Tissue specific expression of 11BHSD and its effects on plasma corticosterone during the stress response.

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Summary Statement

Peripheral enzymes are primarily responsible for enzymatic modulation of the glucocorticoid stress response in songbirds.

Abstract

The hypothalamic-pituitary-adrenal (HPA) axis is under complex regulatory control at multiple levels. Enzymatic regulation plays an important role in both circulating levels and target tissue exposure. Three key enzyme pathways are responsible for the immediate control of glucocorticoids. De novo synthesis of glucocorticoid from cholesterol involves a multistep enzymatic cascade. This cascade terminates with 11β-hydroxylase, responsible for the final conversion of 11 deoxy- precursors into active glucocorticoids. Additionally, 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) controls regeneration of glucocorticoids from inactive metabolites, providing a secondary source of active glucocorticoids. Localized inactivation of glucocorticoids is under the control of Type 2 11β-HSD (11β-HSD2). The function of these enzymes is largely unexplored in wild species, particularly songbirds. Here we aim to explore the contribution of both clearance and generation of glucocorticoids to regulation of the hormonal stress response via use of pharmacological antagonists. Additionally, we mapped 11β-HSD gene expression. We found 11β-HSD1 primarily in liver, kidney, and adrenal glands though it was detectable across all tissue types. 11β-HSD2 was predominately expressed in the adrenal glands and kidney with moderate gonadal and liver expression. Inhibition of glucocorticoid generation by metyrapone was found to decrease levels peripherally, while both peripheral and central DETC administration resulted in elevated concentrations of corticosterone. These data suggest that during the stress response, peripheral antagonism of the 11β-HSD system has a greater impact on circulating glucocorticoid levels than central control. Further studies show aim to elucidate the respective roles of the 11β-HSD and 11β-hydroxylase enzymes.

Introduction

Glucocorticoids are critical for the physiological responses to environmental, both external and internal, perturbations. In response to acute stressors such as food shortage, inclement weather or predators, the organism responds by increasing the synthesis of glucocorticoids from the adrenal glands, giving rise to the classic "stress response". In the short term, elevation of glucocorticoids is adaptive promoting changes in physiology and behavior to promote survival (e.g. Sapolsky et al., 2000; Romero, 2002; Krause et al., 2017). As prolonged or chronic elevation of glucocorticoids can result in a number of pathological conditions, the homeostatic regulation of glucocorticoid levels is essential for maximizing fitness.

Regulation of glucocorticoid synthesis and secretion in response to stressors begins with integration of internal and external cues by the brain. Ultimately, cells in the paraventricular nucleus (PVN) of the hypothalamus are stimulated causing the release of corticotrophin-releasing factor (CRF) and arginine vasopressin or arginine vasotocin depending on species (e.g. Joëls et al., 2008). CRF triggers the release of adrenocorticotropic hormone (ACTH) from the corticotroph cells in the anterior pituitary gland into the systemic circulation. ACTH then acts to stimulate increased synthesis of glucocorticoids in the adrenal glands. Glucocorticoids not only signal to target cells, but also provide negative feedback to the hypothalamo-pituitary-adrenal (HPA) axis regulating further activation of the HPA axis and ultimately returning plasma hormone levels to baseline. These actions occur through two classes of receptors: the high affinity mineralocorticoid (MR) and low affinity glucocorticoid (GR) receptors. GR receptor tends to be bound at high circulating levels of glucocorticoids such as those encountered during a stress response so GR is considered to be the primary mediator of stress effects and negative feedback (Reul et al., 1987). Glucocorticoid signaling generates negative feedback by binding to CRF-neurons in the PVN as well as blocking hippocampal signaling to the CRF neurons, thus reducing CRF release and inhibiting ACTH release through binding at corticotrophs in the anterior pituitary gland (de Kloet, 2014). Recent work has suggested a short negative feedback loop within the adrenal gland itself, with locally produced glucocorticoids serving to suppress further glucocorticoid synthesis as levels rise (Walker et al., 2015). Thus, negative feedback regulation of the stress response can broadly be categorized as either central or peripheral depending upon the site of glucocorticoid signaling involved. Given the importance of

glucocorticoid receptors in mediating negative feedback control, the majority of research to date has focused on variation in distribution and concentration of MR and GR within the HPA axis (Breuner and Orchinik, 2001; Canoine et al., 2007; Harris et al., 2013; de Kloet, 2014; Krause et al., 2015; Cornelius et al., 2018). To date other mechanisms of stress axis modulation have received comparatively little attention.

Of particular interest is enzymatic control of glucocorticoid synthesis/regeneration and localized inactivation. Adrenal generation of active glucocorticoids from cholesterol ends with the conversion of deoxy- forms (deoxycorticosterone or 11-deoxycortisol) to active glucocorticoid by the 11β hydroxylase enzyme. The liver also serves as a secondary source of glucocorticoids through the regeneration of inactive 11 keto-glucocorticoids to glucocorticoids by the enzyme 11β hydroxysteroid dehydrogenase type 1 (11β-HSD1). Indeed in humans regeneration of inactive 11 keto-glucocorticoids has been found to account for up to 40 % of glucocorticoid synthesis (Basu et al., 2004). 11β-HSD1 may also serve to mediate local tissue level exposure as it has been reported in an array of mammalian tissue types including the brain, liver, adipose tissue, fat, gonads, vasculature, multiple brain regions, uterus, and muscle (Diaz et al., 1998; Holmes and Seckl, 2006; Wyrwoll et al., 2011; reviewed in Chapman et al., 2013). Reports of 11β-HSD1 in avian species are limited with a single study reporting it as undetectable in the brain of zebra finches, *Taeniopygia guttata*, (Rensel et al., 2018).

Whereas 11β-HSD1 and 11β hydroxylase act to synthesize and regenerate inactivated glucocorticoids respectively, 11β hydroxysteroid dehydrogenase type 2 (11β-HSD2) serves as a key regulator of local exposure to glucocorticoids at the tissue level by inactivating glucocorticoids to inert metabolites. This is best demonstrated by its well-documented action in the kidneys where inactivation of glucocorticoids by 11β-HSD2 allows aldosterone, instead of glucocorticoids, to bind to the non-selective MR receptors. Similar to 11β-HSD1 in birds, 11β-HSD2 has been reported to have a relatively limited neural distribution in mammals and is most closely associated with protection of developing tissue from excess glucocorticoid signaling and modulation of neural aldosterone signaling in the adult brain (Wyrwoll et al., 2011). In birds 11β-HSD2 has been described in the chicken (Gallus gallus; Klusoňová et al., 2008) and zebra finch (Katz et al., 2010; Rensel et al., 2018) in brain, liver, kidney, colon and gonads. The expression of 11β-HSD enzymes in the brain in particular suggests the potential for altering negative feedback control of the hormonal stress response. Similarly, peripheral 11β-HSD is

expected to impact rates of clearance and regeneration of glucocorticoids. Together, these dual effects may provide a critical mechanism for maintenance of both plasticity and variation (seasonal and inter-individual) in the functional characteristics of the HPA axis. This is supported by studies in 11β-HSD1 knockout mice that have demonstrated increased glucocorticoid secretion in response to restraint stress (Harris et al., 2001).

