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# Gene expression in the female tree swallow brain is associated with inter- and intra-population variation in glucocorticoid levels

Cedric Zimmer [a,b,*], Conor C. Taff [a,c], Daniel R. Ardia [d], Kimberly A. Rosvall [e], Christine Kallenberg [a], Alexandra B. Bentz [e,f], Audrey R. Taylor [g], L. Scott Johnson [h], Maren N. Vitousek [a,c]

[a] Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, NY 14853, USA
[b] Laboratoire d'Ethologie Expérimentale et Comparée, LEEC, Université Sorbonne Paris Nord, UR 4443, 93430 Villetaneuse, France
[c] Cornell Lab of Ornithology, Ithaca, NY 14850, USA
[d] Department of Biology, Franklin and Marshall College, Lancaster, PA 17604, USA
[e] Department of Biology, Indiana University Bloomington, Bloomington, IN 47405, USA
[f] Department of Biology, University of Oklahoma, Norman, OK 73019, USA
[g] Biological Sciences, University of Alaska Anchorage, Anchorage, AK 99508, USA
[h] Department of Biological Sciences, Towson University, Towson, MD 21252, USA

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ABSTRACT

Studies of the evolutionary causes and consequences of variation in circulating glucocorticoids (GCs) have begun to reveal how they are shaped by selection. Yet the extent to which variation in circulating hormones reflects variation in other important regulators of the hypothalamic-pituitary-adrenal (HPA) axis, and whether these relationships vary among populations inhabiting different environments, remain poorly studied. Here, we compare gene expression in the brain of female tree swallows *(Tachycineta bicolor)* from populations that breed in environments that differ in their unpredictability. We find evidence of inter-population variation in the expression of glucocorticoid and mineralocorticoid receptors in the hypothalamus, with the highest gene expression in a population from an extreme environment, and lower expression in a population from a more consistent environment as well as in birds breeding at an environmentally variable high-altitude site that are part of a population that inhabits a mixture of high and low altitude habitats. Within some populations, variation in circulating GCs predicted differences in gene expression, particularly in the hypothalamus. However, some patterns were present in all populations, whereas others were not. These results are consistent with the idea that some combination of local adaptation and phenotypic plasticity may modify components of the HPA axis affecting stress resilience. Our results also underscore that a comprehensive understanding of the function and evolution of the stress response cannot be gained from measuring circulating hormones alone, and that future studies that apply a more explicitly evolutionary approach to important regulatory traits are likely to provide significant insights.

## 1. Introduction

The capacity to cope with challenges can differ substantially within and among populations (Angelier and Wingfield, 2013; Taff and Vitousek, 2016). Successfully navigating these challenges requires an integrated behavioral and physiological response. The ability of individuals to mount an appropriate response can influence their current and future performance (Hau et al., 2016; Wingfield and Sapolsky, 2003). A central coordinator of the stress response in vertebrates is the hypothalamic-pituitary-adrenal (HPA) axis (Hau et al., 2016; Romero, 2002; Sapolsky et al., 2000; Vitousek et al., 2019a) which regulates the production and release of glucocorticoids (GCs) (Hau et al., 2016; Sapolsky et al., 2000; Wingfield et al., 1998). When facing unpredictable challenges, a cascade of reactions is triggered in the brain, beginning with the perception of the stressor in higher brain areas (hippocampus or amygdala), signaling the hypothalamus to secrete corticotropin-

* Corresponding author at: Laboratoire d'Ethologie Expérimentale et Comparée, UR 4443, Université Sorbonne Paris Nord, 99, avenue Jean-Baptiste Clément, 93430 Villetaneuse, France.
*E-mail address:* cedric.zimmer@univ-paris13.fr (C. Zimmer).

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releasing hormone (CRH), resulting in the release of adrenocorticotropic hormone (ACTH) by the pituitary gland which causes the secretion of GCs by the adrenal glands. GCs promote a suite of physiological and behavioral changes that facilitate the successful response to and recovery from challenges by temporarily redirecting resources from inessential activities toward immediate survival (Hau et al., 2016; Sapolsky et al., 2000; Wingfield et al., 1998). GC baseline and stress induced levels have been shown to have a low to moderate heritability and can respond to selection (Bairos-Novak et al., 2017; Béziers et al., 2019; Jenkins et al., 2014; Stedman et al., 2017), though relationships with fitness vary across populations and contexts (Bonier et al., 2009; Breuner and Berk, 2019; Caulfield and Cavigelli, 2020; Schoenle et al., 2021; Schoenle et al., 2018). Although the GC stress response is essential to coping with challenges, prolonged GC elevation can be costly, reducing fitness (Angelier et al., 2018; McEwen, 2008; Wingfield and Sapolsky, 2003). These costs are not only dependent on maximum GC concentrations but also on the duration of exposure to elevated concentrations (Dallman et al., 1992; Romero et al., 2009; Sapolsky, 1983; Vitousek et al., 2019b; Zimmer et al., 2020b; Zimmer et al., 2019). Elevated GCs also trigger negative feedback within the HPA axis that tightly regulates the stress response, ultimately returning GCs to baseline concentrations (Lattin and Kelly, 2020; Romero, 2004; Vitousek et al., 2019b).

Hormones have been assessed widely in ecology and evolution, in part because circulating levels are easy to measure (Ketterson et al., 2009; Vitousek et al., 2018a; Zera et al., 2007; Zimmer et al., 2020a). Despite this overwhelming focus on circulating hormone levels, they are but one component of a complex signaling system. Over the past decade, researchers have begun to measure intra- and inter-population variation in other key aspects of endocrine systems, including receptors and co-factors in wild animals (Abolins-Abols et al., 2018; Bergeon Burns et al., 2014; Fuxjager et al., 2015; Krause et al., 2015; Lattin et al., 2015; Li et al., 2020; Liebl and Martin, 2013; Rosvall et al., 2012; Rosvall et al., 2016; Schuppe and Fuxjager, 2019; Zimmer et al., 2021). However, most of this work has focused on sex steroid signaling (Fuxjager and Schuppe, 2018; Ketterson et al., 2009; Lipshutz et al., 2019); we know far less about how tightly variation in GC levels is linked with variation in upstream and downstream regulatory components, within and across populations (but see Baugh et al., 2017; Krause et al., 2015; Li et al., 2020; Liebl and Martin, 2013). This represents a significant gap in our understanding of how GCs mediate adaptive responses to stress and how HPA axis varies and evolves as populations diverge (Jimeno and Zimmer, 2022; Zimmer et al., 2022).

Because of variation in trait expression, functional trait linkages and trade-offs can differ across scales of organization (Agrawal, 2020; Hau, 2007; Hau et al., 2016). Thus, there may be different relationships between variation in circulating GCs and other components of the HPA axis at the within-individual, among-individual, population, or species level. For instance, at the inter-population level, local adaptation or developmental plasticity might result in differences in patterns of gene expression within the HPA axis. At the intra-population level, variation in gene expression is unlikely to result from local adaptation, but instead may be mechanistically related to within- or among-individual differences in hormone release, as these components are part of an integrated system (but note that hormone levels and other components of endocrine systems can also vary independently: Hau (2007); Ketterson et al. (2009); Lipshutz et al. (2019). These patterns are not visible by measuring circulating hormone levels alone, but studies looking at covariation between GC profiles and expression of upstream and/or downstream factors in natural populations are rare (but see Baugh et al., 2017; Krause et al., 2015; Lane et al., 2021; Li et al., 2020; Liebl and Martin, 2013). Determining the relationships between GC regulation and hormone receptor expression in the regulatory regions of the HPA axis, at the inter- and intra- population levels, is challenging as both traits can change quickly (Bengston et al., 2018). Yet these efforts are important to ultimately revealing how selection drives HPA axis regulation, and whether the history of selection on GC regulation influences the capacity to cope with unpredictable or changing environments (Zimmer et al., 2020b).

