Acute restraint stress does not alter corticosteroid receptor or 11-Hydroxysteroid dehydrogenase gene expression at Hypothalamic-Pituitary-Adrenal axis regulatory sites in captive male white-crowned sparrows (Zonotrichia leucophrys gambelii)

Acute restraint stress does not alter corticosteroid receptors or 11β-hydroxysteroid dehydrogenase gene expression at hypothalamic-pituitary-adrenal axis regulatory sites in captive male white-crowned sparrows (*Zonotrichia leucophrys gambelii*)

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Abstract

Capture-restraint is often used to investigate the acute hypothalamic-pituitary-adrenal axis (HPA) response to stress in wild and captive animals through production of glucocorticoids. Although this approach is useful for understanding changes in glucocorticoids, it overlooks potential changes in the complex regulatory systems associated with the glucocorticoid response, including genomic receptors, steroid metabolizing enzymes, carrier proteins, and downstream target proteins (e.g. gonadotropin-inhibitory hormone; GnIH). The present study in captive male white-crowned sparrows (*Zonotrichia leucophrys*) tests the hypothesis that corticosteroid receptors (mineralocorticoid - MR and glucocorticoid - GR), 11β-hydroxysteroid dehydrogenase 1 (11βHSD1) and 2 (11βHSD2), corticosteroid binding globulin (CBG), and GnIH undergo rapid changes in expression to mediate the glucocorticoid response to acute stress. To determine dynamic changes in gene mRNA expression in the hippocampus, hypothalamus, pituitary gland, and liver, birds were sampled within 3 minutes of entering the room and after 10, 30, and 60 minutes of capture restraint stress in a cloth bag. Restraint stress handling increased CBG and decreased GnIH mRNA expression in the liver and hypothalamus, respectively. MR, GR, 11βHSD1, and 11βHSD2 mRNA expression in the brain, pituitary gland and liver did not change. No correlations were found between gene expression and baseline or stress-induced plasma corticosterone levels. No rapid changes of MR, GR, 11βHSD1, and 11βHSD2 mRNA expression during a standardized acute restraint protocol suggests that tissue level sensitivity may remain constant during acute stressors. However, the observed rise in CBG mRNA expression could act to facilitate transport to target tissues or buffer the rise in circulating glucocorticoids. Further studies on tissue specific sensitivity are warranted.

Introduction

In vertebrates the hypothalamic-pituitary-adrenal (HPA) axis regulates the production of glucocorticoid hormones, cortisol or corticosterone, that circulate through the blood stream bound to corticosteroid binding globulin (CBG) which is produced by the liver (Schoech, Romero et al. 2013). In response to a challenge or stressor, the activity of the HPA axis is modulated, resulting in increased glucocorticoid production, which can be several orders of magnitude higher than initial capture or baseline levels (de Kloet 2014). The actions of glucocorticoids are mediated through two separate classes of receptors: the high affinity mineralocorticoid receptor (MR) and the low affinity glucocorticoid receptor (GR) (de Kloet 2014). The difference in receptor affinity allows for primary activation of the MR at low levels of glucocorticoids, often referred to as 'baseline'. GR is activated as glucocorticoid levels elevate above baseline in response to a challenge or stressor, which results in behavioral and physiological adjustments (Joëls, Karst et al. 2008, de Kloet 2014). The cellular levels of glucocorticoids are further affected by the enzymes 11β-hydroxysteroid dehydrogenase 1 (11βHSD1) and 2 (11βHSD2). 11βHSD2 deactivates corticosterone by metabolizing it to a less biologically active glucocorticoid, while this process is reversed by 11βHSD1 (Seckl and Chapman 1997). Thus, these enzymes act antagonistically to one another to regulate intracellular corticosterone levels which has direct effects on receptor activation. In addition, there is evidence that CBG can mediate glucocorticoid signaling by either facilitating the delivery of steroids to their target tissues, reducing the bioavailability by binding steroids at the target tissue, or controlling the amount of steroid that is free in the plasma (Hammond, Smith et al. 1991, Grasa, Cabot et al. 2001, Breuner and Orchinik 2002, Schoech, Romero et al. 2013, Sivukhina

and Jirikowski 2014, Gulfo, Ledda et al. 2016, Gulfo, Castel et al. 2019). Ultimately, all these factors combine to determine the rate at which MR and/or GR are activated, thus affecting stress physiology.

The hippocampus, the paraventricular nucleus (PVN) of the hypothalamus and the anterior pituitary gland are the major control sites for HPA axis function in mammals (Dallman, Akana et al. 1992, Joëls and Kloet 2017) and birds (Bouillé and Baylé 1975, Bouillé and Baylé 1976, Hodgson, Meddle et al. 2007, Dickens, Romero et al. 2009, Krause, McGuigan et al. 2015, Smulders 2017, Cornelius, Perreau et al. 2018). Through the interactions of MR and GR, the hippocampus controls the amplitude and timing of circadian rhythms for baseline glucocorticoids (Dallman, Akana et al. 1992, Sapolsky, Romero et al. 2000, Smulders 2017). Stress-induced levels of glucocorticoids are thought to provide negative feedback at the level of the PVN and anterior pituitary gland primarily through GR (Sapolsky, Romero et al. 2000, Landys, Ramenofsky et al. 2006). Pharmacological or genetic manipulations of MR, GR, 11βHSD1 and 11βHSD2 at each or all levels of the HPA axis can affect baseline and/or stress-induced plasma glucocorticoids, which suggests a complex and coordinated signaling cascade (Harris, Kotelevtsev et al. 2001, Kolber, Wieczorek et al. 2008, Schmidt, Sterlemann et al. 2009, Harris, Holmes et al. 2013, Pérez, Swanson et al. 2020).

Field biologists often assess acute HPA axis function by using a standard capture restraint protocol for up to 60 minutes and taking serial blood samples to measure circulating glucocorticoids. However, it remains unknown if the corticosteroid receptors or glucocorticoid metabolizing enzymes also change within this standard 60-minute restraint stress protocol. We hypothesized that the regulatory components of the HPA axis undergo rapid changes in expression in order to potentiate glucocorticoid signaling. In order to determine the rapidity at

which gene expression of the regulatory elements of the HPA axis may change, tissues from male captive white-crowned sparrows were sampled within three minutes of entering the room and after 10, 30, and 60 minutes of restraint stress. We predicted that gene expression for MR, GR, and 11βHSD1 would be upregulated and 11βHSD2 downregulated in response to stress in the hippocampus, hypothalamus, pituitary gland, and liver. In addition, we quantified gene expression of two known downstream targets of glucocorticoids; CBG and gonadotropin inhibitory hormone (GnIH) (Calisi, Rizzo et al. 2008, Kirby, Geraghty et al. 2009). We predicted that hepatic CBG expression would be upregulated to buffer the increase in plasma glucocorticoids induced by restraint stress. We also predicted upregulation of gonadotropin-inhibitory hormone (GnIH), because it is elevated by increased GR activation and plays a role in suppressing reproductive pathways (Kirby, Geraghty et al. 2009, Calisi, Díaz-Muñoz et al. 2011, Ernst, Lynn et al. 2016).