To date, the role of regulatory enzymes, both in the periphery and brain, in controlling glucocorticoid levels remains poorly understood in free living animals, particularly birds. Here we seek to understand the relative contribution of both peripheral and central inactivation (via 11β-HSD2) and activation (via 11β-HSD1 regeneration and 11β-hydroxylase synthesis) of glucocorticoids in modulating the hormonal stress response. In order to address these major knowledge gaps, we have taken a multi-step approach utilizing the Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*) - a seasonally breeding songbird species with well-characterized stress physiology. First, we quantified mRNA expression of 11β-HSDs across both central and peripheral tissues in both sexes. To test the importance of both central and peripheral enzyme activity we conducted both peripheral and central ICV administration of pharmacological antagonists targeting both glucocorticoid (hereafter corticosterone, the major glucocorticoid in birds) generation and clearance. To test the contribution of corticosterone synthesis in modulating circulating plasma levels we utilized the well-characterized blocker of corticosterone synthesis metyrapone (MET), shown to block both synthesis by 11β hydroxylase and 11β-HSD1 regeneration of corticosterone. Simultaneously we utilized a previously identified selective inhibitor of 11β-HSD2 (Schweizer et al., 2003), sodium diethyldithiocarbamate trihydrate (DETC; inhibits 11β-HSD2) to block clearance of corticosterone.

Materials and Methods

Animals

Gambel's White-crowned Sparrows were captured on their wintering grounds near Davis, California USA (N 38° 33', W 121°44') between 2016 and 2017 using a combination of seed baited potter traps and Japanese mist nets. In December 2016, photosensitive field caught birds (n= 9 per sex) were euthanized by overdose of isoflurane and following confirmation of death

brain, pituitary gland, gonad, kidney, liver, fat, gastrocnemius muscle, pectoralis muscle, heart, and adrenal glands were collected for subsequent RT-PCR analysis. Collected tissues were immediately fresh frozen on dry ice and stored at - 80°C then shipped to the Roslin Institute on dry ice, and then stored at - 70°C. Time to euthanasia from capture was 146 ± 5 sec and a baseline blood sample (ca 70 µL) was taken from all animals within 87 ± 7 sec of capture to determine baseline levels of corticosterone. Blood was collected by puncture of the alar vein with a 26 gauge needle and surface blood collected by heparinized microcapillary tube (41B501; Kimble Chase, Vineland, NJ USA).

Twenty-eight males and twenty-two females were sampled in the field for Experiment 1 (Peripheral 11β-HSD2 Regulation) from February to March of 2017. In early April 2017 an additional 26 pre-breeding males were captured and transferred to captivity in aviary facilities at the University of California, Davis for use in Experiment 2 (Central Regulation). Sex was determined by PCR followed by gel electrophoresis per (Griffiths et al., 1998) for free living birds and by necropsy for captive birds. All procedures were approved by UC Davis Institutional Animal Care and Use Committee (IACUC), protocol # 19758 and followed UK ARRIVE (ASPA) guidelines. In all experiments birds were randomly assigned to treatment.

Determining 11β-HSD mRNA Expression

RNA was extracted from tissues using Zymo DIRECT-zol RNA miniprep kits (Zymo Research, Irvine CA. USA). Following RNA extraction, total concentration of RNA was determined via nanodrop. RNA input was equalized tissue-wise for reverse transcription to cDNA using a High Capacity cDNA Reverse Transcription Kit (Cat no. 4368814; Life Technologies, Carlsbad, CA USA) prior to quantification. Quantitative polymerase chain reaction (qPCR) using Brilliant III Ultra-Fast Sybr Green (Agilent Technologies, http://www.genomic.agilent.com, Santa Clara, CA USA) in Thermofast 96 well detection plates (AB1100, ThermoFisher, UK) with optical caps (4323032, ThermoFisher, UK) was used to measure 11β-HSD1 and 11β-HSD2 gene expression. Reactions were performed and samples counted on a Stratagene MX 3000 Machine and relative measurements calculated using MxPro software by extrapolation to a standard sample series of defined concentration (standard curve), as previously described (Reid and Dunn, 2018). Manual examination of reaction quality involved

examination of dissociation curves for a single peak, check of standard curve correlation (>0.995) and confirmation of good reaction efficiency (90-110%). Following quality control, sample size for male gastrocnemius muscle was reduced to 8, and both gonad and pituitary gland to 6 samples for final analysis. All qPCR data were normalized to the geometric mean value of two reference genes; 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta (YWHAZ) and NADH:ubiquinone oxidoreductase subunit A1 (NDUFA1). Primers were designed based on NCBI (https://www.ncbi.nlm.nih.gov) database entries for available passerine species: White-throated sparrow (*Zonotrichia albicollis*) and zebra finch (*Taeniopygia guttata*) Two potential sequences were examined for 11β-HSD1 based on a search of NCBI databases and were denoted 11β-HSD1-762 (XM_005495762.2) and 11β-HSD1-865 (XM_005492865.1). Based on sequencing and homology data, candidate 11β-HSD1-865 (hereafter referred to as 11β-HSD1) was determined to correctly represent 11β-HSD1 and used for subsequent tissue analysis. Single sets of primers were designed for 11βHSD2 (XM_014269709.1), YWHAZ (NM_001031343.1) and NDUFA1 (NM_001302115.1). All primers were validated via standard PCR of *Z. leucophrys* cDNA and amplicons sequenced to confirm identity. See Table 1 for details of primers used.