Here, we characterized inter- and intra- population variation in the expression of three components involved in regulating the stress response: mineralocorticoid receptor (MR), glucocorticoid receptor (GR), and corticotropin releasing hormone (CRH). The downstream effects of GCs are mediated primarily through their binding to MR and GR (de Kloet, 2014; de Kloet et al., 1998). Both of these GC receptors are expressed in the main regulatory regions of the HPA axis: the hippocampus, paraventricular nucleus of the hypothalamus, and anterior pituitary gland in mammals and birds (de Kloet, 2014; de Kloet et al., 1998; Dickens et al., 2009; Hodgson et al., 2007; Joëls et al., 2008; Krause et al., 2015; Krause et al., 2021; Senft et al., 2016; Smulders, 2017; Zimmer and Spencer, 2014). MR has a tenfold higher affinity for GCs than GR, resulting in MR being activated at low to moderate GC concentrations and mediating the integrity, stability and sensitivity of the HPA axis (de Kloet, 2014; de Kloet et al., 1998; Joëls et al., 2008). GRs are additionally recruited when GC concentrations increase at daily peaks and in response to unpredictable challenges; this GR binding mediates the phenotypic response associated with the GC stress response and negative feedback (de Kloet, 2014; de Kloet et al., 1998; Joëls et al., 2008; Spencer et al., 2018). GR and MR are each encoded by a single gene and their expression can be shaped by selection as suggested by differences in expression between populations or species (Bauer et al., 2018; Liebl and Martin, 2013; Rosvall et al., 2016). There is also ample evidence of plasticity in the expression of both types of GC receptors. In birds, expression of these receptors within the HPA axis is modulated by early life stress (Banerjee et al., 2012; Zimmer et al., 2017; Zimmer and Spencer, 2014), chronic stress (Dickens et al., 2009), season (Krause et al., 2015; Li et al., 2020; Liebl et al., 2013), social information (Cornelius et al., 2018), acute mate separation (Madison et al., 2018), reproductive timing between subspecies (Bauer et al., 2018) and artificial selection (Hodgson et al., 2007).

CRH is a fundamental part of the HPA axis that acts upstream of glucocorticoids, triggering the pituitary to release ACTH. In response to acute challenges CRH expression increases in the hypothalamus and in extrahypothalamic regions including the hippocampus (Herman et al., 2016; Kovács, 2013; Yao and Denver, 2007). A key element of negative feedback is downregulating CRH expression (Evans et al., 2013; Herman et al., 2016; Kovács, 2013), thus reducing further GC secretion. Acute, chronic, and early life stress usually increase CRH expression; overexpression or deficiency in CRH expression can result in HPA axis dysregulation that affects circulating GC concentrations (Albeck et al., 1997; Evans et al., 2013; Makino et al., 1995; Zito et al., 2017).

We measured gene expression of GR, MR and CRH in female tree swallows *(Tachycineta bicolor)* breeding in three different populations (Alaska, Wyoming, New York). Within each population, we determined whether and how expression of these genes relate to circulating glucocorticoid levels. We have previously used long-term weather data to characterize environmental unpredictability during the breeding period at these sites. That analysis revealed that birds from the Alaska and Wyoming populations face more unpredictable temperatures than birds in New York (Zimmer et al., 2020b). However, the extent to which all birds in the Alaska and Wyoming populations are exposed to highly unpredictable environments likely varies. In Alaska, all tree swallows in the area breed at a similar elevation and thus experience the same unescapable environmental conditions, whereas birds at the high elevation sites we sampled in the Bighorn Mountains of Wyoming appear to be part of a population that breeds along an elevational gradient from low elevation steppe to high elevation plateau. Thus, in Wyoming, individuals may be more or less exposed to the unpredictable conditions of the higher elevations depending on their specific breeding site. It is likely that birds in this population disperse along the elevational gradient as the total distance between high and low elevation sites is ~8 km, which is well within the typical range of natal dispersal for tree swallows (Cohen et al., 1989; Winkler et al., 2005). We recently showed

that environmental unpredictability is associated with variation in GC secretion across these populations. Birds breeding in unpredictable environments (Alaska and Wyoming) have higher baseline GC concentrations, a more robust stress response, and a stronger decrease in GC levels after dexamethasone challenge (i.e., stronger negative feedback) than those breeding in relatively predictable environments (New York and Tennessee) (Zimmer et al., 2020b). Within each of these four populations, the magnitude of the stress response covaries positively with the efficacy of negative feedback (Zimmer et al., 2020b), which could stem from shared regulatory pathways involving CRH and GR effects (Breuner and Orchinik, 2001; de Kloet et al., 1998; Herman et al., 2016; Jeanneteau et al., 2012).

One of the goals of the current study was to determine whether the patterns of expression of these components of the HPA axis (MR, GR and CRH) differ among populations breeding in contrasting environments. We hypothesized that populations could adjust their HPA axis activity to local conditions by modifying the expression of one or more of these components. These adjustments could happen rapidly (through developmental plasticity or reversible phenotypic flexibility) or reflect evolved differences among populations. Based on the general relationships between MR, GR and CRH expression and the different levels of circulating GCs described above, and because baseline and stress-induced GC concentrations are higher and negative feedback is stronger in populations of tree swallows breeding in more unpredictable environments (Zimmer et al., 2020b), we predicted higher levels of expression of both receptors and CRH in more unpredictable environments. Applying this general prediction to the specific study populations suggested a few alternative outcomes. First, if environmental unpredictability at the breeding locations generates differences in brain gene expression, then the AK and WY populations would have higher expression than the NY population. Under this scenario, we would not be able to differentiate whether any population differences stem from plastic or evolved responses to environmental conditions. Second, if environmental unpredictability shapes brain gene expression via local adaptation, then expression would be the highest in AK with lower expression in both WY and NY – because the majority of the WY population breeds at low elevation sites with less unpredictability and thus this population may have limited potential for local adaptation. Finally, if environmental unpredictability does not shape brain gene expression, or only does so via responses to circulating levels of GCs, then we may not see population differences. We acknowledge that environmental unpredictability is far from the only environmental or life history factor that differs among these populations; however, it has frequently been suggested as a primary driver of variation in glucocorticoid levels among populations, which is why we have focused on it here (Guindre-Parker and Rubenstein, 2021; Rubenstein et al., 2016; Schoenle et al., 2018; Wingfield, 2003; Wingfield, 2013; Zimmer et al., 2020b).

A second goal of this study was to determine intra-population variation among components of the HPA axis, as a window into whether and how circulating GC concentrations relate to regulatory components of the HPA axis in free-living animals. We measured baseline and stress-induced MR, GR and CRH mRNA abundance in two of the main regulatory regions of the HPA axis (i.e., hypothalamus, hippocampus) in breeding tree swallow females from these three populations. Overall, we predicted that because GC receptors and CRH are involved in regulating the HPA axis (de Kloet, 2014; Herman et al., 2016; Joëls et al., 2008), and because circulating GC levels can affect expression of these components (Bagamasbad and Denver, 2011), receptors and CRH expression would covary with glucocorticoid hormone levels. As MR mainly regulates baseline GC levels we predicted that individuals with higher MR gene expression would have higher baseline GC concentrations – a pattern that has previously been seen in other species of birds (Baugh et al., 2017; de Kloet et al., 1998; Dickens et al., 2009). As GR is the main mediator of negative feedback, and higher GR expression in the HPA axis has been associated with an attenuated stress response in captive birds (Zimmer et al., 2017; Zimmer and Spencer, 2014), we predicted that GR gene expression would be higher in individuals with stronger negative feedback (those who more quickly return to baseline GC levels after a dexamethasone challenge). We also predicted that MR and GR gene expression would decrease in response to acute stress, as shown in lab rodents (Karandrea et al., 2000; Paskitti et al., 2000). Because CRH acts upstream of GC secretion, and because CRH levels are positively related to baseline GCs in other species (Muglia et al., 1995; Núñez et al., 2008; Stenzel-Poore et al., 1992), we predicted that CRH gene expression would be lower in individuals with lower baseline GC levels. As CRH deficient mice show an impaired GC and CRH increase during an acute stress response (Ginsberg et al., 2003; Muglia et al., 1995), we also expected that CRH would increase in response to an acute stressor, and that this increase would be higher in individuals with stronger GC stress responses. Patterns of gene expression may differ across brain regions (Zimmer and Spencer, 2014); the link between GC levels and patterns of expression may also differ between regions (Inda et al., 2017; Kovács, 2013; Watts, 2005). As the hypothalamus is the primary site of regulatory control of the HPA axis, we predicted tighter correlations between circulating GCs and gene expression in the hypothalamus than in the hippocampus. However, the regulatory effects of MR on the HPA axis occur primarily in the hippocampus as MR is highly expressed in this region (Joëls, 2008; Krause et al., 2021; Sapolsky et al., 2000; Smulders, 2017). Thus, it is possible that the relationship between circulating GCs and MR expression would be stronger in the hippocampus.