Methods

Birds

A total of 35 male Gambel's white-crowned sparrows, (*Zonotrichia leucophrys gambelii*), were caught using mist nets and baited potter traps in late October through early November 2015 in the vicinity of Davis, California (38.5449° N, 121.7405° W). Birds were housed in individual cages (35 cm (w) x 40 cm (l) x 45 cm (h)) in three separate indoor aviaries at 20±2°C. A diet of 60% Mazuri maintenance chow and 40% wild bird seed and water were provided *ad libitum*. Birds were exposed to naturally changing photoperiod for Davis, CA, USA (38.55° N, -121.74° E) during the acclimation and experimentation period. Increasing natural photoperiod results in the activation of physiological cascades for migration and breeding (Farner and Follett 1966). Increasing photoperiod in captive Gambel's white-crowned sparrows

does not significantly affect HPA axis activity as they do not undergo the seasonal changes in baseline and stress-induced corticosterone observed in free-living individuals (Romero and Wingfield 1999, Ramenofsky, Campion et al. 2017).

Blood and Tissue collection

The effect of restraint stress on plasma levels of glucocorticoids was determined on a mid-March photoperiod 12L:12D (lights on: 07:00 and off 19:00) on separate days from tissue collection. Approximately 60µL of blood was collected following venipuncture of the alar vein with a 26-gauge needle and collected into a heparinized glass microcapillary tubes (VWR: 15401-56). Samples were collected within 3 minutes of entering the room and 30 minutes post restraint in an opaque cloth bag. Birds were bled at 09:00 over two consecutive days. Blood was centrifuged at 15,000 *g* for 5 minutes to separate plasma from red blood cells. The plasma was aspirated with a Hamilton syringe, transferred into a labeled microcentrifuge tube, and stored at -30^{o}C until radioimmunoassay for corticosterone.

Birds were euthanized in early July when the photoperiod was 14L:10D (lights on: 07:00 and off 21:00) to ensure they were in a fully reproductive state which was confirmed by assessment of an enlarged cloacal protuberance and large testes size. As determined at the time of euthanasia, the testes had an average length of 6.96 ± 1.28mm and width of 5.2 ± 1.12mm (n=35; mean ± S.D.) and there was no evidence of body or wing feather molt. Birds were sampled between the hours of 09:00 and 11:00 over two consecutive days and were randomly assigned to four groups on each day; a baseline sampled in under 3 minutes (n=8), and three other groups in which the bird was placed into a cloth bag for 10 (n=8), 30 (n=9) or 60 (n=9) minutes. The time points were chosen as they represent the standardized capture restraint time

intervals at which blood samples are collected in stress physiology studies. Birds were euthanized by sedation with isoflurane followed by rapid decapitation. Birds were dissected and tissues were quickly collected and fresh frozen on powdered dry ice, wrapped in aluminum foil and placed in labeled plastic bags and stored at -80°C. In the case of the liver, the left lobe was collected. Samples were later shipped on dry ice to the Roslin Institute, University of Edinburgh where they were stored at -80°C until cDNA preparation. All work was approved by University of California, Davis - Institutional Animal Care and Use Committee (IACUC) under protocol 19758, United States Fish and Wildlife Service permits - Federal MB90026B-0, and California State SC13449.

Preparation of cDNA

Whole frozen brains were warmed to -18°C in a cryostat and the hippocampus (~30mg) and hypothalamus (~80-100mg) were dissected in standardized blocks for isolation of RNA. The hippocampus from both hemispheres was isolated from the dorsal aspect of the telencephalon. Hypothalamic blocks were created by placing the brain into a brain matrix and landmarks were used according to Stokes, Leonard et al. (1974) to make a coronal cut just caudal to the Tractus septomesencephalicus (TrSM) and a rostral coronal cut at the start of the cerebellum. Tissues were homogenized using an Ultra Turrax homogenizer (IKA-Werke GmbH & Co. KG) in conjunction with 1000µL of trizol (Sigma-Aldrich T9424), except for the pituitary gland in which 300µL was used due to the small mass (<3mg). Total RNA was extracted from 250µL of tissue homogenate using Zymo Research Direct-zol 96 well plates (R2057) including the DNA digestion step per the manufacturer's instructions. Total RNA for hippocampus, hypothalamus, pituitary gland, and liver were assessed using a Nanodrop 1000 (Thermo Scientific) and found to have an average yield of 261±112, 254±126, 26 ±10, and 219±89 ng/µL (mean±SD) with an

168 average 260/280 ratio of 2.05±0.05, 2.02±0.02, 2.06±0.16, and 2.08±0.02 (mean±SD),
169 respectively. cDNA was produced by reverse transcribing 250 ng of RNA per sample using the
170 High Capacity Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's
171 guidelines and the final volume was adjusted to 110µL per sample using ultra-pure (Milli-Q)
172 water.

173 *Quantitative polymerase chain reaction (qPCR)*

174 All assays used Brilliant 111 Ultra-fast SYBR Green qPCR Master mix (Agilent
175 Technologies 60083) which was read on an Agilent Mx3005p qPCR system and processed with
176 MxPro software (Agilent Technologies) as previously described (Reid, Wilson et al. 2017). In
177 brief, a 20µL total reaction volume per sample was generated by combining 10µL SYBR Green,
178 8µL cDNA, 0.4µL 20µM forward primer, 0.4 µL 20 µM reverse primer, 0.3µL 1/500 dilution of
179 ROX reference dye solution, and 0.9µL Milli-Q H_2O. Each assay used the following thermal
180 conditions: 50°C; 120s, 95°C; 120s, (40 cycles of 95°C; 15s, 60°C; 30s), then 95°C; 60s, 60°C;
181 30s, 95°C; 15s. Apparent reaction efficiencies were determined by analyses of the standard
182 curves and ranged between 92.4 - 96.8%. The R^2 for standard curves ranged between 0.984-
183 1.000.

184 Primers were designed using the relevant white-throated sparrow coding sequence (cds)
185 unless white-crowned sparrow cds were available (CBG and GnIH). CBG was recently identified
186 in *Taeniopygia guttata* after nearly 40 years of research. Primers were designed against the
187 published because few genome sequences have been updated (Vashchenko, Das et al. 2016).
188 Multiple primer combinations were tested for each gene using the previously mentioned thermal
189 conditions for SYBR reactions and with FastStart PCR (Roche) using a standard PCR thermal
190 conditions [95°C; 240s, (40 cycles of 95°C; 30s, 58°C; 30s, 72°C; 30s), then 72°C; 420s].