Antagonists

2-Methyl-1,2-di-3-pyridyl-1-propanone - 96% (MET, M2696; Sigma-Aldrich, USA) and sodium diethyldithiocarbamate trihydrate (DETC, D3506; Sigma-Aldrich, USA) were freshly prepared in Ringer's Lactated saline (0.9%) to provide an injection volume of 100-200 µL at desired dosages: MET high 30 mg kg^{-1}, MET low 15 mg kg^{-1}, DETC high 400 mg kg^{-1}, and DETC low 200 mg kg^{-1}. DETC has been shown to be a selective inhibitor of 11β-HSD2 with an IC50 of 6.3 ± 3.8 µM and no detectable activity with respect to both reduction and oxidation reactions catalyzed by 11β-HSD1 (Schweizer et al., 2003).

Experiment 1: Effects of peripheral injection of DETC and MET on the corticosterone stress response in free-living white-crowned sparrows

Immediately following capture (within 3 min) a baseline blood sample (ca. 70 µL) was obtained as described above. Birds were weighed by Pesola spring scale to the nearest 0.1 g to determine appropriate drug dosage. Birds were randomly assigned to one of 5 treatment groups receiving either: high dose MET (30 mg kg^{-1}; n = 11), low MET (15 mg kg^{-1}; n =12), high dose DETC (400 mg kg^{-1}; n =13), low dose DETC (200 mg kg^{-1}; n=9), or control (100 µL of Lactated Ringers solution; 13) via IP injection. Birds were held under a standardized capture restraint protocol for 60 min with additional blood samples collected at 10, 30 and 60 mins in an opaque cloth bag (Astheimer et al., 1992). Each bird was banded with a unique US Fish and Wildlife Service band prior to release.

Experiment 2: Effects of central administration of DETC and MET on corticosterone stress response in captive white-crowned sparrows

Birds were initially housed in two large flight aviaries (3×2.5×2m) on a 11L:13D photoperiod for acclimation. A total of 24 birds were utilized in the experiment as described below. A 3:1 mixture of Mazuri Small Bird Maintenance Diet (#56A6; Mazuri, Richmond, IN USA) and mixed wild bird seed along with water and grit were provided *ad libitum*. After 30 days birds were transferred to individual cages (35×25×40 cm) and housed in groups of 3-4 in sound chambers on a fixed photoperiod of 10L:14D for the remaining duration of the experiment.

Birds were cannulated as previously described (Bentley et al., 2006). Birds were food deprived two hours prior to surgery, anesthetized with 2-4% isoflurane with supplemental oxygen (1 L min^{-1}) and placed into a stereotaxic apparatus specially designed for songbirds (MyNeuroLab.com). The intersection of the mid-sagittal and transverse sinuses was located on the skull and served as a reference point. The 11 mm 26 gauge guide cannula (C315G; Plastics One, Roanoke, VA USA) was moved 2.3 mm anterior from the reference point, lowered 6mm below the surface of the skull, bonded in place with dental cement (NC9655090; Stoelting Wood Dale, IL USA) and allowed adequate curing time before the animal was removed from the stereotaxic frame. A 33 gauge dummy cannula (C315DC; Plastics One, Roanoke, Virginia) was inserted into the guide cannula to prevent the development of obstruction. Patency of the cannula was determined by administration of human angiotensin II (A9525-1mg; Sigma-Aldrich, USA)

which rapidly promotes thirst and drinking behavior within 2 min of infusion (Wada et al., 1975; Richardson and Boswell, 1993). Only birds that drank within 2 min following the administration of 1µg of Angiotensin II in 2 µL of sterile LRS were included in experimental treatments.

Birds were divided into three groups (n = 8 per group/round), which initially received one of the following treatments: control (0.9% sterile saline), MET (20 µg), or DETC (200 µg) administered in a 2 µL bolus infusion over 2 min by infusion pump (PHD 2000; Harvard Apparatus, Holliston, MA USA). Central administration was carried out using a 10 µL Hamilton syringe attached to a clear piece of polyethylene tubing marked to show 1 µL volumes. The injection cannula (C315I ; Plastics One, Roanoke, VA USA) protruded 0.5 mm past the end of the guide cannula allowing central administration into the third ventricle. Birds were assigned to initial treatments such that each chamber received a mix of treatments. Birds were subsequently rotated through each treatment condition so all birds experienced each treatment over the 3 week experimental period. Upon opening of each sound chamber a baseline blood sample (approximately 50 µL) was collected from the alar vein within 3 min or less (as previously described), prior to administration of the appropriate intracerebroventricular (ICV) infusion. Additional 50 µL blood samples were collected at 10, 30 and 60 mins post disturbance. Two birds per chamber were infused and sampled each day, such that all birds were sampled in a two day period each week. Sampling order was reversed each week.

Blood sample processing

All blood samples were stored on ice until processing. Plasma was separated from red blood cells by centrifuging at 10,000 rpm for 5 min. The plasma was then aspirated via a Hamilton syringe and placed into microcentrifuge tubes and stored at -30° C until corticosterone quantification.

Corticosterone Radioimmunoassay

Corticosterone levels were measured by a radioimmunoassay as previously described by Wingfield et al. (1992). Briefly, 15 µL of plasma from baseline samples and 10 µL of the post capture time points were assayed. Recovery efficiency was estimated by adding 2000 CPM of tritiated corticosterone (Perkin Elmer NET399250UC, Waltham, MA USA) to each sample prior

to extraction. Corticosterone was extracted from the samples by incubating with 4 mL of re-distilled dichloromethane with regular vortexing (D154-4; Fisher Chemical, Pittsburgh, PA USA). The aqueous phase was then extracted into a clean 10 mL test tube and the samples were dried in a water bath at 35°C under nitrogen gas, prior to being reconstituted using 550 µL of phosphate buffered saline gelatin (PBSG). The reconstituted samples were separated into 200 µL duplicate aliquots for quantification and 100 µL retained for determination of recovery efficacy. Recovery samples were combined with 2 mL of scintillation fluid (Ultima Gold: 6013329; Perkin Elmer, Waltham, MA USA) and counted to determine the percent recovery for each sample. 100 µL of tritiated corticosterone and 100 µL antiserum (07–120016, lot 3R3-PB-20E; MP Biomedical, Santa Ana, CA USA) were added to each duplicate assay tube and incubated overnight at 4°C. 500 µL of dextran-coated charcoal was added to each duplicate and after exactly 12 min, samples were centrifuged at 3000 rpm for 10 min at 4°C. The supernatant was decanted into scintillation vials and combined with 4 mL of scintillation fluid (Perkin Elmer Ultima Gold: 6013329, Waltham, MA USA). Samples were placed on a Beckman 6500 liquid scintillation counter and each vial was counted for 5 min or within 2% accuracy. The corticosterone values were determined from a standard curve and adjusted using the corresponding recovery percentage. Mean recoveries were 82.7% and intra-assay (calculated using C.V. between duplicates) and inter-assay variations were 7.25% and 10.87%, respectively. The detection limit of the assays was 8.85 ± 0.49 pg per tube (~0.7 ng mL^{-1} per tube).