## 2. Methods

### 2.1. Populations

We studied three populations of nest-box breeding tree swallows in Ithaca, New York (NY) (42.5°N, 76.5°W, 340 m elevation), Burgess Junction, Wyoming (WY) (44.4°N, 107,2°W, 2451 m elevation), and Anchorage, Alaska (AK) (61.2°N, 149.8°W, 49 m elevation) in 2017 and 2018. Birds breeding in AK and WY face less predictable weather conditions and shorter breeding seasons (Zimmer et al., 2020b) and differ in life-history strategy, investing more in the current reproductive attempt than in self-maintenance (Ardia, 2005; Zimmer et al., 2020b). These populations also differ in the secretion of circulating glucocorticoids (baseline and stress-induced levels and negative feedback) (Zimmer et al., 2020b).

### 2.2. Experimental treatment and brain collection

Nests were monitored every 1–2 days throughout the breeding season from the initiation of activity at each site. For logistical purposes, we focused on females because they are easily captured, though we acknowledge that sexes and life history stages may vary in the endocrine parameters measured here. Females were first caught on incubation day 6 or 7 from 0700 to 1000 h in NY (between May 23 and June 10) and WY (between June 16 and July 9), and from 0600 to 0900 h in AK (between May 28 and June 2) to account for the earlier start of activity due to increased day length. At this capture, we collected a series of blood samples to measure females' HPA axis activity. First, we took an initial blood sample within 3 min of disturbance to measure baseline circulating corticosterone levels. After 30 min of restraint in a cloth bag, we took a second blood sample to measure stress-induced corticosterone levels. Following this sample, all females were injected intramuscularly with dexamethasone (dex) (0.5 $\mu l.g^{-1}$, Dexamethasone Sodium Phosphate, Mylan Institutional LLC), a synthetic glucocorticoid that binds to receptors within the HPA axis, to induce negative feedback (Zimmer et al., 2019). Finally, a third blood sample was taken 30 min after dex injection to measure the degree of downregulation in circulating corticosterone (a measure of negative feedback). This method of measuring negative feedback was previously validated in this species (Zimmer et al., 2019).

Three days after the first capture, females were recaptured during the

same time interval (as described above) for brain collection. Females were randomly assigned to a control treatment (euthanized within 3 min of initial disturbance; $n = 4$ per population) or a stress-induced treatment (held for 90 min after capture in a cloth bag before being euthanized; $n = 8$ per population). We included more birds in the stress-induced treatment so that we could explore relationships between individual variation in gene expression and in the hormonal response to a stressor. Birds were euthanized by overdose of isoflurane followed by rapid decapitation. Brains were immediately removed from the skull using RNA-free tools, flash frozen on dry ice and stored at −80 °C until dissection. A subset of these samples were also used in another study on population variation in thermal tolerance (Woodruff et al., 2022). It is worth noting that gene expression may have been influenced by the capture-restraint stress protocol to which birds were exposed three days before brain collection. While this standardized protocol was performed in the same way in all populations, we cannot rule out effects on subsequent gene expression. All methods were approved by Cornell Institutional Animal Care and Use Committee (protocol #2001–0051).

### 2.3. Corticosterone assay, brain microdissection and qPCR

Following a triple ethyl acetate extraction, corticosterone levels were determined using an enzyme immunoassay kit (DetectX Corticosterone, Arbor Assays: K014-H5) previously validated for tree swallows (Taff et al., 2019). Samples were run in duplicate and all samples from an individual were run on the same plate. The average extraction efficiency was 92.8 % and detection limit was 0.47 $ng.ml^{-1}$. The intra-assay variation was 8.88 % and the inter-assay variation was 11.1 %.

Brains were placed on RNAse free petri dishes on ice for dissection. Brains were dissected into functional regions following (Soma et al., 1999) and as performed previously in tree swallows (Bentz et al., 2019a). We collected the hippocampus by first removing the cerebellum and then making two parallel cuts 1.5 mm lateral to the midline and 1 mm deep. Samples from both hemispheres were pooled. To collect the hypothalamus, we removed the optic tecta, optic chiasm, and hindbrain, and then isolated the hypothalamus to the depth of the anterior commissure (including the preoptic area and ventromedial hypothalamus). The different brain regions were placed in microtubes on dry ice as soon as they were dissected and kept at −80 °C until RNA extraction

We extracted RNA from the hypothalamus and hippocampus, as they are two of the main regions involved in HPA axis regulation. We extracted RNA using the phenol-chloroform-based Trizol method, following the manufacturer's instructions (Invitrogen, Carlsbad, CA). We quantified total RNA with spectrophotometry and treated 1 μg of RNA with DNAse (Promega, Madison, WI) and RNase Inhibitor (Promega, Madison, WI) for reverse-transcription with oligo dT primers and Superscript III (Invitrogen, Carlsbad, CA). For a few samples for which RNA yield or concentration was too low, we performed the reverse transcription using 400 ng of RNA, later adjusting for this modification to roughly equalize the amount of cDNA loaded onto the qPCR plate (see below). The resulting cDNA was used in qPCR to determine mRNA abundance of GR, MR and CRH in the hypothalamus and hippocampus. After testing different reference genes, we determined that peptidyl-prolyl isomerase A (PPIA) was the best reference gene as it did not differ between populations ($F_{2,35.62} = 0.24$, $p = 0.79$), treatment ($F_{1,35.62} = 0.61$, $p = 0.44$) or tissue ($F_{2,35.43} = 0.25$, $p = 0.62$), and so was used for normalization. These PPIA primers have been previously used in tree swallows (F: AGAAGGGATTTGGCTACAAGG, R: CCATTGTGGCGTGTGAAGT, (Bentz et al., 2019b), which maps to only one product in birds (accession number: XM_033081460.2). We also verified 102.01 % efficiency in tree swallow brain samples. Primers for GR (F: TGAAGAGCCAGTCCCTGTAG, R: CAACCACATCATGCATAGAGTCCAGCA) and MR (F: AAGAGTCGGCCAAACATCCTTGTTCT, R: AAGAAACGGGTGGTCCTAAAATCCCAG) were obtained from Banerjee et al. (2012), and validated in tree swallow brain, with 97.05 and 103.10 % efficiency, respectively. In silico analyses also link each of these primer sets to a single product: XM_033073002.2 for GR, and XM_030270212.3 for MR. We designed CRH primers here (F: CCGTGTACCAAGTGCAGAA, R: CGTAGCGATGGCACTAGAATAA) using sequences from Bentz et al. (2019b), which map to only one product in birds (accession number: XM_049823989.1). We validated these primers using serial dilution (efficiency 103 %), confirming that they meet the assumptions of qPCR.

