiencing some level of developmental organization or lifelong acclimation to urban habitats. In other systems it has been shown that the expression of GR can be organized by early life experiences and may be decreased by exposure to stressors (Dickens et al., 2009; Liu et al., 1997; McCormick et al., 2005; Zimmer and Spencer, 2014). Urban habitats are well-documented to have more frequent disturbance and song sparrows at our study sites experience more frequent human disturbance and higher ambient noise (Akçay et al., 2020; Foltz et al., 2015a, 2015b). Conversely, though urban habitats are characterized as being more stressful, this characterization may be premature. Many urban habitats also provide animals with additional resources (anthropogenic sources of food, water, and nesting substrates) and protection from predators (Reynolds et al., 2019; Shochat et al., 2010; Soulsbury and White, 2016). Urban habitats differ considerably from more native rural habitats, but they may no longer be stressful to animals that have acclimated to living within them. The glucocorticoid stress response assists with recovery from acute stressors, and its activation may decrease with acclimation to the novel stimuli present in urban habitats.

Overall, the current study provides evidence of brain region specific differences in receptor and enzyme abundance between urban and rural adult male song sparrows, even though there are no consistent differences in levels of circulating hormones. We did not detect differences in the termination of the glucocorticoid stress response using a Dex challenge. We did find that urban males have lower stress induced Cort levels, suggesting urban individuals have a dulled glucocorticoid stress response. However, past studies in our lab (Davies et al., 2018) and others (Foltz et al., 2015a) failed to find consistent differences in baseline or stress induced Cort, casting doubt on this conclusion. Finally, we found differences in the HPA axis associating urbanization with significantly reduced hippocampal GR and 11β -HSD2 abundance. Additional studies are needed to determine if these differences result from developmental programming by urban habitats or lifelong acclimation to urban habitats. Importantly, future work also needs to examine the HPA axis response to stressors other than restraint to determine if animals in urban habitats are habituating selectively to urban stressors and do not, in fact, show altered HPA axis function.

CRediT authorship contribution statement

Samuel J. Lane: Conceptualization, Methodology, Investigation, Data curation, Writing - original draft, Supervision, Visualization. **Michael G. Emmerson:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - review & editing. **Isaac J. VanDiest:** Methodology, Investigation, Writing - review & editing. **Catherine Hucul:** Methodology, Investigation. **Michelle L. Beck:** Investigation, Writing - review & editing. **Scott Davies:** Investigation, Writing - review & editing. **Elizabeth R. Gilbert:** Resources, Supervision, Writing - review & editing. **Kendra B. Sewall:** Conceptualization, Methodology, Investigation, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2021.113809.

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