Measurement of 11-dehydrocorticosterone in mice, rats and songbirds: Effects of age, sex and stress

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ABSTRACT

Glucocorticoids (GCs) are secreted into the blood by the adrenal glands and are also locally-produced by organs such as the lymphoid organs (bone marrow, thymus, and spleen). Corticosterone is the primary circulating GC in many species, including mice, rats and birds. Within lymphoid organs, corticosterone can be locally produced from the inactive metabolite, 11-dehydrocorticosterone (DHC). However, very little is known about endogenous DHC levels, and no immunoassays are currently available to measure DHC. Here, we developed an easy-to-use and inexpensive immunoassay to measure DHC that is accurate, precise, sensitive, and specific. The DHC immunoassay was validated in multiple ways, including comparison with a mass spectrometry assay. After assay validations, we demonstrated the usefulness of this immunoassay by measuring DHC (and corticosterone) in mice, rats and song sparrows. Overall, corticosterone levels were higher than DHC levels across species. In Study 1, using mice, we measured steroids in whole blood and lymphoid organs at postnatal day (PND) 5, PND23, and PND90. Corticosterone and DHC showed distinct tissue-specific patterns across development. In Studies 2 and 3, we measured circulating corticosterone and DHC in adult rats and song sparrows, before and after restraint stress. In rats and song sparrows, restraint stress rapidly increased circulating levels of both steroids. This novel DHC immunoassay revealed major changes in DHC concentrations during development and in response to stress, which have important implications for understanding GC physiology, effects of stress on immune function, and regulation of local GC levels.

1. Introduction

Glucocorticoids (GCs) are steroid hormones secreted by the adrenal glands to coordinate a myriad of physiological processes (Landys et al., 2006; Munck et al., 1984; Sapolsky et al., 2000; Toufexis et al., 2014; Wada, 2008). Corticosterone is the predominant circulating GC in many species, including mice, rats, and songbirds (Gong et al., 2015; Landys et al., 2006; Taves et al., 2017; Taves et al., 2016; Taves et al., 2015; van Weerden et al., 1992). Corticosterone can be converted to the inactive 11-dehydrocorticosterone (DHC) within tissues by the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) (Cottrell and Seckl, 2009). Conversely, corticosterone can be regenerated from DHC by 11β-HSD1 (Chapman et al., 2013) (Fig. 1). During development,

11β-HSD1 and 2 show marked changes in expression in tissues, including lymphoid organs (Taves et al., 2016). Furthermore, during neonatal development, rodents display the stress hyporesponsive period (SHRP), in which they have very low levels of circulating GCs and minimal effects of stressors on circulating GCs (Gunnar and Donzella, 2002; Sapolsky and Meaney, 1986; Stanton and Levine, 1990; Wood and Walker, 2015). In mice, the SHRP begins at postnatal day (PND) 2 and ends at approximately PND12 (D'Amato et al., 1992; Schmidt et al., 2003).

GCs are powerful regulators of immunity, and bone marrow, thymus, and spleen are particularly sensitive to the effects of GCs. For example, GCs modulate thymocyte selection *via* glucocorticoid receptor binding (Iwata et al., 1991; Vacchio et al., 1999). In addition, short-
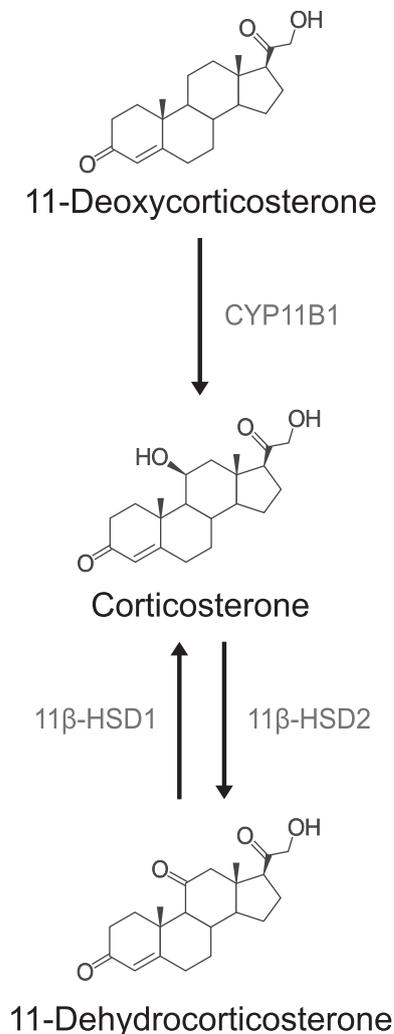

Fig. 1. Simplified glucocorticoid production pathway, with steroid names in black and steroidogenic enzymes in grey.

term increases in systemic GCs enhance immunity by promoting proliferation and trafficking of immune cells in the early stages of an immune response (Bowers et al., 2008; Dhabhar et al., 1996; Wiegers et al., 1994). In contrast, long-term elevation of GCs suppresses the immune system, as demonstrated by involution of the thymus and spleen (Selye, 1936). GCs promote thymic involution, in part, by increasing apoptosis of developing and mature T cells (Ashwell et al., 2000).

In mice, lymphoid organs such as bone marrow, thymus, and spleen locally produce GCs, allowing for tissue-specific regulation of GC levels (Schmidt et al., 2008; Schmidt and Soma, 2008; Taves et al., 2011). Evidence for the local production of corticosterone in mice includes expression of all GC-synthetic and -regenerative enzymes, *in vitro* production of corticosterone by thymic epithelial cells, and local elevation of corticosterone during the SHRP (Mittelstadt et al., 2018; Pazirandeh et al., 1999; Qiao et al., 2009; Taves et al., 2016; Taves et al., 2015; Vacchio et al., 1994). Interestingly, when either the corticosterone precursor, 11-deoxycorticosterone, or the corticosterone metabolite, DHC, are provided *in vitro* at a high concentration (1 μM) to bone marrow, thymus, or spleen, all three tissues produce more corticosterone *via* regeneration from DHC than *via* synthesis from 11-deoxycorticosterone (Taves et al., 2016). However, little is known about endogenous DHC levels in mice during early development, although a few studies have examined adult mice (Alberts et al., 2005; Cobice et al., 2013; Harris et al., 2001; Hundertmark et al., 2002; Tagawa

et al., 2007; Verma et al., 2018; Yau et al., 2001). Thus, it remains unclear to what extent corticosterone is locally regenerated from DHC *in vivo*, especially during the SHRP. Importantly, very little is known about endogenous DHC levels because no immunoassays are currently available to measure DHC.