All qPCR reactions (10 μl) were run in triplicate, alongside no template controls (NTCs), in a QuantStudio 5 Real-Time PCR System (ThermoFisher Scientific, Waltham, MA) using PerfeCta SYBR Green SuperMix with low ROX (Quanta Biosciences, Gaithersburg, MD). Each well contained 3 μl cDNA diluted 1:50 (except for samples with low RNA yield, which used 1:20 in order to end up with the final cDNA concentration), or 3 μl water for NTCs, and primers diluted 1:20 for a total volume of 10 μl. cDNA dilutions were selected to optimize efficiency across all samples within a tissue. Thermocycling conditions were: 10 min at 95 °C, followed by 40 cycles of 95 °C for 30 s, 60 °C for 1 min, and 70 °C for 30 s. A final melting phase of 95 °C for 1 min, 55 °C for 30 s, and 95 °C for 30 s was run to confirm single-product specificity for each gene for each sample.

We used Relative Quantification in Thermo Fisher Cloud to calculate mRNA abundance using the comparative Ct method ($2^{-\Delta\Delta\ Ct}$), which reports mRNA abundance for each gene of interest as the fold change in expression compared to a calibrator sample and normalized to an internal reference gene (Livak and Schmittgen, 2001). The calibrator for each tissue was a pool of control neural tissue cDNA, and we used PPIA as an internal reference. The $2^{-\Delta\Delta\ Ct}$ method allows for comparison of mRNA abundance within genes across treatments, tissues, and individuals.

### 2.4. Data analysis

We used three generalized linear mixed models (GLMMs) fitted with a gamma distribution to compare mRNA abundance of each gene of interest (GR, MR and CRH) between populations, treatments and tissues. Population, treatment, tissue and their interaction were specified as fixed factors and female identity as a random factor in each model.

Within each population, we then asked which aspect(s) of females' HPA axis activity best predicted the relative expression of each gene of interest in both parts of the HPA axis (hypothalamus and hippocampus). With mRNA abundance as the response variable, we compared a set of nine candidate generalized linear models (GLM) fit with a gamma distribution to determine the best predictors of variation in GR, MR and CRH expression in the hypothalamus and hippocampus within each population. Because of the small sample size, the models were limited to one or two factors and their interactions (Table 1); thus we were not able to statistically test whether within-population relationships between gene expression and HPA axis activity differed among populations. The models included a subset of the variables baseline corticosterone, stress-

**Table 1**
List of candidate models to determine the best predictors of intra-population variation in the relative expression of MR, GR and CRH in the hypothalamus and hippocampus.

| Candidate models | k |
|---|---|
| Null | 1 |
| Baseline corticosterone | 2 |
| Stress-induced corticosterone | 2 |
| Post-dex corticosterone | 2 |
| Baseline corticosterone + Stress-induced corticosterone + Baseline corticosterone*Stress-induced corticosterone | 4 |
| Stress-induced corticosterone + Post-dex corticosterone + Stress-induced corticosterone*Post-dex corticosterone | 4 |
| Baseline corticosterone + Post-dex corticosterone + Baseline corticosterone*Post-dex corticosterone | 4 |
| Treatment | 3 |
| Relative Clutch Initiation Day | 2 |

induced corticosterone, and post-dex corticosterone. To account for the possibility that the response to stress is influenced by interactions among different aspects of HPA axis activity (Vitousek et al., 2018b; Zimmer et al., 2019), we constructed models that included two-way interactions between the measured components of HPA axis activity. We also determined whether the different aspects of HPA activity were correlated within each population using Spearman correlations. We considered treatment as a predictor to account for possible differences between baseline and stress-induced expression. Finally, we considered relative clutch initiation date as a predictor, because clutch initiation date has been previously linked with female quality (Winkler and Allen, 1995). Best-fit models were identified by comparing the corrected Akaike information criterion (AICc) scores of the candidate models and models within 2 ΔAICc of the best fit model were considered has having meaningful support.

GLMMs were run using the GLIMMIX procedure and GLMs were run using the GENMOD procedure in SAS University Edition (SAS Institute Inc., Cary, NC, USA). Post-hoc comparisons were performed using Tukey-Kramer multiple comparison adjustment to obtain corrected *p*-values. Probability levels $\leq 0.05$ were considered significant.

## 3. Results

### 3.1. Inter-population differences in gene expression

In the overall model, acute stressor treatment did not significantly affect mRNA abundance for any genes ($F_{2,35.14} \leq 1.84$, $p \geq 0.179$). Abundance of GR mRNA differed among populations and tissues (population*tissue: $F_{2,35.95} = 4.92$, $p = 0.004$). GR relative expression was the highest in the hypothalamus of Alaska females and significantly differed from GR expression in the hypothalamus of both New York and Wyoming females, and from GR expression in the hippocampus in birds from all three populations ($t \geq 2.94$, $p \leq 0.048$; Fig. 1a). GR expression in the hypothalamus did not differ between New York and Wyoming ($t = 0.96$, $p = 0.932$; Fig. 1a) and within these populations did not differ from GR expression in the hippocampus ($t \leq 1.31$, $p \geq 0.781$; Fig. 1a). In the hippocampus, GR expression did not differ among the three populations ($t \leq 0.62$, $p \geq 0.989$; Fig. 1a).

The abundance of MR mRNA differed among populations ($F_{2,35.13} = 19.20$, $p < 0.0001$). MR relative expression was higher in Alaska than in New York and Wyoming in both tissues ($t \geq 4.69$, $p \leq 0.0001$; Fig. 1b). MR relative expression was also higher in the hippocampus than in the hypothalamus ($F_{1,34.95} = 18.14$, $p = 0.0001$; Fig. 1b).

The abundance of CRH mRNA differed among populations and between tissues (population*tissue: $F_{2,33.95} = 6.17$, $p = 0.003$). CRH relative expression was highest in the hippocampus of Wyoming females, where it was significantly higher than in the hippocampus of females in New York or Alaska ($t \geq 3.27$, $p \leq 0.020$; Fig. 1c). CRH expression in the hypothalamus did not differ among the three populations ($t \leq 2.30$, $p \geq 0.206$; Fig. 1c). Within each population, CRH expression did not differ between the hypothalamus and hippocampus ($t \leq 2.81$, $p \geq 0.068$; Fig. 1c).

### 3.2. Predictors of intra-population variation gene expression

We fitted a set of candidate models to determine the best predictors of GR, MR and CRH expression in the hypothalamus and hippocampus within each population (Tables 1, 2). In the three populations, baseline and stress induced corticosterone levels and baseline and post-dex corticosterone levels were not correlated ($r \leq 0.31$, $p \geq 0.331$). However, stress-induced and post-dex corticosterone levels were correlated in New York ($r = 0.71$, $p = 0.012$) and in Wyoming ($r = 0.68$, $p = 0.018$) but not in Alaska ($r = 0.44$, $p = 0.167$).