Products from both PCR and qPCR reactions were visualized using 2% agarose gel electrophoresis. The primer combination for each gene was selected on the amplicons that yielded highest amplifying reaction that lacked visible primer dimers or infidelity signals (Table 1). Once primers were selected, FastStart amplicons were isolated from the agarose gel using a QIAquick Gel Extraction Kit (Qiagen 28704). Amplicons were sent for sequencing and GR, MR, 11βHSD1, 11βHSD2, YWHAZ were found to have sequence homologies with the published white-throated sparrow (*Zonotrichia albicolis*) genome of 98, 95, 100, 99.72, and 98.82% respectively. The CBG amplicon shared 90% identity with *Taeniopygia guttata* CBG, and the GnIH amplicon shared 98% identity with the published *Zonotrichia leucophrys gambelii* GnIH amplicon. Stock amplicons were serially diluted to produce standard curves for qPCR. For each gene assay per tissue, duplicate samples from each bird were all run on a single plate. The purified cDNA was used to generate standard curves by quantification on a Nanodrop and then a serial dilution was conducted from an aliquot of the stock amplicon solution. For each gene assay per tissue, duplicate samples from each bird were all run on a single plate. All genes were normalized to the reference gene YWHAZ due to its reliability in previous avian studies (Olias, Adam et al. 2014, Reid, Wilson et al. 2017).

MxPro software compares Ct values of unknown samples to those of standards with known target concentration to determine an absolute measure of the number of target cDNA copies in each reaction volume, which represented a constant proportion of total RNA. Absolute expression values for each gene of interest were then divided by the corresponding YWHAZ absolute expression value to give a normalized relative measure of expression, with arbitrary units, for each sample. This controlled for any inconsistency in total RNA representation (e.g. difference in total RNA reverse-transcribed per sample), as well as any disproportional

representation caused by large differences in unassessed transcripts (e.g. ribosomal RNAs or tissue-specific genes under certain seasonal conditions) compared to YWHAZ basal expression. Additional retrospective assessment of YWHAZ for reference suitability found expression to be consistent across all samples for each tissue type (see results).

Quantification of plasma corticosterone

Corticosterone concentrations were determined using a previously validated radioimmunoassay for corticosterone (Krause, Pérez et al. 2017). In brief, 15µL from baseline plasma samples and 10µL from the 30-minute samples were combined with 2000CPM of tritiated corticosterone (Perkin Elmer NET399250UC). 4mL of freshly distilled dichloromethane was used to extract corticosterone from the plasma samples. Aspirated dichloromethane was placed into a water bath at 35°C and was dried using nitrogen gas. Dried extracts were reconstituted in 550µL of phosphate buffer saline with gelatin (PBSG). 100µL of reconstituted extract was added to a scintillation vial and combined with 3mL of scintillation fluid (Perkin Elmer Ultima Gold: 6013329) to determine extraction recovery percentages. Next, 200µL of reconstituted extracts were added to duplicate RIA assay tubes with 100µL of tritiated corticosterone (Perkin Elmer NET399250UC) and 100µL of antiserum (MP Biomedical 07–120016, lot 3R3-PB-20E antibody). Unbound steroid was separated from bound steroid using 500µL of dextran coated charcoal solution. Samples were then placed in a centrifuge for 10 minutes at 4°C at 3000 g to separate bound from free steroids in solution. The supernatant containing bound steroids was decanted into scintillation vials and 3.5mL of scintillation fluid were added. Mean recovery was 85.25±4.2% (mean±SD) and intra- (calculated using C.V. between duplicates) and inter-assay variations were 5.05 and 6.14%, respectively.

Statistical Analyses

Statistical analyses of the data were performed in R Statistical Analysis software with R studio version 3.6.3 (R Core Development Team 2018). Gene expression data were analyzed using an analysis of variance (ANOVA) with the main effect of sampling time points. The residuals were checked for normal distribution using Shapiro-Wilks and normality using Levene's test. If the assumptions were not met, the data were log transformed. All post hoc tests were performed using the emmeans package (Lenth, Singmann et al. 2018) to perform Tukey's Honestly Significant Difference (HSD) tests and generate effects size estimates based on Cohen's D. Pearson's correlations were used to investigate the relationship between either baseline or stress-induced corticosterone and gene expression in each of the measured tissues.

Results

Effects of restraint handling stress on gene expression

MR, GR, 11βHSD1 and 11βHSD2 mRNA expressions were not affected by restraint handling in any of the tissues measured (Figs. 1&2; Table 2). However, restraint handling resulted in changes in mRNA expression of GnIH in the hypothalamus and CBG in the liver (Fig. 3; Table 2). Hypothalamic GnIH expression was higher at the 3-minute compared to 10-minute sampling points (t=4.26, P=0.002, df=21, Cohen's d=2.39) and 30 (t=2.82, P=0.04, df=21, Cohen's d=1.38; Fig. 2A). CBG in the liver was higher at the 60-minute sampling point compared to the 3-minute (t=3.04, P=0.02, df=23, Cohen's d=1.57) and 10 minute (t=2.82, P=0.045; Fig. 2B, df=23, Cohen's d=1.50) sampling points.

YWHAZ gene expression was not affected by restraint handling in the hippocampus ($F_{3,22}$=0.25, P=0.85), hypothalamus ($F_{3,24}$=0.007, P=0.99), pituitary gland ($F_{3,19}$=0.04, P=0.98), or liver ($F_{3,23}$=2.21, P=0.11).

Relationships between gene expression and circulating corticosterone

No correlations were found between circulating baseline or stress-induced glucocorticoids and any of the genes measured (Table 2).

Discussion

We tested whether regulatory elements downstream of plasma levels of corticosterone respond rapidly to the stress of a standardized restraint handling protocol. There were no changes in MR, GR, 11βHSD1, or 11βHSD2 mRNA expression, suggesting that with respect to standardized restraint stress protocols lasting 1 hour or less, quantification of plasma corticosteroid levels may be sufficient to characterize HPA axis activity in white-crowned sparrows.. In rock doves, *Columba livia,* transcriptomic analysis showed no changes in MR, GR, 11βHSD1, or 11βHSD2 mRNA expression in the hypothalamus nor pituitary gland following 30 minutes of restraint stress (Calisi, Austin et al. 2017). Our findings along with Calisi et al. 2017, may be explained by the fact that changes in gene expression, particularly for genomic receptors, may occur on a longer time scale and were likely missed in the present study given the maximum observation window of one hour. For instance, in rats, 120 minutes, but not 15, 30, or 60 minutes, of acute stress decreased hippocampal GR expression but not MR (Paskitti, McCreary et al. 2000), upregulated hypothalamic GR expression but not MR, and decreased pituitary GR expression but not MR (Karandrea, Kittas et al. 2000). In birds, MR and GR mRNA expression

vary over longer time periods, for example in response to seasonal changes (Liebl, Shimizu et al. 2013, Krause, McGuigan et al. 2015, Li, Davis et al. 2020), social stress (Cornelius, Perreau et al. 2018), early life stress (Zimmer and Spencer 2014), chronic stress (Dickens, Romero et al. 2009), selection for high HPA axis activity (Hodgson, Meddle et al. 2007), and range expansion (Liebl and Martin 2013).