Statistical Analyses

All statistical analyses were performed in R (R Core Development Team, 2018) using packages: pracma (Borchers, 2017), ggplot2 (Wickham, 2009), lme4 (Bates et al., 2014), lmerTest (Kunznetsova et al., 2014), emmeans (Lenth, 2019) and tidyr (Wickham and Henry, 2018). Normalized gene expression data was analyzed by ANOVA and post-hoc testing performed using Tukey's Honestly Significant Difference tests. Sex differences between tissues were not tested based on the absence of main or interaction effects of sex. For the peripheral injection study the data low and high doses of DETC and MET were compared via linear mixed effects model with a dose by handling time interaction individually to determine any dose effect. As no significant main effect (DETC: $F_{1,66} = 1.77$, $p = 0.188$; MET: $F_{1,35} = 0.18$, $p = 0.674$) of

dose nor any interaction with restraint time (DETC: $F_{1,64} = 0.001$, $p = 0.975$; MET: $F_{1,60} = 0.09$, $p = 0.764$) was found, the dosages were combined for each drug for all subsequent analyses. Subsequently a fully parameterized linear mixed effects model of Treatment, Restraint Time, and Sex and all interactions was tested and no effect of sex nor interaction was detected. This model returned as rank deficient thus a second model with the main effect of Treatment, Restraint Time and Sex as well at the interaction of Treatment and restraint time was tested and again sex was found to be non-significant ($F_{2,51} = 1.83$, $p = 0.172$) and thus sex was excluded from further analyses. A final base model of the interaction of Restraint Time and Treatment (MET, DETC, and saline) with both band number (unique identifier) and corticosterone assay number included as random intercepts to account for repeated sampling and intra-assay variation respectively, was used. The dosage of the drug had no effect for MET ($F_{1,22} = 0.12$, $P = 0.85$) nor for DETC ($F_{1,18} = 2.58$, $P = 0.12$) in field samples, thus in both cases dosages were combined to increase statistical power. Following detection of significant effect in the linear mixed effects model, post hoc tests were performed using estimated marginal means with Tukey's Honestly Significant Difference and Kenward-Roger estimation of degrees of freedom in the emmeans package. Integrated corticosterone was determined by calculation of area under the curve using the function *trapz* in the pracma package in R. Integrated corticosterone was analyzed by linear mixed effects model with fixed effect of Drug and random effects matched to experimental design (see Table 2 for final models). All data are reported as the mean ± standard error of the mean (s.e.m).

Results

11β -HSD Gene Expression

11β-HSD1 and 11β-HSD2 were detected across tissues (Figs 1,2). 11β-HSD1 expression differed significantly between tissues (Fig. 1; $F_{10, 169} = 54.94$, $p < 0.001$) and tissue level expression patterns varied by sex ($F_{10, 169} = 2.60$, $p = 0.006$). Expression of 11βHSD1 was highest in liver for both sexes (Fig. 1), though sexes differed significantly from each other ($F_{1,169} = 0.339$, $p < 0.001$). 11β-HSD1 expression was detected in the hippocampus, hypothalamus and anterior pituitary gland, but was close to the lower limit of detection in all three tissues (Fig. 1). 11β-HSD2 expression also differed significantly between tissues (Fig. 2; $F_{10,170} = 63.24$, $p <$

0.001), but no effect of sex ($F_{1,170} = 1.33$, $p = 0.251$) nor sex by tissue interaction was detected ($F_{10,170} = 1.41$, $p = 0.181$). As with 11β-HSD1 expression, 11β-HSD2 was low but present in the hippocampus, hypothalamus and anterior pituitary (Fig. 2).

Experiment 1: Effects of peripheral antagonist injections on corticosterone concentrations during the stress response in free living white-crowned sparrows

The final model consisted of restraint time, treatment and their interaction with the random effect of bird. Corticosterone was found to increase independent of treatment with duration of restraint stress (Fig. 3A; $F_{1,200} = 103.98$, $p < 0.001$). Treatment also significantly altered plasma corticosterone levels ($F_{2,200} = 7.22$, $p < 0.001$). DETC treatment resulted in significant elevation of corticosterone at 10 min compared to MET (DETC-MET: $t_{171} = 5.47$, $p < 0.001$), at 30 min post capture compared to both Saline and MET groups (DETC-Saline: $t_{169} = 4.36$, $p < 0.001$; DETC-MET: $t_{174} = 9.23$, $p < 0.001$), and at 60 minutes post capture compared to MET ($t_{174} = 3.04$, $p = 0.007$). Peripheral injection of MET resulted in decreased corticosterone compared to Saline at 30 minutes post capture ($t_{171} = -3.36$, $p = 0.003$).

Total integrated corticosterone secreted over the hour restraint period, as calculated by area under the curve, was affected by treatment (Fig. 3B; $F_{2,45} = 25.9$, $p < 0.001$). DETC significantly increased corticosterone compared to Saline ($t_{45} = 3.15$, $p = 0.008$) and MET ($t_{45} = 7.17$, $p< 0.001$). MET treatment reduced total corticosterone secreted compared to Saline controls ($t_{45} = -2.88$, $p = 0.017$).

Experiment 2: Effects of central ICV administration of antagonists on corticosterone stress response in captive white-crowned sparrows

Corticosterone concentrations increased over the 60 min restraint period across treatments (Fig. 3C; $F_{1, 192} = 45.8$, $p <0.001$). The increase of corticosterone concentrations over time was found to be dependent upon drug type infused ($F_{2, 192} = 4.14$, $p = 0.02$). There were no differences between treatments detected at the 0 and 10 min time points. DETC treated birds had significantly higher corticosterone at 30 (Fig.3B; DETC-Saline: $t_{193} = 4.81$, $p < 0.001$; DETC-MET: $t_{188} = 3.38$, $p =0.04$) and 60 min post restraint (DETC-Saline: $t_{193} = 3.37$, $p = 0.02$; DETC-

MET: $t_{188} = 3.53$, $p = 0.026$). Corticosterone concentration in response to MET infusion did not differ from Saline controls at any time point ($p \geq 0.05$).

Total corticosterone secreted showed a trend towards being affected by drug infused (Fig. 3D; $F_{2,31} = 2.68$, $p = 0.084$). However, post-hoc testing detected no difference between treatment groups in total corticosterone secreted, though DETC infusion ($t_{36} = 1.79$, $p = 0.19$) trends towards increasing corticosterone as compared to saline when inspected graphically.

