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Sources of variation in plasma corticosterone and dehydroepiandrosterone in the male northern cardinal (*Cardinalis cardinalis*): I. Seasonal patterns and effects of stress and adrenocorticotropic hormone

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ABSTRACT

The secretion of steroids from the adrenal gland is a classic endocrine response to perturbations that can affect homeostasis. During an acute stress response, glucocorticoids (GC), such as corticosterone (CORT), prepare the metabolic physiology and cognitive abilities of an animal in a manner that promotes survival during changing conditions. Although GC functions during stress are well established, much less is understood concerning how adrenal androgens, namely dehydroepiandrosterone (DHEA) are influenced by stress. I conducted three field studies (one experimental and two descriptive) aimed at identifying how both CORT and DHEA secretion in free-living male northern cardinals (*Cardinalis cardinalis*), vary during acute stress; across different circulations (brachial vs. jugular); in response to ACTH challenge; and during the annual cycle. As predicted, restraint stress increased plasma CORT, but unexpectedly DHEA levels decreased, but the latter effect was only seen for blood sampled from the jugular vein, and not the brachial. The difference in DHEA between circulations may result from increased neural uptake of DHEA during stress. Injection with exogenous adrenocorticotropic hormone (ACTH) increased CORT concentrations, but failed to alter DHEA levels, thus suggesting ACTH is not a direct regulator of DHEA. Monthly field sampling revealed distinct seasonal patterns to both initial and restraint stress CORT and DHEA levels with distinct differences in the steroid milieu between breeding and non-breeding seasons. These data suggest that the CORT response to stress remains relatively consistent, but DHEA secretion is largely independent of the response by CORT. Although CORT functions have been well-studied in wild animals, little research exists for the role of DHEA and their variable relationship sets the stage for future experimental research addressing steroid stress responses.

1. Introduction

The hypothalamic–pituitary–adrenal (HPA) axis is an endocrine cascade in vertebrates that modulates key physiological and behavioral processes that promote survival during situations of stress (Wingfield and Sapolsky, 2003). The HPA axis initiates with the hypothalamic release of corticotropin-releasing hormone (CRH) into a specialized portal system, which binds to pituitary corticotropic cells resulting in secretion of adrenocorticotropic hormone (ACTH) into systemic circulation (Aguilera and Liu, 2012; Fokidis and Deviche, 2011). Acting on the adrenal cortex, ACTH promotes the synthesis and subsequent secretion of glucocorticoids (GCs) into the blood (Sapolsky et al., 2000). Specifically, ACTH interacts with a serpentine membrane bound G-protein coupled receptor on the adrenocortical cells found within the zona fasciculata (middle zone) where it rapidly encourages lipoprotein uptake and delivery to increase cholesterol bioavailability for steroidogenesis (Boyd and Trzeciak, 1973). Concurrently ACTH also initiates the transcription of GC synthesizing enzymes and mitochondrial oxidative phosphorylation systems that provide energy for enhanced cellular metabolism associated with increased steroid synthesis (Boyd and Trzeciak, 1973; Miller, 2013). The dominant bioactive GCs in vertebrate circulation are cortisol (the predominant GC in many mammals, including humans, and fish) and corticosterone (CORT: the dominant GC in birds and reptiles) (Sapolsky et al., 2000; Quinn et al., 2013). Both of these steroids induce a myriad of physiological effects that promote survival in the short-term, such as energy mobilization and vigilance behaviors,

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but can be detrimental with prolonged or chronic overstimulation, known as "allostatic load" (McEwen and Wingfield, 2003). In these situations, GCs can suppress immunity, interfere with reproductive physiology, and can influence the brain by impairing neuronal health and development (McEwen and Wingfield, 2003).