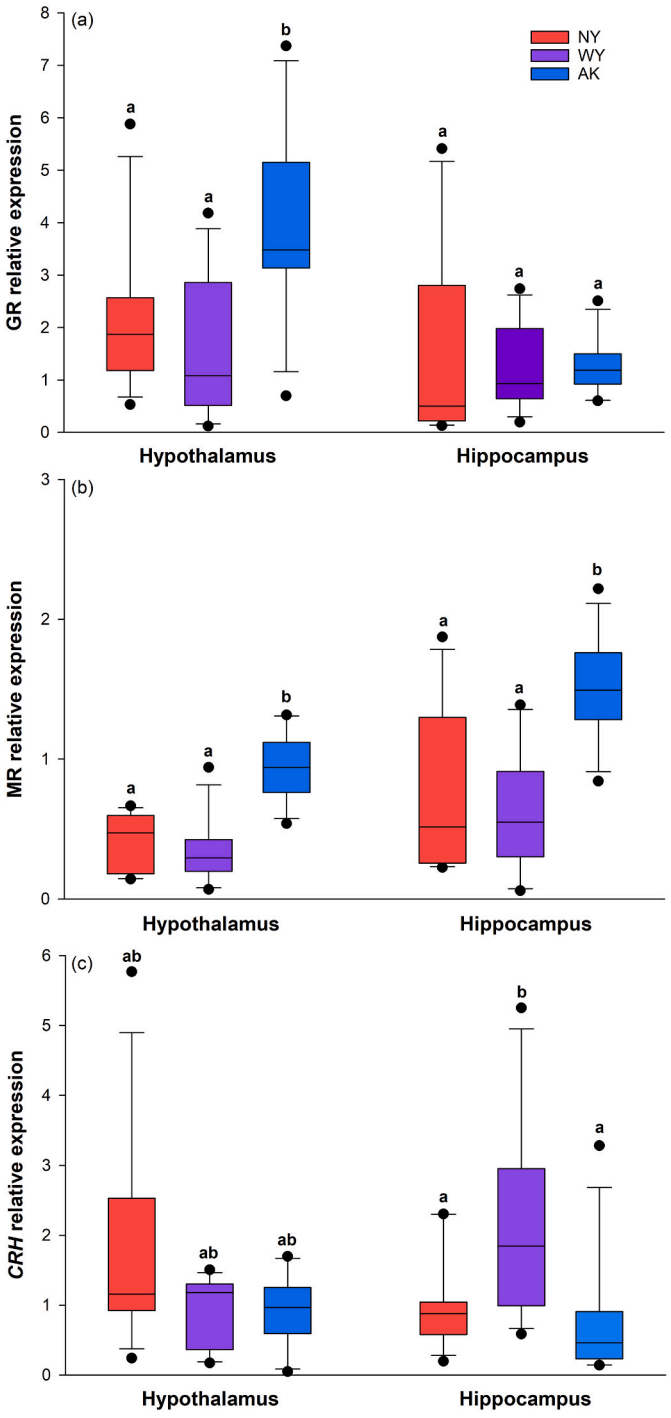


**Fig. 1.** Inter-population differences in the relative expression of (a) the glucocorticoid receptor (GR), (b) the mineralocorticoid receptor (MR) and (c) the corticotropin-release hormone (*CRH*) in the hypothalamus and the hippocampus in females breeding in New York (red bar), Wyoming (purple bar) and Alaska (blue bar). Relative mRNA abundance is a unitless quantity ($2^{-\Delta\Delta Ct}$), which depicts mRNA abundance in each sample normalized to a reference gene (PPIA) and relative to a calibrator sample separately for each gene. Bars show 10th and 90th percentiles and dots show outliers that are outside these percentiles. Different letters indicate significant differences, in (b) they indicate significant differences between populations only as the interaction between population and brain regions is not significant.

**Table 2**
The best fit models of intra-population variation in GR, MR and CRH relative expression in the hypothalamus and hippocampus in females in New York, Wyoming and Alaska. All candidate models within 2 ΔAICc of the best fit model, and the null model, are shown.

| Population | Tissue | Gene | Candidate models | *k* | LL | ΔAICc | Weight |
|---|---|---|---|---|---|---|---|
| New York | Hypothalamus | GR | Post-dex corticosterone | 2 | −10.18 | 0 | 0.61 |
| | | | Null | 1 | −23.12 | 2.78 | 0.15 |
| | | MR | Baseline corticosterone | 2 | −0.3 | 0 | 0.49 |
| | | | Null | 1 | −2.05 | 1.86 | 0.20 |
| | | CRH | Stress-induced corticosterone | 2 | −15.3 | 0 | 0.35 |
| | | | Treatment | 3 | −14.99 | 1.37 | 0.18 |
| | | | Relative Clutch Initiation Day | 2 | −13.42 | 1.51 | 0.17 |
| | | | Null | 1 | −17.43 | 1.59 | 0.16 |
| | Hippocampus | GR | Baseline corticosterone | 2 | −4.83 | 0 | 0.58 |
| | | | Null | 1 | −15.43 | 12.81 | 0.00 |
| | | MR | Null | 1 | −6.66 | 0 | 0.37 |
| | | | Treatment | 3 | −4.61 | 0.57 | 0.28 |
| | | CRH | Null | 1 | −9.34 | 0 | 0.44 |
| Wyoming | Hypothalamus | GR | Post-dex corticosterone | 2 | −10.78 | 0 | 0.74 |
| | | | Null | 1 | −17.24 | 3.54 | 0.13 |
| | | MR | Baseline corticosterone | 2 | −9.34 | 0 | 0.37 |
| | | | Null | 1 | −8.77 | 1.52 | 0.17 |
| | | CRH | Null | 1 | −8.7 | 0 | 0.39 |
| | | | Relative Clutch Initiation Day | 2 | −7.82 | 1.92 | 0.15 |
| | Hippocampus | GR | Null | 1 | −6.12 | 0 | 0.72 |
| | | MR | Null | 1 | −5.57 | 0 | 0.31 |
| | | | Baseline corticosterone | 2 | −4.45 | 1.42 | 0.15 |
| | | | Relative Clutch Initiation Day | 2 | −4.63 | 1.77 | 0.13 |
| | | | Stress-induced corticosterone | 2 | −4.68 | 1.89 | 0.12 |
| | | CRH | Baseline CORT | 2 | −14.69 | 0 | 0.76 |
| | | | Null | 1 | −18.57 | 4.08 | 0.10 |
| Alaska | Hypothalamus | GR | Stress-induced corticosterone *Post-dex corticosterone | 4 | 1.06 | 0 | 0.94 |
| | | | Null | 1 | −18.84 | 12.29 | 0.00 |
| | | MR | Baseline corticosterone | 2 | 2.6 | 0 | 0.39 |
| | | | Null | 1 | 0.44 | 0.39 | 0.32 |
| | | CRH | Baseline corticosterone | 2 | −5.55 | 0 | 0.35 |
| | | | Null | 1 | −8.11 | 0.84 | 0.23 |
| | | | Post-dex corticosterone | 2 | −5.97 | 0.85 | 0.23 |
| | Hippocampus | GR | Null | 1 | −8.04 | 0 | 0.74 |
| | | MR | Treatment | 3 | −2.92 | 0 | 0.48 |
| | | | Null | 1 | −4.79 | 1.08 | 0.28 |
| | | CRH | Baseline corticosterone | 2 | −4.15 | 0 | 0.80 |
| | | | Null | 1 | −8.65 | 5.34 | 0.06 |

Models with interaction also include the main effects. Models are generalized linear models with a gamma distribution. *k* is the number of parameters.

### 3.3. New York

In New York, GR expression in the hypothalamus was best predicted by post-dex corticosterone level ($\chi_{1,10} = 5.86$, $p = 0.016$; Table 2). GR expression decreased with increasing post-dex corticosterone ($\beta = -0.033 \pm 0.013$; Fig. 2a). MR expression in the hypothalamus was best predicted by baseline corticosterone level ($\chi_{1,11} = 6.11$, $p = 0.013$; Table 1). The best-fit model and the null model were within 2ΔAICc but the model including baseline corticosterone still received substantial support (Weight: 0.49 vs. 0.20). In the best-fit model, MR expression increased with increasing baseline corticosterone ($\beta = 0.34 \pm 0.14$). The best predictor of CRH expression in the hypothalamus was stress-induced corticosterone level ($\chi_{1,11} = 4.79$, $p = 0.029$; Table 2); in this model CRH expression increased with increasing stress-induced corticosterone ($\beta = 0.023 \pm 0.010$). However, three other models were within 2ΔAICc of the best-fit model; a model including treatment, a model including clutch initiation day, and the null model (Table 2), suggesting limited support for these predictors.