Discussion:

11β-HSD gene expression

Detection of 11β-HSD expression across tissues supports a functional role in regulation of tissue specific and circulating corticosterone levels in birds. Consistent with previous reports in birds we found 11β-HSD1 expression to be highest in the liver (Rensel et al., 2018) and 11β-HSD2 to be highest in the kidney and detectable levels in the gonads, liver and brain (Klusoňová et al., 2008; Katz et al., 2010; Rensel et al., 2018). This expression across body tissues supports the established major role of 11β-HSD1 in the hepatic generation of active glucocorticoids from circulating precursors (Rensel et al., 2018) and 11β-HSD2 protection of renal aldosterone signaling via protection of MR from corticosterone binding.

For the first time we report expression of both 11β-HSD1 and 11β-HSD2 in the adrenal glands of a bird. While, the presence of 11β-HSD1 in the adrenal gland is expected, given its key role in corticosterone biosynthesis, the presence of 11β-HSD2 is surprising as it inactivates corticosterone. We found 11β-HSD2 levels here to be 2-3 times higher than in the kidney (the second-highest site of expression). Protection of the adrenal glands from toxic levels of corticosterone and modulation of local autocrine or paracrine feedback loops by 11β-HSD2, may explain these findings. This is supported by data from rats and humans showing that adrenal 11β-HSD2 expression is concentrated primarily in the zona fasciculata, where glucocorticoid synthesis occurs, with lower expression into the zona reticularis and medulla (Roland and Funder, 1996; Mazzocchi et al., 1998) and none in the capsule and zona glomerulosa (site of mineralocorticoid synthesis). Mazzocchi and colleagues (1998) also found 11β-HSD2 activity in human adrenal preparations to be indirectly responsive to exogenous ACTH. This modulation of

adrenal 11β-HSD2 and its distribution suggests the possibility of dynamic modulation of glucocorticoid production within the adrenal gland itself over the course of the stress response. Such modulation of glucocorticoid secretion by adrenal 11β-HSD2 remains to be tested in avian systems.

Hypothalamic expression of both 11β-HSD enzymes were low compared to expression in peripheral tissues. These results are consistent with previous studies in zebra finches (Katz et al., 2010; Rensel et al., 2018). Zebra finch 11β-HSD2 expression was found to be widespread across the brain, and has been suggested to be driven by the widespread neural expression of MR in this species as compared to mammals and other birds (Katz et al., 2010). The present study lacks the necessary data to test this hypothesis and whether it is a unique feature of zebra finches.

Peripheral and central MET administration

Our findings suggest that MET's efficacy may be context and species dependent as inly peripheral administration had minimal detectable effect. Previous studies have demonstrated the efficacy of MET in inhibiting corticosterone synthesis in rodents, with a dose of 40 mg/kg generating robust suppression in rats (Herman et al., 1992). Previous studies in birds, utilizing implants to deliver MET in a time released manner, had no prolonged effect on corticosterone levels in house sparrows (Gray et al., 1990; Aharon-Rotman et al., 2017). Though this may be taken to suggest that MET simply lacks efficacy in avian species, the lowest levels of circulating corticosterone was found to occur two days after implant placement with levels rising over the course of the study (Aharon-Rotman et al., 2017). This suggests that MET implants are able to alter basal corticosterone levels, but that these alterations are unable to overcome homeostatic control in the long run. These results combined with the findings of the present study support a limited efficacy of MET in disrupting generation of corticosterone in birds. The single short term bolus injection approach used in this study may have enabled us to better detect METs effects. However, our data also suggest that MET has a very limited ability to alter corticosterone levels in birds. Further interpretation of MET's specific actions is limited. Further studies are necessary to disentangle the role of 11β hydroxylase action from that of 11β-HSD1.

Inhibition of 11β-HSD2 by peripheral and central DETC administration

Peripheral administration of DETC effectively blocked 11β-HSD2 action, as evidenced by the increase in circulating corticosterone (Fig.3A&B) as predicted by previous cell based assays utilizing DETC (Atanasov et al., 2003; Schweizer et al., 2003). This suggests that peripheral 11β-HSD2, in addition to providing localized protection at the level of the target tissue, also serves to modulate circulating levels of corticosterone. Of particular interest in this respect is the high level of 11β-HSD2 expressed in the adrenal glands, previously unreported in songbirds. Contrary to our a priori predictions, central DETC administration also resulted in elevated circulating corticosterone levels over the restraint period (Fig.3C). Blocking of hypothalamic 11β-HSD2 was expected to lead to a local elevation of corticosterone, thereby increasing negative feedback and ultimately lowering the total amount of corticosterone secreted or at least increasing the speed at which corticosterone returned to baseline. Instead we see a trend towards increased total corticosterone secreted in response to DETC infusion into the 3V (Fig. 3D). Examination of the present data in light of the broader literature, including the ability of DETC to cross the blood brain barrier (Frank et al., 1995), supports two broad hypotheses. First, the escape of centrally injected DETC into the periphery to act upon the major sites of corticosterone inactivation, the kidney and liver. While physically possible, the small volume of DETC administered via ICV (representing a comparatively tiny absolute DETC dosage in comparison to the peripheral injections), makes escape of centrally administered DETC to the periphery a highly unlikely explanation. Alternatively, DETC within the brain may inhibit as yet uncharacterized sites of neural stress axis regulation, that in the absence of normal 11β-HSD2 protective action trigger positive feedback supporting the further release of corticosterone. Future studies utilizing labelled corticosterone to determine tissue level processing in response to antagonist treatment will be necessary to elucidate the role 11β-HSD2 in regulation of the stress axis. Furthermore, conditional knockout models may provide a robust alternative, but are presently not widely available in avian systems.

Role of 11β-HSD2 enzymes in regulation of the stress response

Our findings support a critical role for 11β-HSD2 in the regulation of circulating levels of corticosterone. The high peripheral gene expression and clear reduction in systemic clearance observed in response to peripheral blocking of 11β-HSD2 suggest that peripheral 11β-HSD2

action contributes more to regulation of the hormonal stress response than central 11β-HSD2 activity, if this indeed exists. It is critical to recall that the presence of 11β-HSD2 in multiple peripheral tissues may complicate interpretation of plasma levels in response to antagonist treatment due to potentially opposing effects of 11β-HSD2 blockage between tissues. The complexity of 11β-HSD action has been previously highlighted by studies in 11β-HSD1 knockout mice, which display compensatory adrenal hyperplasia and increased basal levels of corticosterone, despite the presumed absence of hepatic corticosterone reactivation from cortisone (Kotelevtsev et al., 1997). Additionally, in this study it was found that the adrenal glands continued to effectively secrete corticosterone in 11β-HSD1 knockout animals, which also displayed increased adrenal sensitivity to ACTH stimulation. While the results of our present study support clear involvement of peripheral tissue 11β-HSD2 in regulation of circulating corticosterone levels, the study design prevents the disentanglement of the contribution of 11β-HSD2 from specific tissues. Development of novel targeted approaches to separate these tissue specific effects will be critical to advancing our understanding of peripheral corticosterone metabolism.