Circulating GC concentration is the typical endpoint measured to assess the acute stress response, usually by comparing a "baseline" level with that after a period of handling stress (Romero and Reed, 2008). This method is widely used and has garnered data concerning patterns of GC secretion and their various roles in wild animals (Deviche et al., 2010; Fokidis et al., 2011; French et al., 2008; Holding et al., 2014; Pereyra and Wingfield, 2003; Pravosudov et al., 2002; Refsnider et al., 2015). Many avian studies of seasonal patterns report higher CORT levels at the onset of breeding where it may serve to mobilize energy stores, and then levels declining post breeding when it may interfere with anabolic processes, such as feather molt (Foltz et al., 2015; Holding et al., 2014; Cornelius et al., 2011; DesRochers et al., 2009; Romero et al., 2009, 2005; Pereyra and Wingfield, 2003; Raja-aho et al., 2013). In some species, plasma CORT levels are reduced during breeding when stress might interfere with limited opportunities for reproduction (e.g., a short breeding season), and thus, yearly CORT patterns often align closely with specific life-history patterns and environmental conditions (Holberton and Wingfield, 2003; Romero et al., 1997).

During stress, the inner most adrenal cortical layer, the zona reticularis, also secretes the androgen precursor, dehydroepiandrosterone (DHEA) (Boonstra et al., 2008; Strous et al., 2006; Soma et al., 2015). In human circulation, DHEA is most prevalent in an inactive sulfated form (DHEAS), although the degree to which this form is present in other species is less understood (Soma et al., 2015). The secretion of DHEA in circulation appears directly regulated by ACTH based on studies in humans, hamsters, and squirrels (Kalimi et al., 1994; Boonstra et al., 2008; Soma et al., 2015), however the essentially undetectable DHEA levels in mice and rats do not change in response to ACTH (van Weerden et al., 1992; Bélanger et al., 1990). Although no studies have tested DHEA responses to exogenous ACTH challenge in a non-mammalian model, in the song sparrow (*Melospiza melodia*) exposure to acute stress did not affect DHEA concentrations (Newman et al., 2008; Soma and Wingfield, 2001). Although, no specific receptor has been clearly identified (Labrie et al., 2001), significant evidence from ligand-binding assays suggests DHEA may interact with a specific membrane-receptor in both bovine aortic and human umbilical vein endothelial cells (Webb et al., 2006; Widstrom and Dillon, 2004). In humans, DHEA in circulation is found in its esterified sulfate form (DHEAS) which is both highly stable and largely thought to be incapable of exerting androgenic effects (Labrie et al., 2001). Data from *in vitro* studies in rats, suggests DHEA can protect neurons and neurogenesis from excessive GC exposure during stress (Kimonides et al., 1999; McNelis et al., 2013; Kalimi et al., 1994). Thus, DHEA may act as an anti-GC hormone within the nervous system however; the mechanisms of action are not clearly understood.

One possibility is that DHEA may be exerting its effects through enzymatic conversion to a bioactive steroid, such as testosterone (T) or estradiol (E_2), and both of these steroids are known to have neuroprotective qualities with the necessary enzymes being present within the brain (Duncan and Saldanha, 2013; Saldanha et al., 2009). This conversion of DHEA to T and E_2 within the brain can also promote territorial aggression in some birds and Siberian hamsters (*Phodopus sungorus*), especially when circulating sex steroids are low (e.g., nonbreeding season) (Schmidt et al., 2008; Soma et al., 2015). The conversion of circulating steroids by enzymes within the brain have prompted comparisons of CORT and DHEA levels between brachial and jugular circulations, particularly in response to handling stress (Newman and Soma, 2011; Newman et al., 2008). Brachial blood sampling may represent systemic hormone concentrations directly secreted by the adrenal gland, whereas jugular sampling may represent hormone levels after depletion and/or enrichment by the brain (Newman and Soma, 2011). Although several studies have compared both brachial and jugular DHEA levels in vertebrates between the breeding and nonbreeding condition (Boonstra et al., 2008; Hau et al., 2004; Soma and Wingfield, 2001), complete monthly-sampled profiles over the course of the year is limited in the literature (but see Hamlin et al., 2014). Furthermore, the secretory patterns of CORT and DHEA are not always congruent and their relationship can vary with seasonality (Newman and Soma, 2009).