In the hippocampus, GR expression was best predicted by baseline corticosterone level ($\chi_{1,10} = 5.85$, $p = 0.015$; Table 2). GR expression decreased with increasing baseline corticosterone ($\beta = -0.23 \pm 0.09$). The best model for MR and CRH expression in the hippocampus was the null model (Table 2).

### 3.4. Wyoming

In Wyoming, GR expression in the hypothalamus was best predicted by post-dex corticosterone level ($\chi_{1,10} = 3.97$, $p = 0.047$; Table 2). GR expression decreased with increasing post-dex corticosterone ($\beta = -0.05 \pm 0.028$; Fig. 2b). MR expression in the hypothalamus was best predicted by baseline corticosterone level ($\chi_{1,11} = 6.68$, $p = 0.010$; Table 2). MR expression increased with increasing baseline corticosterone ($\beta = 0.20 \pm 0.08$). The best-fit model for CRH expression in the hypothalamus was the null model (Table 2).

In the hippocampus, the best-fit model for GR and MR expression was the null model (Table 2). CRH expression in the hippocampus was best predicted by baseline corticosterone level ($\chi_{1,11} = 14.34$, $p = 0.0002$; Table 2). CRH expression in the hippocampus increased with increasing baseline corticosterone ($\beta = 0.037 \pm 0.009$).

### 3.5. Alaska

In Alaska, the best-fit model for GR expression in the hypothalamus included stress-induced corticosterone, post-dex corticosterone levels, and their interaction (Table 2). GR expression was the greatest in females exhibiting both elevated stress-induced corticosterone and a low post-dex corticosterone level (Table 3, Fig. 2c). MR and CRH expression were best predicted by baseline corticosterone level (Table 2). In both cases, the null model and the best-fit model were within 2ΔAICc and both received about the same support (Table 2). In the best-fit model, MR expression increased with increasing baseline corticosterone ($\beta = 0.042 \pm 0.05$, $\chi_{1,10} = 1.36$, $p = 0.243$) while CRH expression decreased with increasing baseline corticosterone ($\beta = -0.62 \pm 0.27$, $\chi_{1,9} = 5.36$, $p = 0.021$).

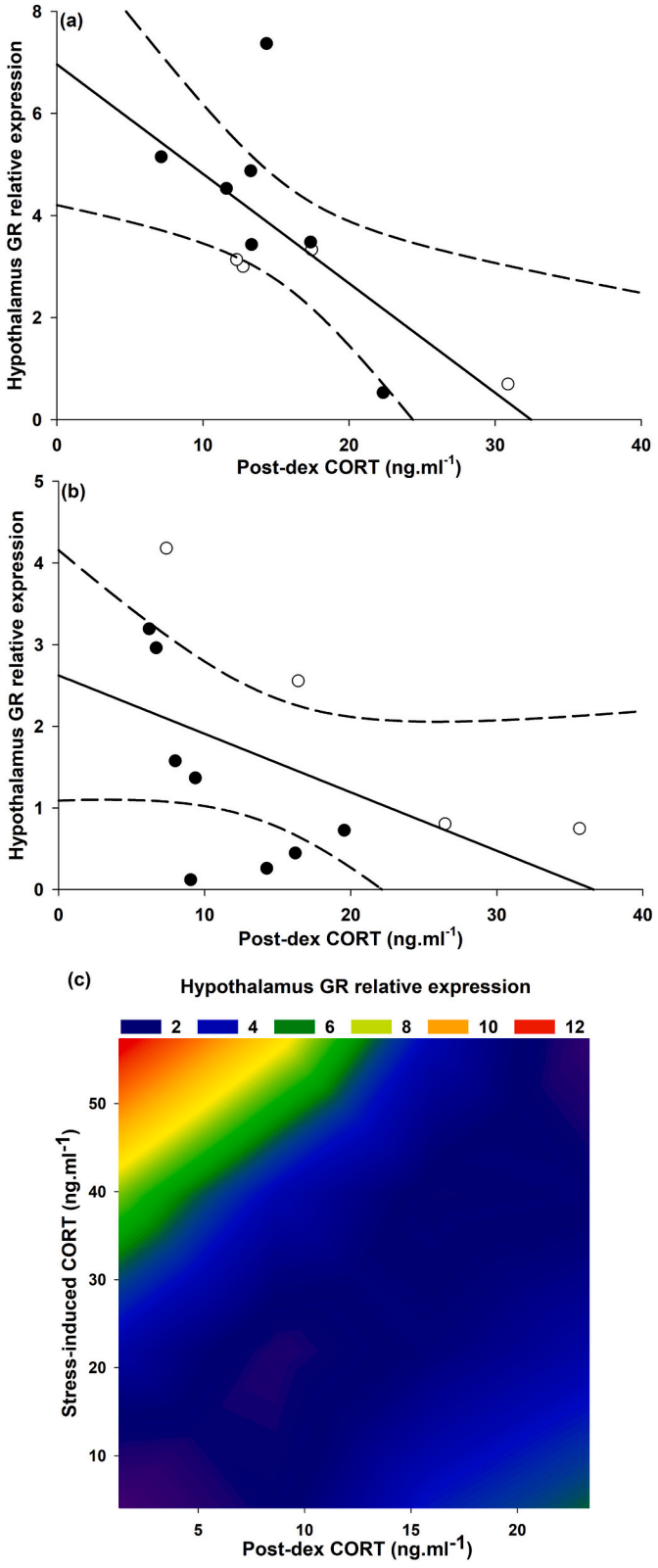


**Fig. 2.** Relationship between intra-population variation in GR relative expression in the hypothalamus and post-dex CORT in females breeding (a) in New York and (b) in Wyoming. Open circles show individuals euthanized at baseline and closed circles show individuals euthanized after 90 min of restraint. (c) Contour plot showing the relationship between GR relative expression in the hypothalamus and stress-induced and post-dex CORT levels in females breeding in Alaska. Warmer colors indicate higher relative expression.

**Table 3**
Best fit model of intra-population variation in GR expression in the hypothalamus in Alaska.

| Predictor | Estimate | SE | $\chi^2$ | p-value |
|---|---|---|---|---|
| Intercept | 1.3544 | 0.1118 | 146.64 | <0.0001 |
| Stress-induced corticosterone | 0.0171 | 0.0027 | 39.01 | <0.0001 |
| Post-dex corticosterone | −0.0135 | 0.0096 | 1.97 | 0.161 |
| Stress-induced corticosterone *Post-dex corticosterone | −0.0009 | 0.0003 | 11.24 | 0.0008 |

In the hippocampus, the best-fit model for GR expression was the null model. MR expression was best predicted by the experimental treatment ($\chi_{1,11} = 4.03$, $p = 0.045$; Table 2). However, the best fit model and the null model were within 2ΔAICc. In the best-fit model, MR expression was higher in the stress-induced group (1.65 ± 0.11) than in the control group (1.24 ± 0.19). CRH expression was best predicted by baseline corticosterone ($\chi_{1,11} = 14.47$, p = 0.0001; Table 2). CRH expression increased with increasing baseline corticosterone levels (β = 0.56 ± 0.15).

## 4. Discussion

Tree swallows that breed in different environments differed in gene expression of both GR and MR in the brain. Birds from one population breeding in an unpredictable environment (Alaska) showed elevated expression of GR and MR. In contrast, birds from a population that inhabits a predictable environment (New York), and birds from a population that ranges across unpredictable high elevation habitat and predictable low elevation habitat (Wyoming), showed similarly low levels of GR and MR expression in the brain. We also found that, as predicted, intra-population differences in circulating GCs are associated with intra-population differences in the expression of genes involved in regulating the HPA axis. Although these patterns were, to a certain extent, similar across populations, there were also cases in which trait linkages differed across populations. To the degree that these transcriptional patterns predict function, our results suggest that differences in GC levels can reflect underlying differences in regulatory components; however, they also suggest flexibility in endocrine trait linkages that may diverge among populations.