In addition to mice, rats and songbirds are important animal models for the study of stress physiology. Numerous studies in rats and birds have demonstrated that circulating corticosterone levels rise in response to acute stressors (Romero, 2002; Sapolsky et al., 2000), but few studies have measured DHC in rats (Hay and Mormède, 1997; Hundertmark et al., 2002; Obut et al., 2004; Tagawa et al., 2007), and no study has measured DHC in birds.

Here, we developed and validated a sensitive and specific enzyme-linked immunosorbent assay (ELISA) to measure DHC. After assay validation, we measured DHC in circulation and in tissues of mice, rats, and songbirds. In Study 1, corticosterone and DHC were measured in whole blood (hereafter "blood") and lymphoid organs of mice at three ages across development. In Studies 2 and 3, circulating corticosterone and DHC were measured before and after restraint stress in adult rats and songbirds, respectively, to determine whether an acute stressor increases DHC levels.

2. Materials and methods

2.1. DHC assay materials

Sheep anti-11-dehydrocorticosterone-3-carboxymethoxyoxime-thyroglobin (anti-DHC) (Antibody Registry: AB_2801550) and biotin-labeled DHC were produced by Dr. Celso E. Gomez-Sanchez (available upon request). DHC (Q3690-000) was purchased from Steraloids Inc. (Newport, USA) and dissolved in 100% ethanol (and stored at −80 °C). High-binding microtiter plates (82050–720) were purchased from VWR (Edmonton, Canada). Boric acid (B6768), bovine serum albumin (BSA) (A1933), tetramethylbenzidine liquid substrate solution (TMB) (T4444), sulfuric acid (28105), Tween 20 (P1379), ProClin 200 (48171-U), and sodium chloride (S5886) were purchased from Sigma-Aldrich (Oakville, Canada). Tris (161–0716) was purchased from Bio-Rad Laboratories (Mississauga, Canada). Avidin horseradish peroxidase (HRP-Avidin) (A-2014) was purchased from Vector Labs (Burlingame, USA). Sheep IgG (7409005) and donkey anti-sheep IgG (7453800) were purchased from Lampire Biological Laboratories (Pipersville, USA).

2.2. DHC assay development and validation

We validated the DHC immunoassay for sensitivity, accuracy, precision, and specificity using multiple approaches.

2.2.1. Standard curve development
To develop a standard curve with excellent sensitivity, various concentrations of DHC, primary and secondary antibodies, and biotin-labeled DHC were tested. The standards in the standard curve were 0, 1, 2, 4, 8, 16, 32, and 64 pg/well.

2.2.2. Accuracy and intra- and inter-assay variation
To assess accuracy and precision, we extracted (using solid-phase extraction (SPE), see Section 2.9 below) and measured two quality controls (QCs) (2 pg and 16 pg, n = 10 each) across multiple assays (n = 10).

2.2.3. Antibody cross-reactivity
Cross-reactivity of the anti-DHC antibody was tested for closely-related steroids: progesterone, 11-deoxycorticosterone, corticosterone, 11-deoxycortisol, cortisol, and cortisone (Table 1). Steroids were dissolved in ethanol, diluted to 5% ethanol using ELISA buffer (see Section 2.3), and then extracted and resuspended as described in Section 2.9.

Table 1
Cross-reactivities of the anti-11-dehydrocorticosterone (anti-DHC) antibody.

	Progesterone	11-Deoxycorticosterone	11-Deoxycortisol	Corticosterone	Cortisol	Cortisone
100 pg	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1000 pg	n.d.	5.10	n.d.	3.56	n.d.	4.89
Cross-reactivity	< 0.1%	0.51%	< 0.1%	0.36%	< 0.1%	0.49%

Data represent the mean (n = 5) relative to DHC binding (100%), n.d. = not detectable.

2.2.4. Recovery

Matrix effects were assessed by creating blood, serum, plasma, and tissue pools and then comparing unspiked and spiked samples from the same pool (Table 2). Blood, serum, plasma and tissue pools were divided into 2 groups, unspiked and spiked. Exogenous DHC and corticosterone were added to spiked pools; vehicle was added to unspiked pools. Pools were then extracted and resuspended as described in Section 2.9.

2.2.5. Serum serial dilution

Matrix effects were also assessed by serial diluting one pool of mouse serum and examining parallelism with the standard curve (n = 2/dilution). Serially diluted serum was extracted via SPE and resuspended as described in Section 2.9.

2.2.6. Comparison with liquid chromatography tandem mass spectrometry (LC-MS/MS)

We compared the immunoassay with a LC-MS/MS assay. We serially diluted one pool of mouse serum, and serum was extracted via SPE, as described in Section 2.9. DHC was measured by both ELISA and LC-MS/MS (n = 2/method/dilution). For LC-MS/MS analysis, dried SPE eluates were resuspended in 50 μl 50% MeOH and injected into a Nexera X2 UHPLC system, as previously described (Tobiansky et al., 2018). Steroids were measured by scheduled multiple reaction monitoring, and data were acquired on a Sciex 6500 Qtrap triple quadrupole tandem mass spectrometer (Tobiansky et al., 2018). Picograms of DHC measured by both ELISA and LC-MS/MS were compared. The DHC concentration at each serum dilution was also calculated (n = 8/method).

2.3. DHC assay procedure

High-binding 96-well microtiter plates were coated with 100 μl/well sheep IgG (5 μg/μl) in coating buffer (deionized H_2O, 0.1 M borate, pH 9.6). Plates were placed on an orbital shaker for 1 h at room temperature, and then left overnight at 4 °C. Plates were washed three times using a plate washer with 200 μl/well washing buffer (phosphate buffered saline (PBS), 0.05% Tween 20). Plates were then blocked with 200 μl/well blocking buffer (PBS, 3% BSA) and incubated for 1 h at room temperature. Blocking buffer was removed, and 100 μl/well donkey anti-sheep IgG in ELISA buffer (deionized H_2O, 1.2% Tris, 0.05% Tween 20, 0.05% ProClin 200, 0.29% NaCl, 0.25% BSA, pH 7.4) was added and plates were incubated for 1 h at room temperature. Plates were washed three times with washing buffer. Then 50 μl/well of anti-DHC diluted in ELISA buffer was added to all wells, except for the non-specific binding (NSB) wells, in which 50 μl/well of ELISA buffer was added. Afterwards, 50 μl/well of standards, controls, and samples were added to appropriate wells. Blank and NSB wells received 50 μl/well of ELISA buffer. Next 50 μl/well of biotin-labeled DHC in ELISA buffer was added to all wells. Plates were shaken for 1 h at room temperature. Plates were washed three times with washing buffer. Then, 100 μl/well HRP-Avidin in ELISA buffer was added to all wells and plates were shaken for 30 min at room temperature. Plates went through a final wash as above. 100 μl/well TMB solution was added to all wells and plates were incubated in the dark for 30 min at room temperature. The reaction was halted by 100 μl/well 1 N sulfuric acid. Absorbance at 450 nm was read using a plate reader (Victor[3] multilabel reader, Perkin Elmer) within 10 min of stopping the reaction.