Here, I investigated plasma CORT and DHEA concentrations in free-living adult male northern cardinals (*Cardinalis cardinalis*) during exposure to capture and handling stress across the course of the year. I also compared concentrations between the jugular and systemic circulations and in response to injection with exogenous ACTH. Northern cardinals are common, sedentary and sexually dimorphic songbirds that exhibit a substantial amount of behavioral similarity between the sexes (Nealen and Breitwisch, 1997; Jawor and MacDougall-Shackleton, 2008; DeVries et al., 2014). The endocrine research on this species has largely focused on the regulation of gonadal T in both sexes (DeVries et al., 2011; DeVries and Jawor, 2013; DeVries et al., 2015; Jawor, 2007; Jawor et al., 2014). Artificial activation of the reproductive axis may increase CORT levels, however CORT does not appear to affect T levels in cardinals (DeVries et al., 2011). Levels of CORT have been examined in other studies of this species (Barron et al., 2012; Owen et al., 2012; DeVries and Jawor, 2013) and this research provides additional insight into the regulation of and relative associations between these adrenal steroids.

2. Materials and methods

2.1. Ethics statement

All studies were conducted under United States Geological Survey Bird Banding Laboratory permit # 23847, Florida Fish, and Wildlife Commission scientific collecting permit #LSSC-13-00057, and with relevant access permits from the appropriate agencies. The Institutional Animal Care and Use Committee at Rollins College approved all experimental procedures (protocols # 2513B and 2514).

2.2. Study 1: variation between jugular and systemic circulation

Blood sampled from the brachial vein represents systemic levels of steroids that are secreted from classic steroidogenic organs (e.g., adrenals, gonads), whereas jugular blood may be enriched by or depleted of steroids by the brain. To test differences in CORT and DHEA between these veins, adult male cardinals were sampled during their nonbreeding season from Jan 26 to Feb 8, 2014 at the Split Oak Wildlife Mitigation Park, in Central Florida using mist nets coupled to conspecific song playback. I recorded the time it took for the bird to respond to the playback (i.e., either show up or sing in response), and the time it took for them to strike the net and be captured.

Within 2 min of capture, an *initial* blood sample (300 μl) was collected from either the right brachial vein ($N = 8$) using a sterile 26 gauge needle and heparinized microhematocrit tubes or the right jugular vein ($N = 10$) using a 28 gauge heparinized needle and a 3 cc insulin syringe. Concurrent research on this species has suggested that limited exposure (<30 min) to conspecific playback recordings does not alter CORT or DHEA levels (Wright and

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Fokidis, 2016), and time within 2 min does not correlate with concentrations of either hormone ($r \leqslant 0.038$, $P = 0.631$). After initial sampling, birds were kept in an opaque cloth bag for 30 min, to induce an acute stress response after which another *restraint* blood sample was collected from the same vein as before. For a subset of birds ($N = 5$), repeated blood samples from the jugular vein were taken at 2, 10, 20, 30 and 45 min post-capture to examine a time frame for circulating changes in CORT and DHEA concentrations. Blood was kept on ice until returned to the laboratory and centrifuged to separate the plasma, which was then stored at −80 °C, until assayed for CORT and DHEA. As with all birds sampled in this research, I collected additional measurements including: tarsus and beak (nares to tip) lengths (to nearest 0.1 mm); the length of the wing chord (to nearest 1 mm); width of the cloacal protuberance (CP: androgen-dependent secondary sex characteristic); pectoralis muscle and furcular fat scores (5 point visual scale); and body mass (to the nearest 0.1 g). Additionally, the molt status, the presence of wing or body feather replacement was recorded, and lastly, subjects obtained a uniquely numbered US Geological Survey aluminum leg band and released at the site of capture.