Another possible mechanism for rapidly modulating tissue level availability of corticosterone is through glucocorticoid regulatory enzymes. 11β-hydroxysteroid dehydrogenase 1 (11βHSD1) catalyzes the reactivation of corticosterone from inactive metabolites, and 11β-hydroxysteroid dehydrogenase 2 (11βHSD2) catalyzes the deactivation corticosterone to inactive metabolites. In the current study, we did not detect any rapid effects of stress on the expression of 11βHSD1 or 11βHSD2. A study in rodents found upregulation of 11βHSD1 mRNA in the hippocampus at 1 and 2 hours post physiological stressor, but no other brain regions involved with HPA axis signaling were investigated (Spiers, Chen et al. 2016). 11βHSD2 is not highly expressed in the brain of mammals (unlike in birds) thus no comparative studies could be found investigating the effects of stress (Chapman, Holmes et al. 2013). In white-crowned sparrows, pharmacological blockade of 11βHSD1 and 11βHSD2 enzyme function during both systemic administration and infusion into the third ventricle of the brain (11βHSD2, only) altered levels of plasma corticosteroids over the course of a standardized restraint stressor (Pérez, Swanson et al. 2020). The central administration of pharmacological antagonists to the third ventricle suggested that 11βHSD2 was involved in regulating negative feedback control at PVN of the hypothalamus. While, systemic injections targeted the liver, kidneys, and pituitary gland suggested that 11βHSD2 and 11βHSD1 are important for balancing net deactivation of corticosterone by the kidney and net reactivation by the liver and/or possible negative feedback

303 at the pituitary gland (Pérez, Swanson et al. 2020). However, regulation of gene expression is not
304 the only way to modulate enzyme kinetics. Both enzymes are dependent upon cofactors, NAD+
305 and NADPH, for proper functioning and thus these could potentially regulate their activity.
306 Studies on 3β-hydroxysteroid dehydrogenase (3βHSD) have been shown to undergo rapid
307 modulation, by changes of NAD+ cofactors, in songbirds (Pradhan, Newman et al. 2010). Our
308 data suggest that rapid regulation of expression is not occurring, but further studies will need to
309 elucidate if either 11βHSD1 or 2 undergoes a rapid response to cofactors.

310 Binding assays in birds have verified the presence of both MR and GR in the liver
311 (Lattin, Waldron-Francis et al. 2012), but we found no effect on liver GR or MR mRNA
312 expression in response to restraint handling stress. We have not found any published studies to
313 date that have investigated the effects of short-term acute stress on corticosteroid receptor gene
314 expression in the liver of any species. This represents a serious gap in our knowledge for how the
315 liver responds to acute stress. In house sparrows, *Passer domesticus*, a chronic stress paradigm
316 also had no effect on MR or GR in the liver when analyzed using receptor binding assays (Lattin
317 and Romero 2014). However in fish and rodents, treatment of hepatocytes or whole animals with
318 glucocorticoids increased GR mRNA expression after 24 h of exposure but not at 1, 4, or 8h post
319 treatment (Sathiyaa and Vijayan 2003, Liu, Nakagawa et al. 2005). In addition, 6 h of chronic
320 immobilization for 1 day in rodents resulted in upregulation of 11βHSD1 and down regulation of
321 11βHSD2 (Chen 2020). Thus, changes in gene expression associated with glucocorticoid
322 signaling appear to be context and duration dependent.

323 Rapid changes in the expression of downstream targets of corticosteroid were observed
324 following restraint stress. CBG expression in the liver increased in as little as 30 minutes in
325 response to acute restraint stress. The current literature shows mixed results for the effects of

restraint handling on circulating plasma CBG in birds; some species show a decline while others are unaffected (Breuner, Lynn et al. 2006). Gambel's white-crowned sparrows do not show a decline in circulating CBG after 1 hour of restraint (Breuner, Lynn et al. 2006). The binding capacity of CBG in songbirds has been reported to be relatively high, allowing binding of additional steroids or potentially buffering the loss of cleaved CBG (Charlier, Underhill et al. 2009, Li, Sun et al. 2017) so that a substantial increase in circulating CBG may not be necessary to regulate HPA axis activity. Based on our data, a longer time course than that used previously by Breuner et al., (2006) may be required to detect a corresponding change in circulating CBG. Alternatively, increased CBG transcript production may be keeping pace with increased CBG cleavage and clearance during a stressor. Long-term stressors, such as food removal or restriction studies, have shown that plasma CBG levels decline in white-crowned sparrows within 22 hours (Lynn, Breuner et al. 2003).

GnIH is important for reducing the activity of the hypothalamic-pituitary-gonadal (HPG) axis, but contrary to our predictions, GnIH expression was markedly reduced after 10 and 30 minutes of restraint stress. Hypothalamic GnIH peptide and mRNA typically increase with stress, and glucocorticoid receptors colocalize with GnIH neurons (Calisi, Rizzo et al. 2008, Kirby, Geraghty et al. 2009, Gojska and Belsham 2014). Nonetheless studies in captive breeding zebra finches have shown GnIH immunoreactive cells to decrease in response to restraint stress and food removal (Ernst, Lynn et al. 2016, Wilsterman, Alonge et al. 2020). Studies in European starlings and rats have shown that GnIH levels in both sexes peak following clutch completion, which may serve to suppress HPG axis function in the event that the clutch is lost the reproductive system can reactivate (Calisi, Geraghty et al. 2016). Thus, it is difficult to determine the functional significance of the observed decline in GnIH, given its transient occurrence

(Kirby, Geraghty et al. 2009, Calisi, Díaz-Muñoz et al. 2011). Based on our existing knowledge of GnIH the decrease in expression may play a functional role in altering either HPG axis activity or sexual behavior in response to acute environmental stressors.

Conclusion

This is the first study in birds to test whether or not rapid changes in corticosteroid receptors or the 11β Hydroxysteroid dehydrogenase system occur at multiple levels within the HPA axis and at target tissues during a standardized restraint handling sampling period. Our findings suggest that neither the expression of corticosteroid receptors nor the 11β-hydroxysteroid dehydrogenase system change over the course of a standardized restraint handling protocol at any regulatory point within HPA axis. However, hepatic mRNA expression of CBG was rapidly upregulated which may serve to buffer circulating levels of glucocorticoids and may also be important for delivering corticosterone to target tissues. Thus, genes that are regulated by glucocorticoid signaling - GnIH and CBG, undergo rapid changes. Our data suggest that tissue level sensitivity for glucocorticoids as accessed by corticosteroid receptors and 11βHSD enzymes suggests that these elements are stable on the acute time scale. Long-term stress studies are required to understand if these regulatory elements change over longer time periods.

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References

Bouillé, C. and J. D. Baylé (1975). "Influence of Septal Nuclei on Basal Pituitary-Adrenocortical Function in Birds." Neuroendocrinology **18**(4): 281-289.

Bouillé, C. and J. D. Baylé (1976). "Comparison between Hypothalamic, Hippocampal and Septal Multiple Unit Activity and Basal Corticotropic Function in Unrestrained, Unanesthetized Resting Pigeons." Neuroendocrinology **22**(2): 164-174.

Breuner, C. and M. Orchinik (2002). "Plasma binding proteins as mediators of corticosteroid action in vertebrates." Journal of Endocrinology **175**(1): 99-112.