Table 2
Steroid recovery from different sample types.

	Whole Blood	Serum/Plasma	Bone Marrow	Thymus	Spleen
Mouse PND5 Corticosterone	129%	n.a.	64%	103%	94%
Mouse PND23 and PND90 Corticosterone	81%	n.a.	81%	76%	90%
Rat Corticosterone	n.a.	50%	n.a.	n.a.	n.a.
Song sparrow Corticosterone	n.a.	63%	n.a.	n.a.	n.a.
Mouse DHC	94%	79%	124%	106%	97%
Rat DHC	n.a.	49%	n.a.	n.a.	n.a.
Song sparrow DHC	n.a.	47%	n.a.	n.a.	n.a.

Data represent the mean steroid recovery (n = 3). PND = postnatal day; DHC = 11-dehydrocorticosterone; n.a. = not applicable. For mouse DHC, values represent recovery for all ages.

2.4. Corticosterone assay

Corticosterone was quantified using a radioimmunoassay (07120103) from MP Biomedicals (Santa Ana, USA). The lowest standard was further diluted to increase assay sensitivity to 1.56 pg/tube (Schmidt and Soma, 2008). Corticosterone recovery was estimated by creating blood, serum, plasma, and tissue pools and then comparing unspiked and spiked samples from the same pool (Table 2). Due to the low concentration of corticosterone in PND5 mice, recovery was estimated separately because a greater tissue amount was used than for PND23 and PND90 mice (Table 2).

2.5. Subjects

2.5.1. Study 1

Subjects were male and female C57BL/6J mice at PND5, PND23, and PND90 (PND86-90) (n = 10 for PND5 and PND23, n = 9 for PND90), with PND0 defined as the first day pups were present in the cage. PND5 was selected because it is within the SHRP, and at PND5, corticosterone levels are higher in lymphoid organs than in blood (Taves et al., 2015). Sex of PND5 mice was determined *via* genotyping at the University of British Columbia Genotyping Facility. After weaning at PND19, mice were housed with 2–5 same-sex mice per cage. Mice were housed in a specific pathogen-free colony in the Centre for Disease Modeling at the University of British Columbia. Colony rooms were maintained between 20 and 22 °C with 40–70% relative humidity. Mice were housed in filter top cages, with beta-chip bedding, under a 14:10 light:dark cycle (lights on 0600–2000 h), with free access to water (purified by reverse osmosis and sterilized by chlorination) and food (Teklad Diet 2919 for breeders and Teklad Diet 2918 after weaning at PND19). A red translucent hut and nestlet were placed in each cage for enrichment.

2.5.2. Study 2

Subjects were adult (9-weeks-old) male and female Long-Evans rats purchased from Charles River (n = 9 and 10, respectively). Rats were housed in a conventional animal facility at the University of British Columbia. Rats were housed in same-sex pairs in polyurethane cages, with aspen-chip bedding, under a 12:12 light:dark cycle (lights on 0700–1900 h), with free access to tap water and food (Rat Diet 4012, Land O'Lakes, Inc). A small polyvinyl chloride tube was placed in each cage for enrichment.

2.5.3. Study 3

Subjects were free-living adult male song sparrows, *Melospiza melodia* (n = 7). They were captured using conspecific song playback and mist nets in October to November (nonbreeding season) in Vancouver, BC (49° 12 N, 123° 01 W).

All procedures were in compliance with the Canadian Council on Animal Care and protocols were approved by the University of British Columbia Animal Care Committee.

2.6. Study 1. Mouse tissue collection

Mouse tissues were collected between 0900 and 1100 h to reduce diurnal variation in steroids. Mice were rapidly and deeply anesthetized with 5% isoflurane (2 L/min) and euthanized by rapid decapitation (< 3 min from touching the cage). Blood, femurs, tibiae, thymus, and spleen were collected in microcentrifuge tubes and immediately frozen on dry ice. Tissues were stored at −80 °C until steroids were extracted. Due to the low concentrations of DHC, separate age-matched cohorts of mice were used to quantify corticosterone and DHC. Steroids were extracted from 0.42 to 25 mg or μl of tissue or blood, depending on the age of the subject and the steroid being measured. Bone marrow was extracted from femurs and tibiae prior to steroid extraction.

2.7. Study 2. Rat restraint stress and serum collection

Animals were handled 5 min/day for 5 days before serum collection. Prior to restraint, rats were moved to a separate holding room and allowed to habituate for 2 h. Individual cages were then transported to an adjacent room, where rats were placed into acrylic restraint tubes (PLAS Labs, Lansing, MI). Restraint tubes for males (6.35 cm inner diameter, 21.6 cm length) were larger than restraint tubes for females (5.1 cm inner diameter, 20.3 cm length). Blood samples were obtained by placing the rat's tail in warm water, making a small incision on a visible tail vein using a scalpel (No. 11), and collecting blood in microcentrifuge tubes. Baseline blood was collected after rats were put into restraint tubes, but within 3 min from approaching the cage. Rats remained in restraint rubes, were placed back into their home cages and moved to another room for the duration of the restraint period. An additional blood sample was obtained for all animals after 60 min of restraint. Serum was isolated by centrifuging blood at 10,000*g* for 1 min and frozen within 30 min of collection, then stored at −80 °C until steroids were extracted. Steroids were extracted from 0.1 to 3 μl of serum, depending on the sex of the subject and steroid being measured.

2.8. Study 3. Song sparrow restraint stress and plasma collection

Blood samples were obtained from the brachial vein by venipuncture using a sterile needle into heparinized capillary tubes as previously described (Newman et al., 2008). Initial blood samples were collected within 3 min from the time of capture. Birds were then restrained in an opaque cloth bag for 30 min and a second blood sample was collected. Capillary tubes were kept cool until centrifugation (within 5 h). After centrifugation, plasma was stored at −80 °C until steroids were extracted. Steroids were extracted from 2 to 4 μl of plasma, depending on the steroid being measured.