### *4.1. Inter-population differences in gene expression*

Previous work has shown that tree swallows breeding in both Alaska and Wyoming maintain higher GC levels than birds that breed in more predictable environments (Zimmer et al., 2020b). Here, we found that MR and GR gene expression did not follow the same pattern. Instead, birds in Alaska had higher MR expression in the hypothalamus and hippocampus, and higher GR expression in the hypothalamus, than birds breeding in either Wyoming or in New York. These patterns could result from local adaptation to environmental unpredictability. The birds sampled in Wyoming, which showed relatively low GR and MR expression despite breeding in a harsh and unpredictable environment, are part of a population that breeds more extensively in low elevation, more environmentally consistent steppe regions, but also ranges up to the harsher, high elevation site that we sampled. Because the distance between these sites (~8 km) is smaller than the standard natal dispersal distance of tree swallows (Cohen et al., 1989; Winkler et al., 2005), we expect that birds in this population are exposed to more or less unpredictable conditions depending on their specific nesting site, and thus would be unlikely to show significant local adaptation to highly unpredictable conditions, even if such local adaptation is possible. As thermal unpredictability was similar in Alaska and Wyoming, plasticity generated by exposure to unpredictable weather would be expected to result in similarly high levels of gene expression in both populations. Thus, the observed inter-population patterns in gene expression are

unlikely to result from a plastic response to thermal unpredictability. However, these differences could also result from developmental plasticity or reversible phenotypic flexibility induced by other experiential factors – and we expect that overall, variation in gene expression is influenced both by genetic background and environment.

Interestingly, inter-population differences in gene expression differ from previously observed inter-population differences in baseline, stress-induced, and post-dex (a measure of negative feedback) corticosterone levels. These levels were previously shown to be best predicted by current environment: birds in both Alaska and Wyoming have higher GC levels and stronger negative feedback than birds in more predictable environments (New York and Tennessee) (Zimmer et al., 2020b). Taken together these findings indicate that birds in Wyoming maintain elevated GC levels without a concomitant increase in neural GR and MR gene expression. This semi-independent regulation of hormone and receptor mRNA suggests that these components of the HPA axis may vary, and be shaped by, different external processes. For instance, GC levels may be more related to recent or developmental environments (Zimmer et al., 2020b), but GR and MR, which are each encoded by a single gene, might instead be shaped by local adaptation, consistent with other studies indicating population or interspecific differences in expression of these genes (Bauer et al., 2018; Liebl and Martin, 2013; Rosvall et al., 2016). Nonetheless, there is also ample evidence of plasticity in the expression of both types of GC receptors (Cornelius et al., 2018; Dickens et al., 2009; Krause et al., 2015; Li et al., 2020; Liebl et al., 2013; Madison et al., 2018; Zimmer et al., 2017; Zimmer and Spencer, 2014). Determining the extent to which GR and MR expression are heritable traits that can respond to selection, and the extent to which their expression evolves in response to environmental unpredictability, or other aspects of the physical and social environment, are important future directions. Additionally, broader-scale comparisons with replicate sites could provide particularly powerful tests of the role environmental unpredictability in driving gene expression.

A primary role of GCs binding to GR is to trigger negative feedback, thereby preventing the stress response from overshooting (de Kloet, 2014; de Kloet et al., 2019). Thus, elevated GR expression in unpredictable environments such as Alaska could help birds respond efficiently to and recover faster from challenges by increasing negative feedback efficacy. It has been previously shown in captive quail that higher GR expression in the HPA axis is associated with stronger negative feedback, resulting in better performance under challenging conditions (Zimmer et al., 2013; Zimmer et al., 2017; Zimmer and Spencer, 2014). GCs binding at MR receptors contribute to regulating foraging and metabolism (Landys et al., 2006). Thus, it stands to reason that the energetically challenging and unpredictable environment of Alaska may favor higher MR expression, like what we observed in our data. Similarly, baseline GC concentrations are often elevated in individuals or populations facing more energetically challenging conditions or engaged in energetically demanding activities such as investment in reproduction (Apfelbeck et al., 2017; Bonier et al., 2011; Hau et al., 2010; Jessop et al., 2013; Vitousek et al., 2019a).

CRH gene expression also differed at the inter-population level, although the observed patterns were not concordant with environmental unpredictability: females breeding in Wyoming exhibited higher CRH expression in the hippocampus than females breeding in Alaska or New York. One possible explanation for this pattern is that during the time birds in this study were incubating, the Wyoming population experienced a 7-day period of inclement weather that reduced the body mass of females and increased nest abandonment (Zimmer et al., 2020b). CRH in the limbic structure, including the hippocampus and amygdala, is involved in modulating the behavioral response to challenges and may have anxiolytic and appetitive effects (Dedic et al., 2019; Dedic et al., 2018; Inda et al., 2017; Paretkar and Dimitrov, 2018). Thus, the higher expression of CRH in the hippocampus in Wyoming females could reflect a response to these recent environmental conditions. It is also important to note that we measured mRNA abundance, not protein abundance or hormone-receptor binding. Follow-up work will be important to discern whether the observed expression differences persist at the post-translational level.

### 4.2. Individual predictors of gene expression at the intra-population level

We evaluated the best predictors of GR, MR and CRH expression within each population. We predicted that higher GR gene expression would be most strongly associated with negative feedback (lower post-dex GC levels), and that this pattern would generally be stronger in the hypothalamus than in the hippocampus. Negative feedback is primarily triggered by GCs binding to GR within the HPA axis, particularly in the hypothalamus, which is the main regulatory region for negative feedback in the brain and highly expresses GR (Baugh et al., 2017; Cornelius et al., 2018; de Kloet, 2014; Sapolsky et al., 2000; Senft et al., 2016; Zimmer and Spencer, 2014). Within all three of our study populations, females with higher GR expression in the hypothalamus had stronger negative feedback. This relationship has been previously shown in captive birds (Zimmer et al., 2017; Zimmer and Spencer, 2014), but to the best of our knowledge, this is the first study showing that higher expression of GR in the hypothalamus is associated with stronger negative feedback in a free-living bird.

Among tree swallows breeding in Alaska, individuals with higher GR gene expression in the hypothalamus showed stronger negative feedback but also a stronger GC stress response (Fig. 2c). Note that in Alaska, unlike in NY and WY, these traits were not correlated. We have previously suggested that this phenotype, where a strong stress response is associated with strong negative feedback, may allow individuals to respond appropriately to unpredictable challenges while limiting the costs associated with exposure to high GC concentrations and thus may be selected for in harsh environments (Zimmer et al., 2020b). Tree swallows breeding in Alaska have stronger stress responses and stronger negative feedback than birds breeding in New York or Wyoming during chick rearing, and stronger stress responses and negative feedback than birds breeding in New York during incubation (Zimmer et al., 2020b). Additionally, long-term studies in New York have shown that females exhibiting a robust stress response followed by strong negative feedback are more stress resilient – less likely to abandon their nest when exposed to challenging conditions – than individuals that do not show this combination of traits (Zimmer et al., 2019). This phenotype, where a strong stress response is associated with strong negative feedback, could result from functional links between peak GC secretion and negative feedback (Zimmer et al., 2019), which is triggered by GC-GR binding in the hypothalamus (de Kloet, 2014); thus, in response to a challenge, higher GC concentrations should recruit more GRs resulting in quicker induction and stronger negative feedback. The strength of negative feedback is likely to be heightened in individuals expressing high levels of GR in the hypothalamus (Jimeno and Zimmer, 2022; Lattin et al., 2016; Romero, 2004; Zimmer et al., 2019). Over time, elevated GC levels could also have causal effects on GR expression (Dickens et al., 2009; Zimmer and Spencer, 2014). These patterns suggest that elevated GR expression in the hypothalamus may be an important adaptation for coping with unpredictable environments by allowing the fine-tuning of HPA axis regulation in response to unpredictable challenges.