Breuner, C. W., S. E. Lynn, G. E. Julian, J. M. Cornelius, B. J. Heidinger, O. P. Love, R. S. Sprague, H. Wada and B. A. Whitman (2006). "Plasma-binding Globulins and Acute Stress Response." Horm Metab Res **38**(04): 260,268.

Calisi, R., S. Austin, A. S. Lang and M. D. MacManes (2017). "Sex-biased transcriptomic response of the reproductive axis to stress." bioRxiv.

Calisi, R. M., S. L. Díaz-Muñoz, J. C. Wingfield and G. E. Bentley (2011). "Social and breeding status are associated with the expression of GnIH." Genes, Brain and Behavior **10**(5): 557-564.

Calisi, R. M., A. C. Geraghty, A. Avila, D. Kaufer, G. E. Bentley and J. C. Wingfield (2016). "Patterns of hypothalamic GnIH change over the reproductive period in starlings and rats." General and Comparative Endocrinology **237**: 140-146.

Calisi, R. M., N. O. Rizzo and G. E. Bentley (2008). "Seasonal differences in hypothalamic EGR-1 and GnIH expression following capture-handling stress in house sparrows (Passer domesticus)." General and Comparative Endocrinology **157**(3): 283-287.

Chapman, K., M. Holmes and J. Seckl (2013). 11β-Hydroxysteroid Dehydrogenases: Intracellular Gate-Keepers of Tissue Glucocorticoid Action.

Charlier, T. D., C. Underhill, G. L. Hammond and K. K. Soma (2009). "Effects of aggressive encounters on plasma corticosteroid-binding globulin and its ligands in white-crowned sparrows." Hormones and Behavior **56**(3): 339-347.

Chen, H.-J. C. Y., T.; Lee, J.K.; Juliani, J.; Sernia, C.; Hill, A.F.; Lavidis, N.A.; Spiers, J.G. (2020). "Restraint Stress Alters Expression of Glucocorticoid Bioavailability Mediators, Suppresses Nrf2, and Promotes Oxidative Stress in Liver Tissue. ." Antioxidants **9**(853).

Cornelius, J. M., G. Perreau, V. R. Bishop, J. S. Krause, R. Smith, T. P. Hahn and S. L. Meddle (2018). "Social information changes stress hormone receptor expression in the songbird brain." Hormones and Behavior **97**: 31-38.

Dallman, M. F., S. F. Akana, K. A. Scribner, M. J. Bradbury, C. D. Walker, A. M. Strack and C. S. Cascio (1992). "Stress, Feedback and Facilitation in the Hypothalamo-Pituitary-Adrenal Axis." Journal of Neuroendocrinology **4**(5): 517-526.

de Kloet, E. R. (2014). "From receptor balance to rational glucocorticoid therapy." Endocrinology **155**(8): 2754-2769.

Dickens, M., L. M. Romero, N. E. Cyr, I. C. Dunn and S. L. Meddle (2009). "Chronic stress alters glucocorticoid receptor and mineralocorticoid receptor mRNA expression in the European starling (*Sturnus vulgaris*) brain." Journal of Neuroendocrinology **21**(10): 832-840.

Ernst, D. K., S. E. Lynn and G. E. Bentley (2016). "Differential response of GnIH in the brain and gonads following acute stress in a songbird." General and Comparative Endocrinology **227**: 51-57.

Farner, D. S. and B. K. Follett (1966). "Light and other environmental factors affecting avian reproduction." Journal of Animal Science **25**(Supplement): 90-115.

Gojska, N. M. and D. D. Belsham (2014). "Glucocorticoid receptor-mediated regulation of Rfrp (GnIH) and Gpr147 (GnIH-R) synthesis in immortalized hypothalamic neurons." Molecular and Cellular Endocrinology **384**(1): 23-31.

Grasa, M. M., C. Cabot, J. A. Fernández-López, X. Remesar and M. Alemany (2001). "Modulation of corticosterone availability to white adipose tissue of lean and obese Zucker rats by corticosteroid-binding globulin." Hormone and metabolic research **33**(7): 407.

Gulfo, J., R. Castel, A. Ledda, M. d. M. Romero, M. Esteve and M. Grasa (2019). "Corticosteroid-Binding Globulin is expressed in the adrenal gland and its absence impairs corticosterone synthesis and secretion in a sex-dependent manner." Scientific Reports **9**(1): 14018.

Gulfo, J., A. Ledda, E. Serra, C. Cabot, M. Esteve and M. Grasa (2016). "Altered lipid partitioning and glucocorticoid availability in CBG-deficient male mice with diet-induced obesity." Obesity **24**(8): 1677-1686.

Hammond, G. L., C. L. Smith and D. A. Underhill (1991). "Molecular studies of corticosteroid binding globulin structure, biosynthesis and function." The Journal of Steroid Biochemistry and Molecular Biology **40**(4–6): 755-762.

Harris, A. P., M. C. Holmes, E. R. de Kloet, K. E. Chapman and J. R. Seckl (2013). "Mineralocorticoid and glucocorticoid receptor balance in control of HPA axis and behaviour." Psychoneuroendocrinology **38**(5): 648-658.

Harris, H. J., Y. Kotelevtsev, J. J. Mullins, J. R. Seckl and M. C. Holmes (2001). "Intracellular Regeneration of Glucocorticoids by 11β-Hydroxysteroid Dehydrogenase (11β-HSD)-1 Plays a Key Role in Regulation of the Hypothalamic-Pituitary-Adrenal Axis: Analysis of 11β-HSD-1-Deficient Mice." Endocrinology **142**(1): 114-120.

Hodgson, Z. G., S. L. Meddle, M. L. Roberts, K. L. Buchanan, M. R. Evans, R. Metzdorf, M. Gahr and S. D. Healy (2007). "Spatial ability is impaired and hippocampal mineralocorticoid receptor mRNA expression reduced in zebra finches (Taeniopygia guttata) selected for acute high corticosterone response to stress." Proceedings of the Royal Society B: Biological Sciences **274**(1607): 239-245.

Joëls, M., H. Karst, R. DeRijk and E. R. de Kloet (2008). "The coming out of the brain mineralocorticoid receptor." Trends in Neurosciences **31**(1): 1-7.

Joëls, M. and E. R. d. Kloet (2017). "30 YEARS OF THE MINERALOCORTICOID RECEPTOR: The brain mineralocorticoid receptor: a saga in three episodes." **234**(1): T49.

Karandrea, D., C. Kittas and E. Kitraki (2000). "Contribution of sex and cellular context in the regulation of brain corticosteroid receptors following restraint stress." Neuroendocrinology **71**(6): 343-353.

Kirby, E. D., A. C. Geraghty, T. Ubuka, G. E. Bentley and D. Kaufer (2009). "Stress increases putative gonadotropin inhibitory hormone and decreases luteinizing hormone in male rats." Proceedings of the National Academy of Sciences **106**(27): 11324-11329.