2.9. Steroid extraction

Steroids were extracted from all samples by SPE using C_{18} columns as previously described in detail (Tobiansky et al., 2018). Briefly, tissue samples were weighed and then homogenized in acetonitrile with 0.01% formic acid using a bead mill homogenizer at 4 m/s for 30sec. After centrifugation of homogenates, supernatants were transferred to 7 mL glass scintillation vials, dried at 40 °C in a vacuum centrifuge, and resuspended in 500 μl HPLC-grade methanol. SPE columns were primed with 3 mL HPLC-grade hexane, 3 mL HPLC-grade acetone, and then 3 mL HPLC-grade methanol prior to sample loading. Samples (500 μl) were loaded onto columns, and the eluates were collected into glass vials. Then 2 mL HPLC-grade methanol was added to the columns, and the eluates were collected into the same vials. The total eluate (2.5 mL) was dried at 40 °C in a vacuum centrifuge. Samples were resuspended by adding ethanol directly to the dried residues, vortexed briefly, and diluted with buffer to 5% ethanol. Resuspended samples were stored at −20 °C overnight. Corticosterone was quantified using a radio-immunoassay (07120103) from MP Biomedicals (Santa Ana, USA), and DHC was quantified using an enzyme-linked immunosorbent assay (ELISA) as described above.

2.10. Statistical analysis

Non-detectable samples (samples below the lowest standard on the standard curve) were set to the average of the water blanks + 2 standard deviations (Taves et al., 2015). To make comparisons between tissues, 1 mL of blood was considered to weigh 1 g (Schmidt and Soma, 2008; Taves et al., 2011). The correlation between ELISA and LC-MS/MS values of DHC was determined by Pearson correlation. When necessary, data were log transformed prior to analysis. In Study 1, male and female mice showed similar steroid levels, so sexes were pooled and subjects were analyzed by one-way analysis of variance separately at each age, followed by Dunnett's multiple comparison test. In Study 2, data were analyzed by two-way analysis of variance, and in Study 3, data were analyzed by paired t-tests. All data were analyzed using GraphPad Prism 6 and R version 3.3.3 (Another Canoe). All graphs are presented using the non-transformed data.

3. Results

3.1. DHC ELISA development and validations

To develop the DHC ELISA, we established a standard curve, and then validated our assay by examining intra- and inter-assay variation, cross-reactivity, recovery, serum serial dilution, and comparison with LC-MS/MS.

3.1.1. Standard curve development

The range of the standard curve was optimized to be 1 to 64 pg/well (Fig. 2), which demonstrates excellent sensitivity. The extracted water blanks across all assays (n = 10) were consistently below the lowest standard (1 pg/well).

3.1.2. Intra- and inter- assay variation

We used two QCs (2 pg and 16 pg) and observed average values of 2.08 pg and 15.59 pg, demonstrating excellent accuracy even for the low QC. The intra-assay variation for the two QCs was 5% and 9%, respectively. To assess inter-assay variation, we used the same two QCs and measured them across multiple assays. Inter-assay variation for the two QCs was 15% and 10%, respectively.

3.1.3. Antibody cross-reactivity

Cross-reactivity was tested for six closely-related steroids: progesterone, 11-deoxycorticosterone, corticosterone, 11-deoxycortisol, cortisol, and cortisone, at 100 pg and 1000 pg. These steroids were all non-
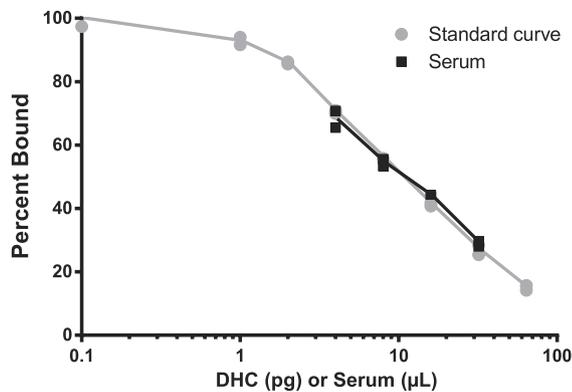
Fig. 2. Representative 11-dehydrocorticosterone (DHC) standard curve and serum serial dilution. Curves were generated by plotting the percent of biotin-labeled DHC bound versus the concentration of DHC/well on a logarithmic scale. Standard curves and serum were run in duplicate and mean percent bound was determined for each concentration. The lowest standard was 1 pg/well, demonstrating excellent assay sensitivity. Serial diluted serum was parallel to the DHC standard curve, indicating a lack of matrix effects. n = 2/dilution for both the standard curve and serum serial dilution.

detectable at 100 pg. At 1000 pg, we detected slight cross-reactivity for 11-deoxycorticosterone (0.51%), corticosterone (0.36%), and cortisone (0.49%) (Table 1).

3.1.4. Recovery

Recovery was calculated by subtracting unspiked tissue values from spiked tissue values and dividing by the amount of exogenous steroid added. Corticosterone and DHC recovery was species- and tissue-specific (Table 2), as seen previously (Taves et al., 2015).

3.1.5. Serum serial dilution

Serial diluted serum was plotted as percent bound and was parallel to the standard curve, demonstrating a lack of matrix effects (Fig. 2).

3.1.6. Comparison with liquid chromatography tandem mass spectrometry (LC-MS/MS)

Analysis of DHC in mouse serum by ELISA and LC-MS/MS showed a significant positive linear correlation between the methods ($r^2 = 0.9928$; $p = 0.0036$; Fig. 3A). The serum DHC concentration as measured by ELISA (0.65 ± 0.04 ng/mL) was slightly higher than the serum DHC concentration as measured by LC-MS/MS (0.39 ± 0.01 ng/mL) (Fig. 3B). This is consistent with previous studies that compare immunoassays and LC-MS/MS for other steroids (Fanelli et al., 2011; Moal et al., 2007). The primary antibody used in the ELISA shows a slight cross-reactivity with 11-deoxycorticosterone (0.51%), corticosterone (0.36%), and cortisone (0.49%). Only corticosterone (76.4 ± 1.5 ng/mL) and 11-deoxycorticosterone (4.5 ± 0.03 ng/mL) levels were high enough in this pool of serum, as measured by LC-MS/MS, to significantly affect DHC ELISA values. Cortisone was non-detectable. This explains the slight difference in DHC values between LC-MS/MS and ELISA.

3.2. Study 1: Systemic and local levels of corticosterone and DHC in mice

In PND5 mice, as expected, corticosterone levels were higher in bone marrow, thymus and spleen than in blood ($F_{3,27} = 90.53$; $p < 0.0001$; Fig. 4A). Also at PND5, DHC levels were higher in bone marrow than in blood, and DHC levels were lower in thymus than in blood ($F_{3,27} = 79.70$; $p < 0.0001$; Fig. 4B).