11β-HSD1 and 11β-HSD2 expression were low in the brain and this is consistent with previous studies in chickens and zebra finches. However, the contribution of 11β-HSD activity in modulating neural negative feedback to the hypothalamus remains unclear. In rats, 11β-HSD2 has been co-localized with GR and MR in the brain, strongly suggests important local regulatory action by these enzymes (Whorwood et al., 1992). Similar data is lacking in free living species; such co-localization data is needed to clarify the potential of 11β-HSD enzymes to modulate corticosterone activity. Existing data from avian studies of neural distribution of MR and GR receptors may provide limited insight, but must be interpreted with caution. Both MR and GR appear to be generally widely distributed within the avian brain (Senft et al., 2016; Rensel et al., 2018). In white-crowned sparrows, GR was strongly expressed in hypothalamus but concentrated primarily in the PVN and pre-optic areas (POA) (Krause et al., 2015). However, caution must be taken in extrapolating as these data are drawn from observation of breeding as opposed to wintering (present study) white-crowned sparrows and brain-wide localization of 11β-HSD1 and 11β-HSD2 remains lacking in this species. Regional co-localization by RT-PCR of both MR and GR expression with 11β-HSD2 expression has been found in zebra finches (Rensel et al., 2018). While this supports an active role for 11β-HSD2 in modulating local corticosterone

concentrations within the avian brain, the lack of neuroanatomical co-localization precludes determination of a functional relationship between the two proteins.

The present work adds to a growing body of literature that supports a significant role for 11β-HSD2 regulatory enzyme action in mediating localized tissue exposure and basal glucocorticoid levels, in addition to modulation in response to stressors. While complete elucidation of role of 11β-HSD2 enzymatic actions in HPA axis modulation remains unexplained, it is clear that 11β-HSD2 is vital in the regulation of tissue specific exposure to glucocorticoids. Future work addressing tissue and brain region specific functional contributions of 11β-HSD enzymes are required to fully explain the roles played in HPA axis regulation.

List of Abbreviations

11β-HSD: 11 beta-hydroxysteroid dehydrogenase; HPA axis: Hypothalamic-Pituitary-Adrenal axis; MR: mineralocorticoid receptor; GR: glucocorticoid; DETC: sodium diethyldithiocarbamate trihydrate; MET: 2-Methyl-1,2-di-3-pyridyl-1-propanone; ICV: intracerebroventricular; 3V: third ventricle; PVN: paraventricular nucleus; ACTH: adrenocorticotropin; CRF: corticotrophin releasing factor.

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Competing Interests

The authors have no competing interests to declare.

Author Contributions

Conceptualization: JHP, JSK, RES, HJL JCW SLM. Sample collection: JHP, JSK, KS, RES, HJL. Avian surgery: JHP and JSK. Molecular biology: JSK, JHP, AR, VRB, JC. Data Analysis: JHP and RES. Original draft and primary writer: JHP and RES. Review & Editing: JHP, JSK, KS, SLM, AR, JCW. Project management: JHP, JSK, JCW, SLM. Funding Acquisition: JCW and SLM.

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Data Availability

All data is available directly upon request from the authors.

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Table 1. qPCR Primers utilized in this study.

Target	Accession No.	Forward Primer	Reverse Primer
11β-HSD1	XM_005492865.1	5'GCTCATCCTCAACCACATCG	5'CCATCTAGGGCGAACTTGGT
11β-HSD2	XM_014269709.1	5'ATATCCAGGCCCACACCAAC	5'CACGTTGTCCCTGTTTTGTAGT
YHWAZ	NM_001031343.1	5'GTGGAGCAATCACAACAGGC	5'GCGTGCGTCTTTGTATGACTC
NDUFA1	NM_001302115.1	5'ATGTGGTACGAGATCCTGCC	5'TTCTCCAGACCCTTGGACAC

Fig. 1. Relative expression of 11βHSD1 mRNA as measured by qPCR for female (black) and male (grey) non-breeding Gambel's white-crowned sparrows across multiple tissues. Letters that are different from one another indicate significant differences ($p < 0.05$) between tissues as determined by post-hoc testing (Tukey's HSD). N = 9 per sex per tissue, except for male gastrocnemius muscle n= 8, gonad n= 6, and anterior pituitary n= 6. Expression was standardized against the geometric mean of YWHAZ and NDUFA reference gene expression. Values are expressed as means $\pm$ SEM.