Kolber, B. J., L. Wieczorek and L. J. Muglia (2008). "Hypothalamic–pituitary–adrenal axis dysregulation and behavioral analysis of mouse mutants with altered glucocorticoid or mineralocorticoid receptor function." Stress **11**(5): 321-338.
Krause, J. S., M. A. McGuigan, V. R. Bishop, J. C. Wingfield and S. L. Meddle (2015). "Decreases in mineralocorticoid but not glucocorticoid receptor mRNA expression during the short Arctic breeding season in free-Living Gambel's white-crowned sparrow (*Zonotrichia leucophrys gambelii*)." Journal of Neuroendocrinology **27**(1): 66-75.
Krause, J. S., J. H. Pérez, S. L. Meddle and J. C. Wingfield (2017). "Effects of short-term fasting on stress physiology, body condition, and locomotor activity in wintering male white-crowned sparrows." Physiology & Behavior **177**: 282-290.
Landys, M. M., M. Ramenofsky and J. C. Wingfield (2006). "Actions of glucocorticoids at a seasonal baseline as compared to stress-related levels in the regulation of periodic life processes." General and Comparative Endocrinology **148**(2): 132-149.
Lattin, C. R. and L. M. Romero (2014). "Chronic stress alters concentrations of corticosterone receptors in a tissue-specific manner in wild house sparrows (Passer domesticus)." The Journal of Experimental Biology **217**(14): 2601-2608.
Lattin, C. R., K. Waldron-Francis, J. W. Richardson, R. de Bruijn, C. M. Bauer, C. W. Breuner and L. Michael Romero (2012). "Pharmacological characterization of intracellular glucocorticoid receptors in nine tissues from house sparrow (Passer domesticus)." General and Comparative Endocrinology **179**(2): 214-220.
Lenth, R., H. Singmann, J. Love, P. Buerkner and M. Herve (2018). "Emmeans: Estimated marginal means, aka least-squares means." R package version **1**(1): 3.
Li, D., J. E. Davis, G. Wang, G. Nabi, V. R. Bishop, Y. Sun, S. L. Meddle, J. C. Wingfield and F. Lei (2020). "Coping with extremes: Remarkably blunt adrenocortical responses to acute stress in two sympatric snow finches on the Qinghai-Tibet Plateau during winter relative to other seasons." General and Comparative Endocrinology: 113434.
Li, Y., Y. Sun, J. S. Krause, M. Li, X. Liu, W. Zhu, Y. Yao, Y. Wu and D. Li (2017). "Dynamic interactions between corticosterone, corticosteroid binding globulin and testosterone in response to capture stress in male breeding Eurasian tree sparrows." Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology **205**: 41-47.
Liebl, A. L. and L. B. Martin (2013). "Stress hormone receptors change as range expansion progresses in house sparrows." Biology Letters **9**(3).
Liebl, A. L., T. Shimizu and L. B. Martin (2013). "Covariation among glucocorticoid regulatory elements varies seasonally in house sparrows." General and Comparative Endocrinology **183**(0): 32-37.
Liu, Y., Y. Nakagawa, Y. Wang, R. Sakurai, P. V. Tripathi, K. Lutfy and T. C. Friedman (2005). "Increased Glucocorticoid Receptor and 11β-Hydroxysteroid Dehydrogenase Type 1 Expression in Hepatocytes May Contribute to the Phenotype of Type 2 Diabetes in db/db Mice." Diabetes **54**(1): 32-40.
Lynn, S. E., C. W. Breuner and J. C. Wingfield (2003). "Short-term fasting affects locomotor activity, corticosterone, and corticosterone binding globulin in a migratory songbird." Hormones and Behavior **43**(1): 150-157.
Olias, P., I. Adam, A. Meyer, C. Scharff and A. D. Gruber (2014). "Reference genes for quantitative gene expression studies in multiple avian species." PLOS ONE **9**(6): e99678.
Paskitti, M. E., B. J. McCreary and J. P. Herman (2000). "Stress regulation of adrenocorticosteroid receptor gene transcription and mRNA expression in rat hippocampus: time-course analysis." Molecular Brain Research **80**(2): 142-152.
Pérez, J. H., R. E. Swanson, H. J. Lau, J. Cheah, V. R. Bishop, K. R. S. Snell, A. M. A. Reid, S. L. Meddle, J. C. Wingfield and J. S. Krause (2020). "Tissue-specific expression of 11β-HSD and its effects on plasma corticosterone during the stress response." The Journal of Experimental Biology **223**(1): jeb209346.

Pradhan, D. S., A. E. M. Newman, D. W. Wacker, J. C. Wingfield, B. A. Schlinger and K. K. Soma (2010). "Aggressive interactions rapidly increase androgen synthesis in the brain during the non-breeding season." Hormones and Behavior **57**(4-5): 381-389.

R Core Development Team (2018). R: a language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria.

Ramenofsky, M., A. W. Campion, J. H. Pérez, J. S. Krause and Z. Németh (2017). "Behavioral and physiological traits of migrant and resident white-crowned sparrows: a common garden approach." The Journal of Experimental Biology **220**(7): 1330-1340.

Reid, A. M. A., P. W. Wilson, S. D. Caughey, L. M. Dixon, R. B. D'Eath, V. Sandilands, T. Boswell and I. C. Dunn (2017). "Pancreatic PYY but not PPY expression is responsive to short-term nutritional state and the pancreas constitutes the major site of PYY mRNA expression in chickens." General and Comparative Endocrinology **252**: 226-235.

Romero, L. M. and J. C. Wingfield (1999). "Alterations in hypothalamic–pituitary–adrenal function associated with captivity in Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*)." Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology **122**(1): 13-20.

Sapolsky, R. M., L. M. Romero and A. U. Munck (2000). "How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions." Endocrine Reviews **21**(1): 55-89.

Sathiyaa, R. and M. M. Vijayan (2003). "Autoregulation of glucocorticoid receptor by cortisol in rainbow trout hepatocytes." American Journal of Physiology-Cell Physiology **284**(6): C1508-C1515.

Schmidt, M. V., V. Sterlemann, K. Wagner, B. Niederleitner, K. Ganea, C. Liebl, J. M. Deussing, S. Berger, G. n. Schütz, F. Holsboer and M. B. Müller (2009). "Postnatal Glucocorticoid Excess Due to Pituitary Glucocorticoid Receptor Deficiency: Differential Short- and Long-Term Consequences." Endocrinology **150**(6): 2709-2716.

Schoech, S. J., L. M. Romero, I. T. Moore and F. Bonier (2013). "Constraints, concerns and considerations about the necessity of estimating free glucocorticoid concentrations for field endocrine studies." Functional Ecology **27**(5): 1100-1106.

Seckl, J. R. and K. E. Chapman (1997). "Medical and physiological aspects of the 11beta-hydroxysteroid dehydrogenase system." European journal of biochemistry / FEBS **249**(2): 361-364.

Sivukhina, E. V. and G. F. Jirikowski (2014). "Adrenal steroids in the brain: Role of the intrinsic expression of corticosteroid-binding globulin (CBG) in the stress response." Steroids **81**: 70-73.

Smulders, T. V. (2017). "The avian hippocampal formation and the stress response." Brain, Behavior and Evolution **90**(1): 81-91.