In PND23 mice, corticosterone levels were higher in blood than in bone marrow, thymus, and spleen ($F_{3,27} = 92.41$; $p < 0.0001$; Fig. 4C). Also at PND23, DHC levels were higher in bone marrow and lower in spleen than in blood ($F_{3,27} = 18.29$; $p < 0.0001$; Fig. 4D).
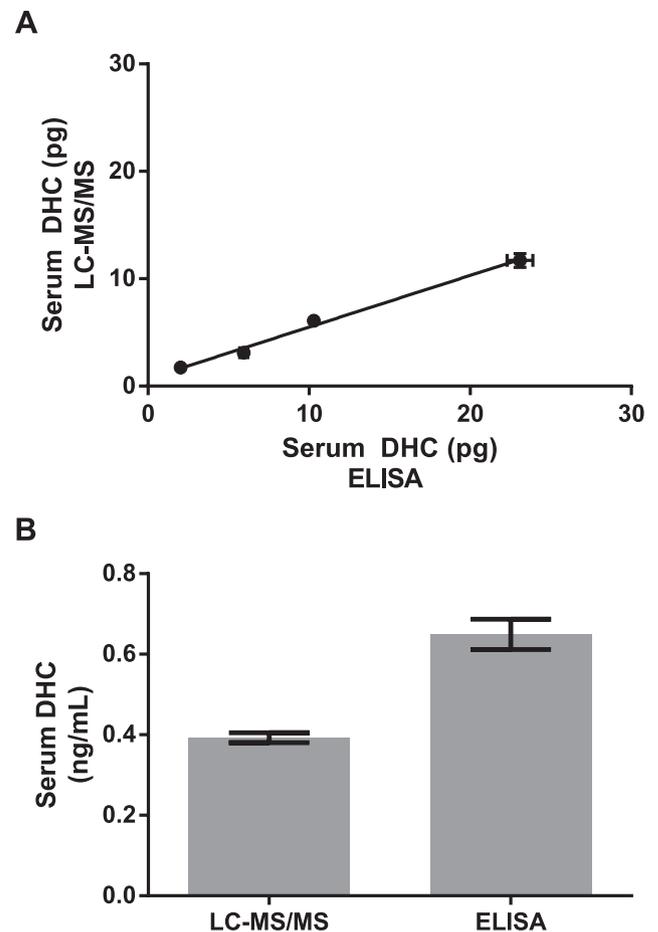

Fig. 3. Picograms of 11-dehydrocorticosterone (DHC) in serial diluted mouse serum measured by LC-MS/MS and ELISA (n = 2/method/dilution) (A) and concentration of DHC in the same mouse serum measured by LC-MS/MS and ELISA (n = 8/method) (B). DHC concentration for each method was determined by first calculating the average concentration of each volume of serum volume and then calculating the average of all serum DHC concentrations. Data are expressed as mean $\pm$ SEM.

In PND90 mice, corticosterone levels were higher in blood than in bone marrow ($F_{3,24} = 44.41$; $p < 0.0001$; Fig. 4E). Also at PND90, DHC levels were higher in bone marrow and thymus than in blood ($F_{3,24} = 10.87$; $p < 0.0001$; Fig. 4F).

Circulating corticosterone levels were lowest at PND5, which is consistent with PND5 being in the SHRP (Fig. 4A, C, E). Unlike corticosterone, DHC levels in blood and tissues generally decreased with age (Fig. 4B, D, F).

Corticosterone concentrations were generally higher than DHC concentrations. At PND5, corticosterone was approximately 1.5-fold higher than DHC in blood and 2- to 5-fold higher than DHC in lymphoid organs. At PND23, corticosterone was approximately 85-fold higher than DHC in blood and 18- to 53-fold higher than DHC in lymphoid organs. At PND90, corticosterone was approximately 110-fold higher than DHC in blood and 12- to 37-fold higher than DHC in lymphoid organs. Thus, the relationship between corticosterone and DHC is tissue- and age-specific.