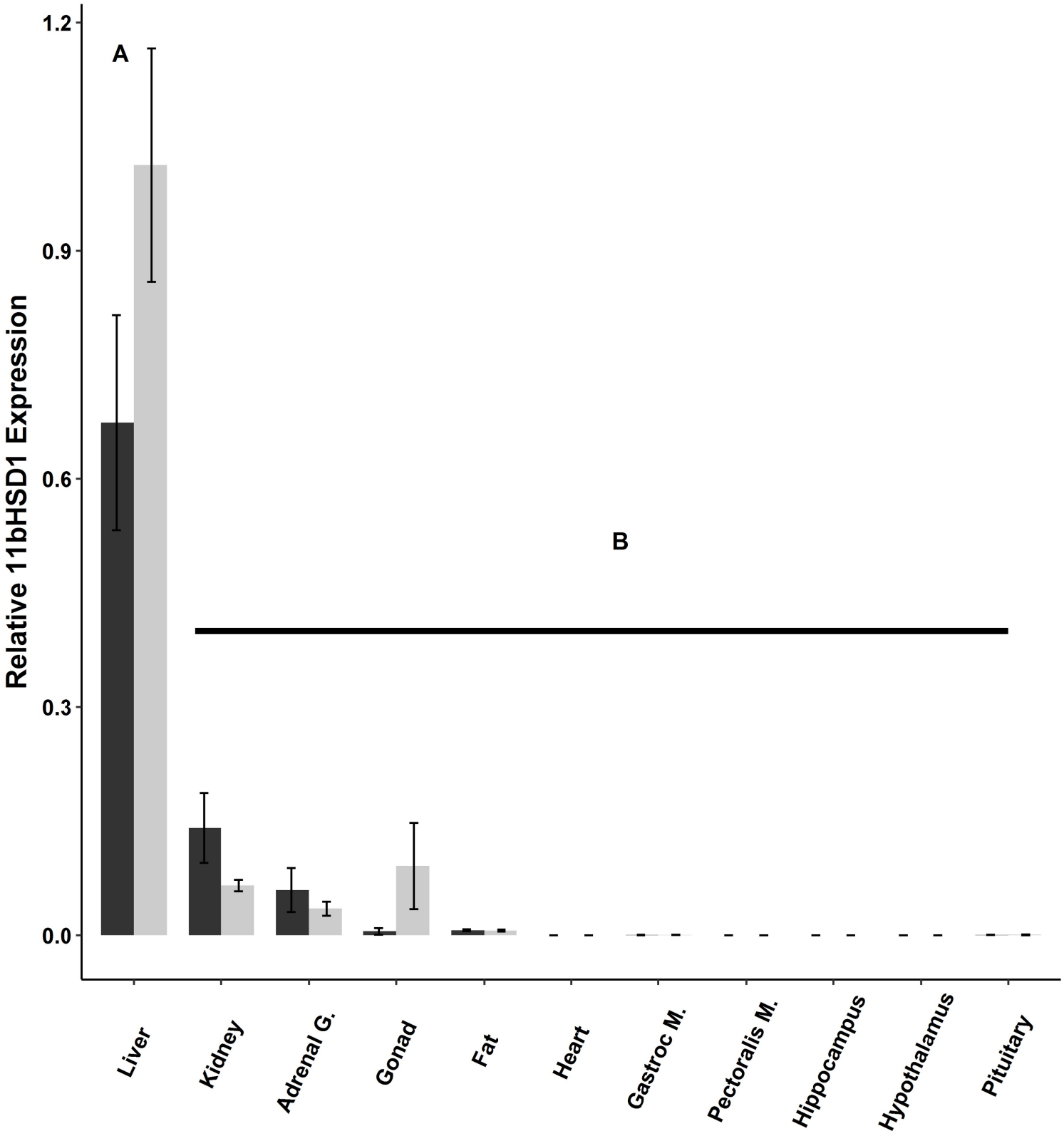
Fig. 2. Relative expression of putative 11βHSD2 mRNA as measured by qPCR for female (black) and male (grey) non-breeding Gambel's white-crowned sparrows across multiple tissues. Letters that are different from one another indicate significant differences ($p < 0.05$) differences between tissues as determined by post-hoc testing. N = 9 per sex per tissue, except for male gastrocnemius muscle n= 8, gonad n= 7, and anterior pituitary n= 6. Expression was standardized against YWHAZ and NDUFA reference gene expression. Values are expressed as means $\pm$ SEM.

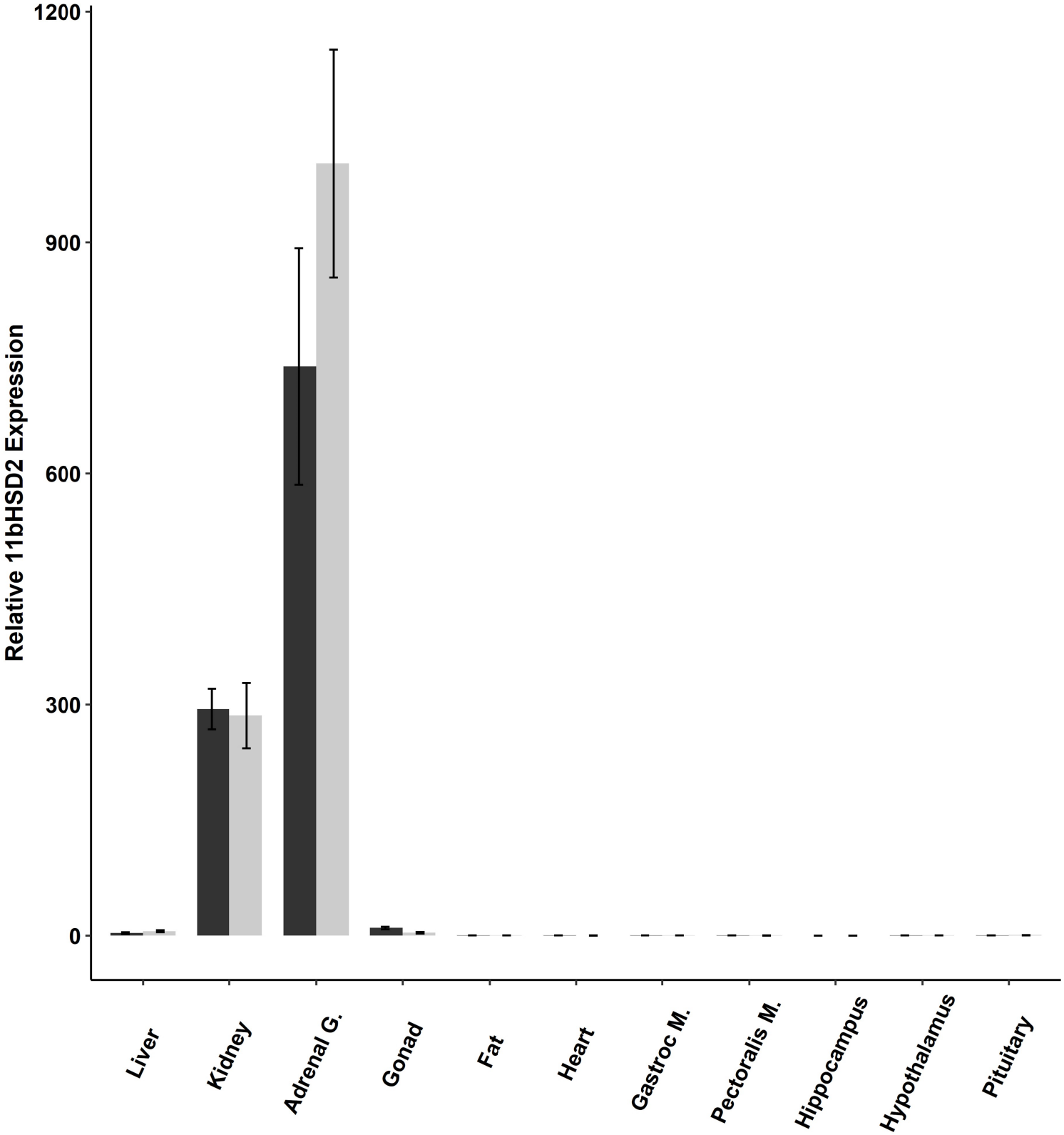
Fig. 3. The effects pharmacological specific inhibition of CORT synthesis using MET and inhibition of 11β-HSD2 clearance of CORT using DETC on plasma concentrations of