MR is mainly involved in controlling HPA axis stability and baseline levels of GCs (de Kloet, 2014; Sapolsky et al., 2000; Smulders, 2017). Thus, we predicted that higher MR gene expression would be associated with higher baseline GC concentrations, and again, that these relationships would be stronger in the hypothalamus than in the hippocampus. Consistent with this view, in all three populations, we found that MR expression in the hypothalamus was best predicted by baseline GC concentrations with higher expression associated with higher baseline GCs. However, unlike with GR, these relationships were generally weak. MR gene expression in the hippocampus did not predict baseline GCs in any of the populations, though the effects of MR on HPA axis regulation are thought to occur primarily in the hippocampus as MR is highly

expressed in this region (Krause et al., 2021; Sapolsky et al., 2000; Smulders, 2017). In birds, however, while MR expression is higher in the hippocampus than in the hypothalamus, MR is also expressed in the hypothalamus (this study; Senft et al., 2016; Zimmer and Spencer, 2014). It is possible that even subtle variations in MR expression in tissues where it is moderately expressed (e.g. hypothalamus) could result in changes in GC regulation, whereas larger changes would be necessary to see a similar effect in tissues with high MR expression, as previously suggested for other genes such as *FKBP5* (Scharf et al., 2011).

CRH is a fundamental part of the HPA axis, triggering the pituitary to release ACTH. Its expression increases in the hypothalamus and in extrahypothalamic regions including the hippocampus in response to acute challenges (Herman et al., 2016; Kovács, 2013; Yao and Denver, 2007). Thus, we predicted that CRH gene expression would be more tightly linked with baseline levels under non-stressed conditions and with stressed levels under acute stress conditions. However, as discussed below, we did not detect evidence of acute stress-induced changes in gene expression. Consistent with our predictions for baseline conditions, in both Wyoming and Alaska, females with higher CRH gene expression in the hippocampus showed higher baseline GC concentrations. Additionally, in Alaska only, low CRH gene expression in the hypothalamus was associated with high baseline GC levels. These opposite relationships between CRH and baseline GC levels in the hypothalamus and the hippocampus are consistent with the inhibiting effect of GCs on CRH release in the hypothalamus and the stimulating effect of GCs on CRH in extrahypothalamic regions (Inda et al., 2017; Kovács, 2013; Watts, 2005). These same patterns were not found in the New York population, in which we saw that CRH was not linked to baseline but to stress-induced GC levels: females with higher CRH gene expression in the hypothalamus showed higher stress-induced GC levels. Thus, unlike for MR and GR, where the patterns were largely consistent across populations within tissue types, the relationships between CRH expression and GC levels differed across populations. Because there are several regulatory steps in between the release of CRH and the release of GCs, within- or among-population variation in these components could affect the degree to which variation in CRH affects circulating GCs.

It is important to note that hormone and gene expression data in our study were collected three days apart to allow for measurement of both stress responses and brain collection under baseline conditions. We cannot rule out lingering effects of the first capture experience on the expression of CRH or other genes. Nevertheless, these findings may also have implications for the long-standing question in evolutionary endocrinology of whether hormonal pleiotropy facilitates evolution or acts as an evolutionary constraint. The differing relationships between CRH expression and GC levels seen within and across populations suggest flexible trait linkages that could shape evolutionary trajectories (Hau, 2007; Ketterson et al., 2009).

### 4.3. Measuring the effects of acute stress on gene expression

We predicted that gene expression of MR and GR would be downregulated and CRH upregulated in individuals exposed to a restraint stress for 90 min. However, at the inter-population level we saw no effect of stressor treatment on the expression levels of any genes except in models with weak support. Similarly, at the intra-population level, treatment was not identified as a predictor of the expression of any genes, except in models with weak support. This general lack of effect of restraint stress may relate to our limited sample sizes, or it may be the result of the time required for changes in gene expression to occur. Some previous studies in other bird species did not find an effect of 30 or 60 min of restraint stress on GR and MR gene expression in the hippocampus and hypothalamus (Calisi et al., 2018; Krause et al., 2021), whereas 90 min of restraint downregulated GR gene expression in gonadal tissue (Abolins-Abols et al., 2018). In lab rodents, results vary: some studies have detected changes in GR and/or MR gene expression 60–75 min after the onset of an acute (Karandrea et al., 2000; Morsink et al., 2006; Romeo et al., 2008), while some have found no evidence of changes until 120 min after exposure (Paskitti et al., 2000). In our study, we aimed to minimize distress by selecting the shortest interval in which changes might be detectable, and we also knew from our standard capture and release protocols that wild tree swallows tolerate 90 min of restraint well. It is possible that this interval may not have been long enough to determine how acute stress affects the expression of GR and MR genes in tree swallows. As noted above, it is also possible that gene expression patterns were affected by experiencing a standardized stressor three days prior to brain collection (capture and restraint for 60 min to measure GC levels). Since all individuals experienced this prior stressor, it is possible that further changes were not induced by this later acute challenge on the day of tissue collection. It is not clear for how long disturbances affect gene expression in birds. On the one hand, a recent study in tree swallows found social competition affects hypothalamic regulatory networks and methylation for hundreds of genes, including GR, CRH, and others related to hormone signaling, some of which were still affected for at least two days after competition subsided (Bentz et al., 2021). On the other hand, longer-term stress does not appear to affect GR and MR across most tissues in house sparrows, including in the brain (Lattin and Romero, 2014).

### 4.4. Implications and future directions

These results reveal that components of the HPA axis exhibit marked within- and among-population variation, at least some of which maps onto differences in local environments and GC secretion. These findings suggest that local adaptation and/or phenotypic plasticity modify different components of the HPA axis to produce different GC phenotypes in different environments. Our findings also underscore the insights that can be gained by field studies that measure circulating hormone concentrations alongside other endocrine traits, particularly since there may be multiple mechanistic paths to achieving a particular endocrine trait (sensu Rosvall, 2022). A long-standing question in evolutionary endocrinology is whether selection acts equivalently on circulating hormone levels and receptors; alternatively, one type of trait (e.g., receptors) may be the primary target of selection but other traits (e.g., circulating hormones) could also predict fitness as a result of phenotypic correlations with the trait under selection (e.g., Ball and Balthazart, 2008; Fuxjager and Schuppe, 2018; Hau, 2007; Ketterson et al., 2009; Lipshutz et al., 2019). This question cannot be resolved by measuring only hormones, or only receptors. Instead, addressing it will require taking a more rigorous evolutionary approach to the study of multiple endocrine regulatory traits, including by estimating the heritability of receptors (and other mediators and cofactors), their genetic vs. phenotypic correlations with other traits, and their relationships with fitness. Significant strides have been made in recent years in determining the heritability of different measures of circulating GCs, the extent to which they are genetically correlated, and the degree to which they predict fitness (e.g., Bairos-Novak et al., 2017; Béziers et al., 2019; Bonier et al., 2009; Breuner and Berk, 2019; Jenkins et al., 2014; Schoenle et al., 2021; Schoenle et al., 2018; Stedman et al., 2017); however, similar studies on receptors and other regulatory components are lacking (but see, e.g., Patterson et al. 2014). To some extent this stems from the logistical constraints involved in robustly measuring heritability and genetic correlations, alongside measuring the performance and fitness correlates of traits that may require terminal sampling (e.g., receptor expression in the brain). Future field studies that take an explicitly evolutionary approach to HPA axis function, using larger numbers of populations and species to test how different regulatory components covary, and how they are shaped by selection, are likely to yield significant insights. We are hopeful that such insights will contribute to moving our field away from the false dichotomy of whether variation in hormones or receptors are *more* important to endocrine function, and toward a systemic approach to understanding how variation in multiple components of these complex systems

contributes to variation in endocrine function – and ultimately in the ability to survive and thrive in challenging environments.

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