3.3. Study 2: Systemic levels of corticosterone and DHC in rats

For serum corticosterone levels in rats, there were main effects of Sex ($F_{1,16} = 30.85$; $p < 0.0001$) and Restraint ($F_{1,16} = 119.8$; $p < 0.0001$) and there was a Sex × Restraint interaction ($F_{1,16} = 6.403$; $p = 0.0223$; Fig. 5A). Corticosterone levels were higher
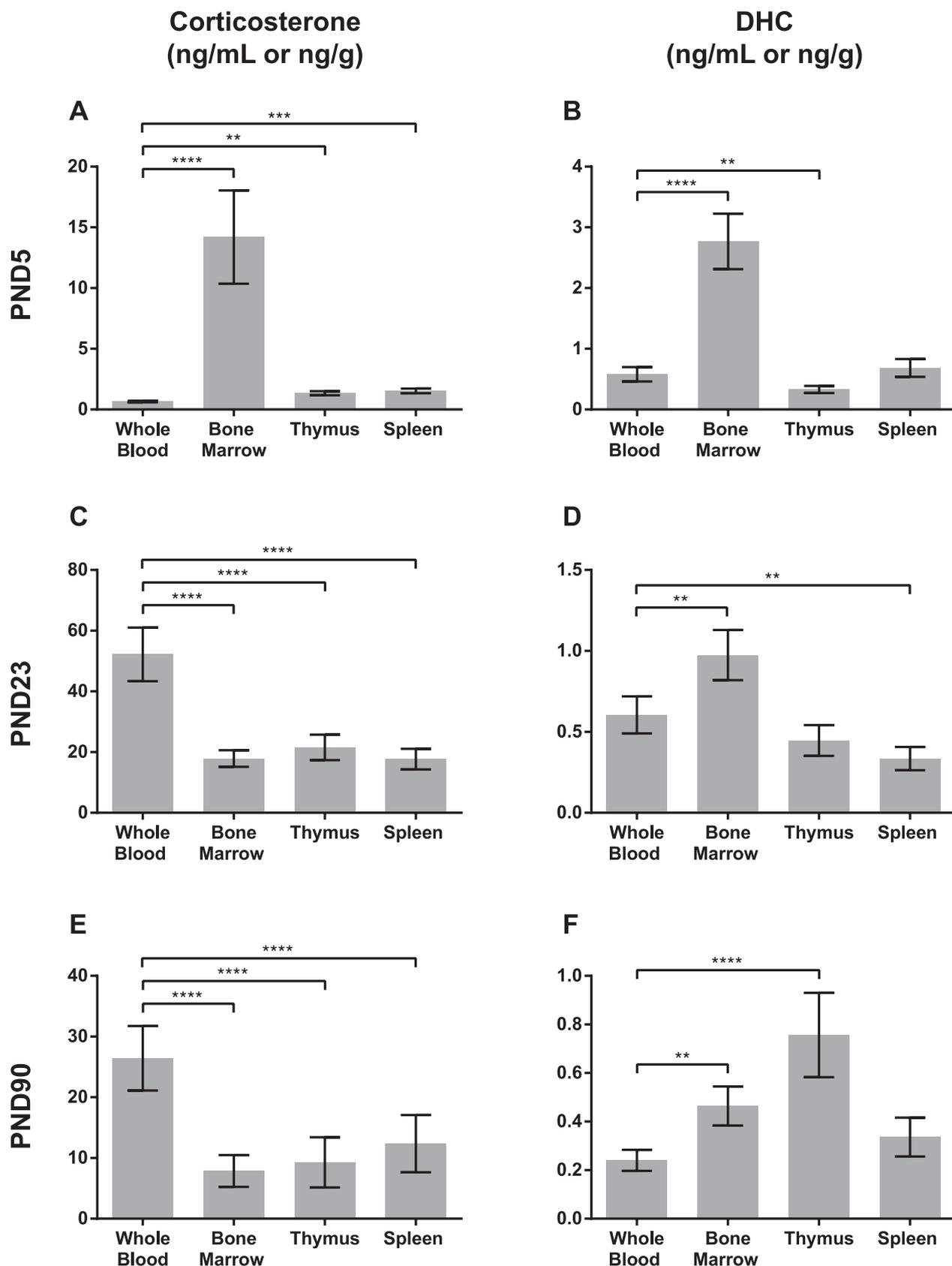
Fig. 4. Concentrations of corticosterone (A, C, E) or 11-dehydrocorticosterone (DHC) (B, D, F) in whole blood, bone marrow, thymus, and spleen of mice at PND5 (A and B), PND23 (C and D), and PND90 (E and F). Data are shown as mean ± SEM and tissue levels different from blood levels are indicated as follows * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. n = 10 for both steroids and all tissues at PND5 and PND23, n = 9 for both steroids and all tissues at PND90.
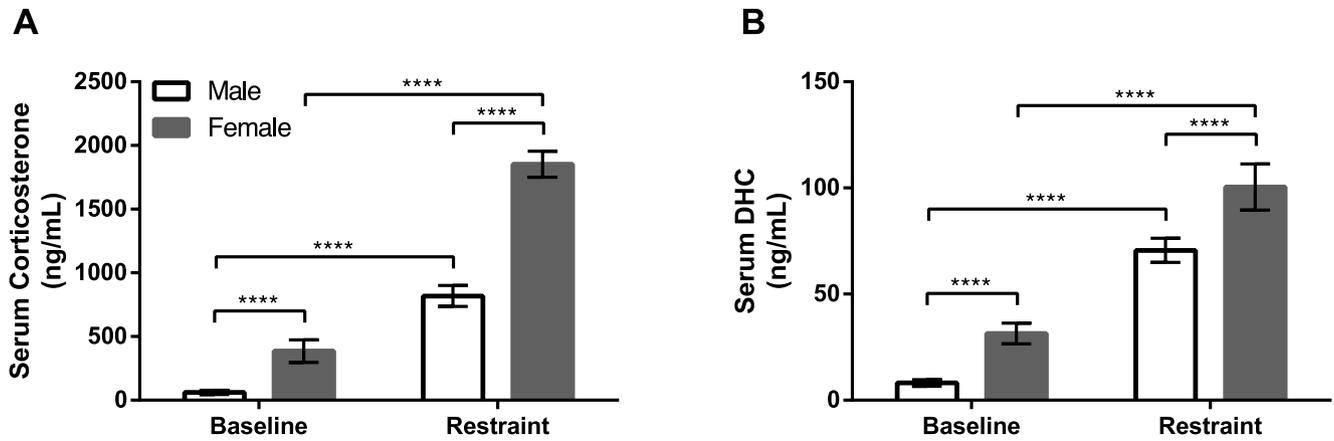
A

B

Fig. 5. The effects of sex and restraint for 60 min on concentrations of corticosterone (A) or 11-dehydrocorticosterone (DHC) (B) in serum of adult rats. Data are shown as mean ± SEM. **** p ≤ 0.0001. n = 9 for males and females for baseline and restraint.

in females than in males and increased more in females than in males after restraint (Fig. 5A).

For serum DHC levels in rats, there were main effects of Sex ($F_{1,16} = 42.04$; p < 0.0001) and Restraint ($F_{1,16} = 148.4$; p < 0.0001) and there was a Sex × Restraint interaction ($F_{1,16} = 14.61$; p = 0.0015; Fig. 5B). DHC levels were higher in females than in males and increased more in females than in males after restraint (Fig. 5B).

At baseline, corticosterone levels were approximately 7-fold higher than DHC levels in male rats and 13-fold higher than DHC levels in female rats. After restraint, corticosterone levels were approximately 12-fold higher than DHC levels in male rats and 20-fold higher than DHC levels in female rats.

3.4. Study 3: Systemic levels of corticosterone and DHC in song sparrows

Plasma corticosterone levels in male song sparrows increased in response to restraint stress ($t_6 = 11.08$; p < 0.0001; Fig. 6A). Plasma DHC levels also increased in response to restraint stress ($t_6 = 11.07$; p < 0.0001; Fig. 6B). Overall, corticosterone levels were approximately 5-fold higher than DHC levels at baseline and approximately 9-fold higher than DHC levels after restraint stress.

4. Discussion

Despite the importance of DHC for local regeneration of corticosterone and thus for stress physiology, DHC levels in circulation have rarely been reported in rodents and have never been reported in birds.

No immunoassays are currently available to measure DHC. Here, we developed and validated a sensitive and specific ELISA to measure DHC. In Study 1, we quantified corticosterone and DHC in mouse blood, bone marrow, thymus, and spleen across three ages. Interestingly, each organ showed a specific pattern of local steroid regulation. In Study 2, we measured circulating corticosterone and DHC in rats at baseline and after restraint stress. Corticosterone levels were higher than DHC levels in both males and females, and both corticosterone and DHC levels increased more in females than in males after restraint. In Study 3, we measured circulating corticosterone and DHC in male songbirds at baseline and after restraint stress. Both corticosterone and DHC levels increased after restraint. To our knowledge, this is the first study to report 1) DHC levels in neonatal mice, 2) DHC levels in lymphoid organs, 3) local regulation of DHC levels, 4) a sex-specific increase in DHC levels in response to an acute stressor in rats, and 5) DHC levels in any bird.