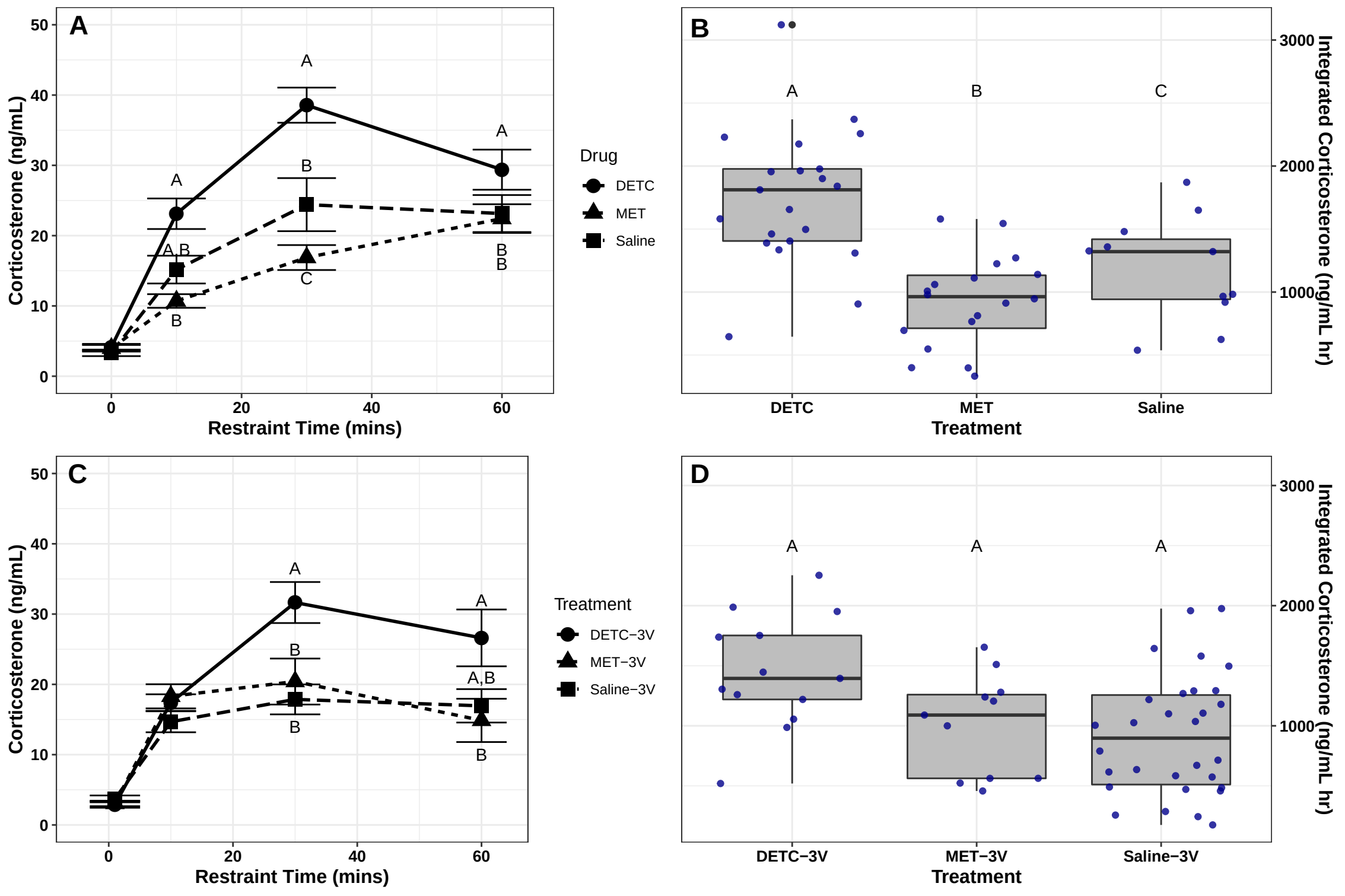
corticosterone. Effects of a single bolus peripheral injections of MET (combined 15 & 30 mg/kg; n = 21) and DETC (combined 200 & 400 mg/kg; n = 21) versus controls (100 µL of Lactated Ringers solution; n=12) on plasma corticosterone concentrations over a A) one hour handling restraint sampling period and B) integrated hormonal response using integrated area under the curve (AUC). Dose had no effect on corticosterone so samples were pooled. Effects of central infusion of Lactated Ringers solution (n = 30), MET (90 nmol; n = 11) and DETC (900 nmol; n = 13) into the third ventricle on corticosterone concentrations over a C) one hour sampling period and D) Integrated area under the curve over the same period. An initial blood sample was taken and then birds were immediately injected with the drug. Data analyses by linear mixed effects model, with Tukey's HSD post-hoc testing. Letters indicate significant differences ($P<0.05$) between treatments at given time point. Values represent means ± SEM.

Model	Fixed Effects	Random Effects
Peripheral Time Series	Time X Drug	Bird ID, Assay
Peripheral Area Under the Curve	Drug	Assay
Central Tme Series	Time X Drug	Bird ID, Assay
Central Area Under the Curve	Drug	Bird ID, Assay

Table 2. Summary of final mixed effects models used in statistical analyses.


Relative 11bHSD1 Expression
1.2
0.9
0.6
0.3
0.0
A
B
Liver
Kidney
Adrenal G.
Gonad
Fat
Heart
Gastroc M.
Pectoralis M.
Hippocampus
Hypothalamus
Pituitary


Relative 11bHSD2 Expression
1200
900
600
300
0
Liver
Kidney
Adrenal G.
Gonad
Fat
Heart
Gastroc M.
Pectoralis M.
Hippocampus
Hypothalamus
Pituitary


A
Corticosterone (ng/mL)
Restraint Time (mins)
Drug
DETC
MET
Saline
B
Integrated Corticosterone (ng/mL hr)
DETC
MET
Saline
Treatment
C
Treatment
DETC−3V
MET−3V
Saline−3V
D
DETC−3V
MET−3V
Saline−3V