Spiers, J. G., H.-J. C. Chen, J. S. M. Cuffe, C. Sernia and N. A. Lavidis (2016). "Acute restraint stress induces rapid changes in central redox status and protective antioxidant genes in rats." Psychoneuroendocrinology **67**: 104-112.

Stokes, T. M., C. M. Leonard and F. Nottebohm (1974). "The telencephalon, diencephalon, and mesencephalon of the canary, *Serinus canaria*, in stereotaxic coordinates." The Journal of Comparative Neurology **156**(3): 337-374.

Vashchenko, G., S. Das, K.-M. Moon, J. C. Rogalski, M. D. Taves, K. K. Soma, F. Van Petegem, L. J. Foster and G. L. Hammond (2016). "Identification of Avian Corticosteroid-binding Globulin (SerpinA6) Reveals the Molecular Basis of Evolutionary Adaptations in SerpinA6 Structure and Function as a Steroid-binding Protein." Journal of Biological Chemistry **291**(21): 11300-11312.

Wilsterman, K., M. M. Alonge, X. Bao, K. A. Conner and G. E. Bentley (2020). "Food access modifies GnIH, but not CRH, cell number in the hypothalamus in a female songbird." General and Comparative Endocrinology: 113438.

Zimmer, C. and K. A. Spencer (2014). "Modifications of Glucocorticoid Receptors mRNA Expression in the Hypothalamic-Pituitary-Adrenal Axis in Response to Early-life Stress in Female Japanese Quail." Journal of Neuroendocrinology **26**(12): 853-860.

Table and Figure Legends

Table 1.

Primers that were utilized in this study.

Gene	Accession Number	Primer	Primer Seq
Corticosteron Binding Globulin (CBG)	KU180443.1	F	GCAGATCTCTCTGGCATCAC
		R	GAGGAAGGGATGGCTGAATTC
Glucocorticoid Receptor (GR)	XM_002192952.3	F	TGAAGAGCCAGTCCCTGTTCGAG
		R	CAACCACATCATGCATAGAGTCCAGCA
Mineralocorticoid Receptor (MR)	NM_001076690.1	F	AAGAGTCGGCCAAACATCCTTGTTCT
		R	AAGAAACGGGTGGTCCTAAAATCCCAG
Gonadotropin Inhibitory Hormone (GnIH)	AB128164.1	F	CTGCCTCGAGACATTCAGGA
		R	GACCACCACTGAAGCAAACT
11β-Hydroxysteroid dehydrogenase 1 (11βHSD1)	XM_005492865.1	F	GCTCATCCTCAACCACATCG
		R	CCATCTAGGGCGAACTTGGT
11β-Hydroxysteroid dehydrogenase 2 (11βHSD2)	XM_005490743.3	F	ATATCCAGGCCCACACCAAC
		R	CACGTTGTCCCTGTTTTGTAGT
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ)	NM_001031343.1	F	GTGGAGCAATCACAACAGGC
		R	GCGTGCGTCTTTGTATGACTC

Table 2.

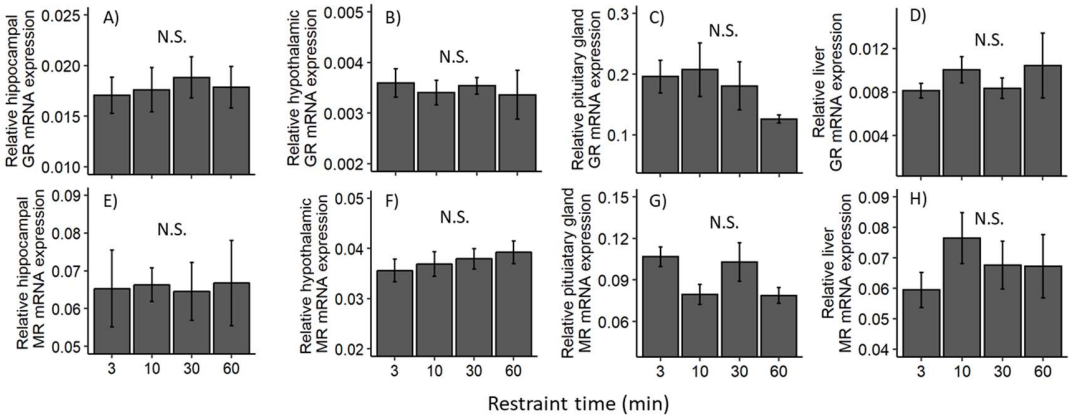
Analyses of variance results investigating the effects of acute capture restraint stress on gene expression and Pearson's correlations between baseline and stress-induced corticosterone and gene expression of glucocorticoid receptor (GR), mineralocorticoid receptor (MR), 11β-hydroxysteroid dehydrogenase 1 (11βHSD1), 11β-hydroxysteroid dehydrogenase 2 (11βHSD2), corticosterone binding globulin

(CBG) and gonadotropin inhibitory hormone (GnIH) mRNA in tissues including the hippocampus, hypothalamus, pituitary gland, and liver of male white crowned sparrows.

	Gene expression			Basline CORT Correlation			Stress-induced CORT Correlation		
Mineralocorticoiod receptor (MR)	*DF*	*F*	*P*	*DF*	*r*	*P*	DF	*r*	*P*
Hippocampus	3,22	0.01	0.98	24	0.28	0.10	24	0.22	0.26
Hypothalamus	3,24	0.73	0.54	2	0.24	0.21	26	0.13	0.48
Pituitary gland	3,14	3.13	0.06	16	0.05	0.81	16	0.02	0.92
Liver	3,22	0.86	0.48	24	0.27	0.17	24	0.14	0.46
Glucocorticoid receptor (GR)									
Hippocampus	3,22	0.13	0.94	24	0.22	0.27	24	0.13	0.51
Hypothalamus	3,24	0.12	0.94	26	0.06	0.73	26	0.28	0.15
Pituitary gland	3,14	1.20	0.34	15	0.11	0.66	15	0.17	0.49
Liver	3,22	0.45	0.71	24	0.02	0.88	24	0.28	0.16
11β-Hydroxysteroid dehydrogenase 1 (11βHSD1)									
Hippocampus	3,21	1.24	0.31	23	-0.05	0.8	23	0.08	0.68
Hypothalamus	3,23	0.23	0.87	25	-0.01	0.92	25	0.03	0.87
Pituitary gland	3,16	0.39	0.76	18	0.43	0.06	18	0.26	0.25
Liver	3,22	0.77	0.51	25	0.33	0.63	25	0.22	0.26
11β-Hydroxysteroid dehydrogenase 2 (11βHSD2)									
Hippocampus	3,21	1.95	0.15	23	0.01	0.93	23	-0.07	0.71
Hypothalamus	3,22	1.84	0.16	24	-0.27	0.16	24	-0.04	0.81
Pituitary gland	3,16	0.13	0.93	18	0.52	0.01	18	0.16	0.5
Liver	3,22	0.77	0.52	24	0.21	0.29	24	0.35	0.07
Corticosterone binding globulin (CBG)									
Liver	**3,23**	**22.85**	**0.02**	25	0.06	0.75	25	0.16	0.41
Gonadotropin-inhibitory hormone									
Hypothalamus	**3,21**	**8.76**	**<0.0001**	23	0.03	0.88	23	0.16	0.56

Figure 1.