4.1. DHC ELISA development and validations

We validated our DHC ELISA on four key elements: accuracy, precision, sensitivity, and specificity. To assess accuracy, we measured two standards (2 pg and 16 pg) and observed values within 4% of expected values. To assess precision, we measured the same two standards repeatedly. The intra-assay variation was 5% and 9% for the 2 pg and 16 pg standards, respectively, and the inter-assay variation was 15% and 10%, respectively. The sensitivity of our ELISA is indicated by the low detection limit of 1 pg/well (Fig. 2). To assess specificity, we used six closely-related steroids. We detected low cross-reactivity to 11-
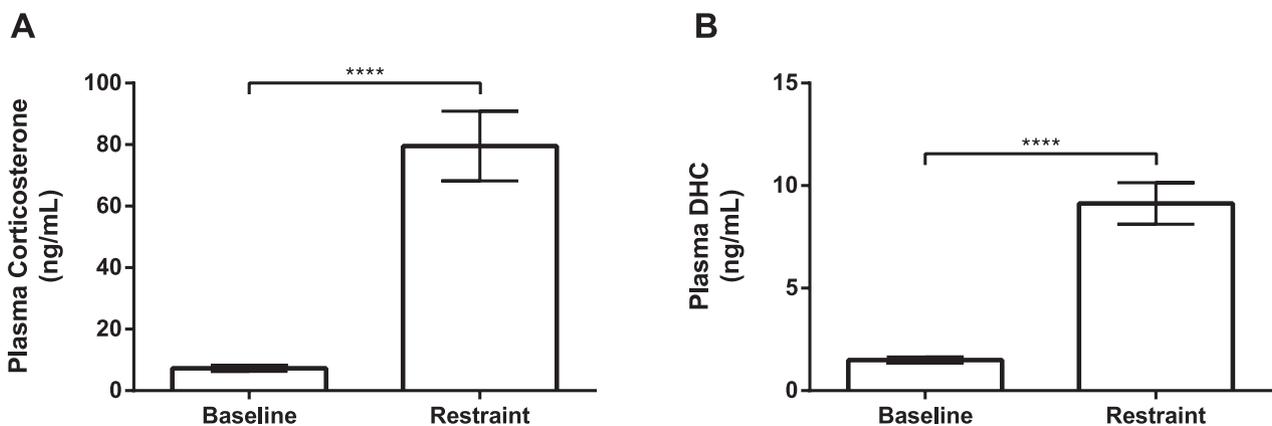
A

B

Fig. 6. The effect of restraint for 30 min on concentrations of corticosterone (A) or 11-dehydrocorticosterone (DHC) (B) in plasma of free-living non-breeding male song sparrows. Data are shown as mean ± SEM. **** p ≤ 0.0001. n = 7 for both baseline and restraint.

deoxycorticosterone, corticosterone, and cortisone at 0.51%, 0.36%, and 0.49%, respectively. Progesterone, 11-deoxycortisol, and cortisol were non-detectable, even at the 1000 pg level (Table 1). Moreover, a serial dilution of serum showed parallelism with the standard curve, demonstrating that the extraction protocol effectively removed possible matrix effects.

To further assess the accuracy, precision, and specificity of our ELISA, we directly compared it against a LC-MS/MS assay. One pool of mouse serum was serial diluted and measured by both LC-MS/MS and ELISA. The amount of DHC in serum samples as measured by both methods showed a significant positive linear correlation ($r^2 = 0.9928$; p = 0.0036). As expected, the DHC concentration in mouse serum was slightly higher when measured by ELISA, relative to LC-MS/MS, as seen in comparisons of other steroid assays (Fanelli et al., 2011; Moal et al., 2007). Higher DHC concentrations obtained by ELISA than LC-MS/MS may be explained by the slight cross-reactivity of the primary antibody with corticosterone (0.36%), which is present at high levels, and with 11-deoxycorticosterone (0.51%), which is present at low levels. Nonetheless, all of these validations taken together indicate that the DHC ELISA is accurate, precise, sensitive, and specific, and it is a useful tool for measuring DHC levels. This immunoassay is especially useful because there are currently no other DHC immunoassays available.

4.2. Study 1: Local regulation of corticosterone and DHC in mouse immune organs

Local production of GCs may be of particular importance during the SHRP, when circulating GCs are low and tissues are rapidly developing. A growing body of evidence suggests many tissues locally regulate GCs (Taves et al., 2017). GCs play an important role in lymphocyte development, most commonly studied in T-cells. Studies over the last three decades have shown that GCs modulate T-cell affinity for antigens, responsiveness, and proliferation (Mittelstadt et al., 2012; Pazirandeh et al., 1999; Vacchio and Ashwell, 1997; Vacchio et al., 1999). Furthermore, recent studies with mice lacking CYP11B1 in either thymocytes or thymic epithelial cells reveal that thymic epithelial cells, rather than thymocytes, are responsible for most of the locally-produced, bioactive corticosterone. However, this study examined only synthesis of corticosterone from 11-deoxycorticosterone, not regeneration of corticosterone from DHC (Mittelstadt et al., 2018). GCs are less studied in B-cell development and function, but GCs modulate B-cell development and cause apoptosis of B-cells at high concentrations (Cortez et al., 1996; Garvy et al., 1993; Gruver-Yates et al., 2014; Trottier et al., 2008). Finally, *in vitro* experiments administering a high concentration (1 μM) of either 11-deoxycortiocsterone or DHC suggest that corticosterone is preferentially produced from DHC rather than 11-deoxycorticosterone (Taves et al., 2016), but DHC levels in blood and lymphoid organs were previously unknown.

The current data demonstrate that DHC is present in the blood and lymphoid organs of mice. In bone marrow, both corticosterone and DHC were locally elevated at PND5, but only DHC was locally elevated at PND23 and neither were locally elevated at PND90. Corticosterone levels were lower in bone marrow than blood at PND23 and PND90. In thymus, corticosterone was locally elevated only at PND5 and was lower than in blood at PND23 and PND90. In contrast, DHC levels were lower in thymus than in blood at PND5. DHC was also elevated in thymus at PND90. In spleen, corticosterone was locally elevated at only PND5 and lower than blood at all other ages. DHC was not locally elevated in spleen at any age and was lower in spleen than blood at PND90 (Fig. 4). The local elevation of corticosterone in all lymphoid organs at PND5 is consistent with previous findings (Taves et al., 2015). Additionally, our DHC levels at PND90 are consistent with recently reported DHC levels in circulation of adult mice measured by LC-MS/MS and our own LC-MS/MS data (Verma et al., 2018).

The local elevation of DHC in bone marrow and the local reduction of DHC in thymus at PND5 could be explained in three different ways.

1) Tissues might increase local DHC levels by sequestering DHC from blood, in order to be locally regenerated to corticosterone. In this scenario, tissues create a "reservoir" of DHC that can be locally regenerated to corticosterone *via* 11β-HSD1. However, there are no known mechanisms to sequester DHC. 2) Local DHC levels might be a result of local corticosterone synthesis and subsequent metabolism to DHC *via* 11β-HSD2. In this scenario, high local levels of DHC simply reflect high local inactivation of corticosterone. 3) Local DHC levels might be a result of both uptake of DHC from blood and local corticosterone synthesis with subsequent metabolism to DHC *via* 11β-HSD2. This scenario allows for the greatest control over local corticosterone levels by providing a mechanism for both local synthesis and regeneration of corticosterone.

In vitro experiments suggest that tissues increasingly regenerate corticosterone from DHC with age (Taves et al., 2016), but until now, DHC levels across age in mice were unknown. As expected, baseline corticosterone levels in the blood were very low at PND5 and increased approximately 75-fold at PND23. Surprisingly, DHC levels did not increase in the blood or in tissues after PND5 and were lowest in blood at PND90. These data indicate that DHC is present in circulation and tissues, but might not be at high enough levels outside of the SHRP to substantially contribute to local corticosterone production. It is possible that tissues rely more heavily on regeneration from DHC to contribute to local elevation of corticosterone during the SHRP and more on synthesis afterwards. These data are also in agreement with the idea that the SHRP reduces the harmful effects of circulating GCs on developing tissues, and thus allows for tissue-specific upregulation of corticosterone based on need (Sapolsky and Meaney, 1986; Taves et al., 2015).

Corticosterone locally synthesized from 11-deoxycorticosterone by thymic epithelial cells modulates T-cell development (Mittelstadt et al., 2018), but no study has examined whether corticosterone locally regenerated from DHC is also biologically active. Studies using genetic models, such as 11β-HSD1$^{-/-}$ mice, will be helpful in determining if intracellular regeneration is important for lymphocyte selection (Cobice et al., 2013). Additionally, studies using a combination of genetically altered mice and labeled GC precursors or DHC will be beneficial in determining if locally-derived GCs are a result of synthesis, regeneration, or both.

4.3. Study 2: Corticosterone and DHC levels after restraint stress in rats

Despite the widespread use of rats in studies of stress physiology, few studies have measured DHC in rats and only one has measured DHC after a stressor (Hundertmark et al., 2002; Obut et al., 2004; Tagawa et al., 2007). Here, we present data on circulating corticosterone and DHC in rats at baseline and after 60 min of restraint stress (Fig. 5). Our data demonstrate that both corticosterone and DHC levels increase in response to restraint in a sex-specific fashion. Similar to corticosterone, DHC increased in response to stress and increased to a greater extent in females than in males. Additionally, our reported DHC levels at baseline are in line with previously reported values in rats of the same age (Tagawa et al., 2007).

These data indicate that DHC increases in serum in response to restraint stress. Systemic DHC levels may increase in response to stress for two reasons: 1) increased conversion of corticosterone to the inactive DHC, thus protecting tissues from harmful effects of corticosterone or 2) provide a reservoir for specific tissues to intracellularly regenerate DHC to corticosterone. Evidence for the latter includes high levels of 11β-HSD1 in the rat brain (Diaz et al., 1998; Holmes and Seckl, 2006; Wyrwoll et al., 2011) and prolonged stress-induced increases in blood corticosterone in 11β-HSD1-deficient mice (Harris et al., 2001). Together, the data suggest that 11β-HSD1 plays a pivotal role in hypothalamic-pituitary-adrenal axis regulation by utilizing systemic DHC to intracellularly regenerate corticosterone, to provide increased negative feedback on the hypothalamic-pituitary-adrenal axis and prevent a heightened and prolonged stress response.

4.4. Study 3: Corticosterone and DHC levels after restraint stress in song sparrows

Song sparrows show increases in corticosterone levels in response to restraint stress (Newman et al., 2008; Newman and Soma, 2009). While the corticosterone response to restraint is well characterized in song sparrows and many other birds, DHC has yet to be measured in any bird species. Here, we present data on both corticosterone and DHC in male song sparrows at baseline and 30 min after restraint. Corticosterone levels were higher than DHC levels at baseline and restraint, and both corticosterone and DHC increased in response to restraint stress (Fig. 6).

Little is known about 11β-HSD1 in song sparrow tissues, but a recent study in zebra finches was unable to detect 11β-HSD1 mRNA in adult brain, while mRNAs for 11β-HSD2, mineralocorticoid receptor, and glucocorticoid receptors were all detected in brain regions (Rensel et al., 2018). Another study in birds found no evidence of local GC production in the brain of European starlings but did report evidence of local GC production in lymphoid organs of zebra finches (Schmidt et al., 2009). These data may indicate that inactivation of corticosterone to DHC by 11β-HSD2, to keep local corticosterone levels low, may be important in the brain of birds, but not in immune tissues. Additional studies in birds of DHC across season, age, and species will be useful.

4.5. Possible implications for human health

Cortisol, and its metabolite cortisone, are the predominant circulating GCs in humans (Levine et al., 2007). A wide array of human tissues express 11β-HSD1—including liver, adipose tissue, and brain—and intracellular regeneration of cortisol from cortisone via 11β-HSD1 might be important in type 2 diabetes mellitus and neurodegenerative diseases (Rask et al., 2001; Walker, 2007). Interestingly, corticosterone and DHC are also present at low levels in human blood, and human neuronal cells can convert DHC to corticosterone *in vitro* (Branchaud et al., 1969; Morris and Williams, 1953; Rajan et al., 1996; Weichselbaum and Margraf, 1960). These data raise the possibility that DHC and corticosterone are relevant for human health.

4.6. Conclusions

We developed and validated a sensitive and specific ELISA for measurement of DHC and compared our immunoassay with a LC-MS/MS assay. After validation, we used this DHC immunoassay to measure circulating and local DHC levels in mice and circulating DHC levels in rats and song sparrows. In mice, DHC levels in the blood and lymphoid organs vary by age and tissue. For example, at PND5, DHC was locally elevated in bone marrow, relative to the blood. DHC levels decreased with age, in contrast to corticosterone. These data suggest that age-related changes in DHC levels impact local regeneration of corticosterone from DHC in lymphoid organs. In rats and song sparrows, circulating DHC and corticosterone levels increase in response to restraint stress. This novel assay and these fundamental data on DHC levels provide a basis for future studies of DHC, an important but relatively neglected component of GC physiology. We welcome collaborations, and those interested can contact the corresponding author regarding DHC immunoassay reagents or LC-MS/MS.

Declaration of Competing Interest

None.

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