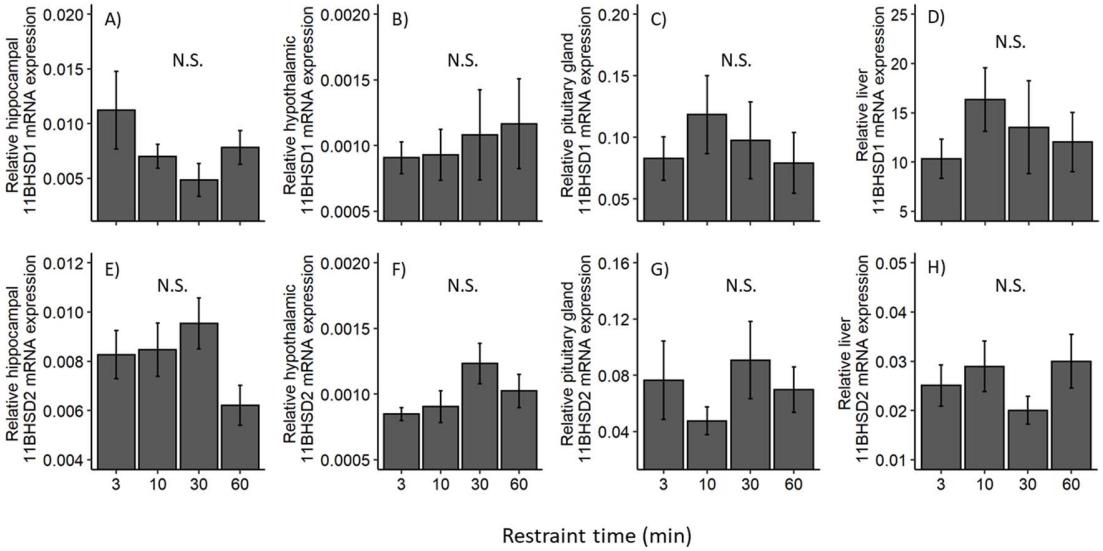
Effects of capture restraint stress on the expression of glucocorticoid (GR; A, B, C, D) and mineralocorticoid (MR; E, F, G, H) receptor mRNA in the hippocampus, hypothalamus, pituitary gland, and liver from time of capture in male white-crowned sparrows. Data were normalized relative to the house keeping gene YWHAZ. Letters that are different from one another indicate a significant difference using contrasts with a Tukey's correction. Values represent means ±SEM.



576

577 **Figure 2**.

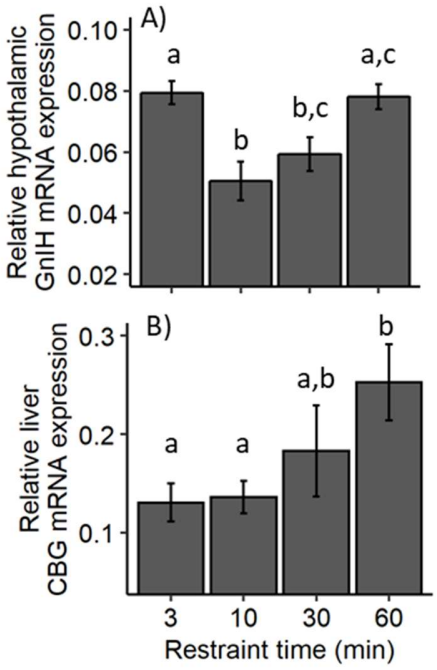
578 Effects of capture restraint stress on the expression of 11β-hydroxysteroid dehydrogenase 1 (11βHSD1;
579 A, B, C, D) and 11β-hydroxysteroid dehydrogenase 2 (11βHSD2; E, F, G, H) mRNA in the hippocampus,
580 hypothalamus, pituitary gland, and liver from time of capture in male white-crowned sparrows. Data were
581 normalized relative to the house keeping gene YWHAZ. Letters that are different from one another
582 indicate a significant difference using contrasts with a Tukey's correction. Values represent means ±SEM.



583

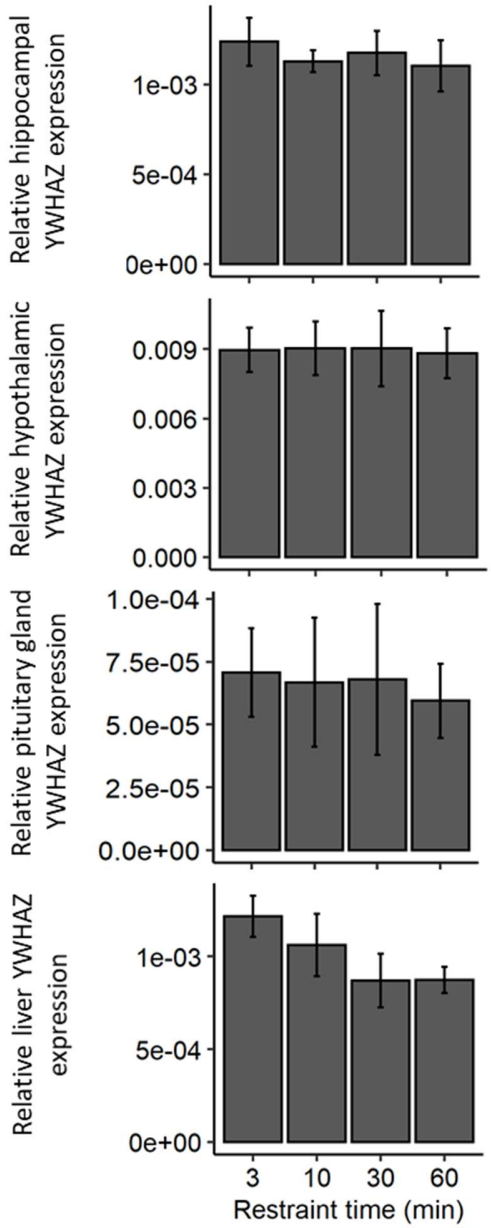
584 **Figure 3**.

585 Effects of restraint stress on the expression of (A) hypothalamic gonadotropin inhibitory hormone (GnIH)
586 and (B) liver corticosteroid binding globulin (CBG) mRNA. Data were normalized relative to the house
587 keeping gene YWHAZ. Letters that are different from one another indicate a significant difference using
588 contrasts with a Tukey's correction. Values represent means ± SEM.



589

590 Supplemental Figure 1. YWHAZ reference gene expression in hippocampus, hypothalamus, pituitary
591 gland and liver of white crowned sparrows exposed to restraint handling.


Relative hippocampal YWHAZ expression
1e-03
5e-04
0e+00
Relative hypothalamic YWHAZ expression
0.009
0.006
0.003
0.000
Relative pituitary gland YWHAZ expression
1.0e-04
7.5e-05
5.0e-05
2.5e-05
0.0e+00
Relative liver YWHAZ expression
1e-03
5e-04
0e+00
3
10
30
60
Restraint time (min)

592