2.3. Study 2: variation in response to exogenous ACTH

Pituitary-derived ACTH primarily regulates synthesis and secretion of adrenocortical steroids (Aguilera and Liu, 2012; Fokidis and Deviche, 2011). Challenging individuals with exogenous ACTH provides the opportunity to observe the maximal capacity of the adrenal gland to secrete steroids, which often exceeds the endogenous levels secreted under normal stress conditions (Astheimer et al., 1994; Boonstra et al., 2008). Previous research has demonstrated that ACTH directly regulates CORT in birds; however, to our knowledge no avian study has tested the effects of ACTH on DHEA.

To this end, adult male Cardinals were captured at the Tosohatchee Wildlife Management Area, near Christmas, FL from Sep 13 to 28, 2014 and a *pre-treatment* blood sample was collected from the jugular vein. After the pre-treatment sample was collected birds were given an intraperitoneal injection (i.p.) of 100 IU/kg dose of porcine ACTH (Sigma-Aldrich Co. LLC. Cat. A6303, St. Louis, MO, USA, $N = 8$) delivered in 100 µl of avian saline (0.75% sodium chloride) vehicle to stimulate adrenocortical steroid secretion. This ACTH has been previously used to elicit significant adrenal CORT responses in songbirds (Romero et al., 1998; Romero, 2006; Fokidis and Deviche, 2011). As a negative control ($N = 7$), another group of male birds were given an i.p. injection of 100 µl of avian saline vehicle. After 30 min post-injection with ACTH or vehicle, a second *post-injection* blood sample was collected from the jugular vein. Birds were then measured and banded, and blood was stored as in study 1, with birds released on site.

2.4. Study 3: variation across an annual cycle

Many steroids exhibit distinct annual patterns in circulation that may coincide with their functions during specific life-history stages. Much of this research has focused on sex steroids (T, E_2), and GCs, yet variation in DHEA remains understudied. To investigate annual patterns in adrenal steroid secretion, Cardinals (monthly $N = 2$–12, total $N = 79$) were sampled every month from Jan 26 to Dec 7, 2014 at the Split Oak Wildlife Mitigation Park, in Central Florida. Birds were captured and the subjected to the same restraint protocol, jugular blood sampling, and measurements as described in study 1.

2.5. CORT enzyme-linked immunoassay

Plasma total CORT concentrations were measured using the DetectX CORT enzyme-linked immunoassay kit (ELISA: Arbor Assays. Inc., Ann Arbor, MI, USA). This ELISA has been previously used in birds, including cardinals (DeVries et al., 2015; Pryor and Casto, 2015). Here I present validations by demonstrating parallelism between serially diluted plasma and the CORT standard curve (Fig. 1A) and by demonstrating high recoveries from plasma of a dose of exogenous CORT in sample plasma (88–95%). Furthermore, plasma stripped of steroid with dextran-coated charcoal (3–9% of CORT recovered). All plasma samples were assayed in duplicate, with samples from the same individuals (i.e., initial and restraint, or pre- and post-treatment) run on the same 96-well plate, but with samples from the same experiment often spanning multiple plates. Plasma CORT concentrations were then calculated by interpolation from the standard curves present on each plate using GraphPad Prism version 4 (La Jolla, CA, USA). The sensitivity of the CORT assay ranged from 4.6 to 6.7 pg/mL and the mean intra-assay and inter-assay coefficients of variation were 9.5% and 14.3%, respectively ($N = 3$ plates, 112 samples total).

2.6. DHEA enzyme-linked immunoassay

Plasma concentrations of DHEA are considerably lower than that of CORT and thus solid phase extraction (SPE) was used to purify the steroid content in samples prior to the DHEA assay. Plasma samples were first pre-diluted using 10 ml of deionized water. Then C18 carbon-bonded silica cartridges (6 cc – 500 mg: Agilent Technologies Inc., Santa Clara, CA, USA) were primed with 3 ml of 100% ethanol and subsequently equilibrated with 10 ml of

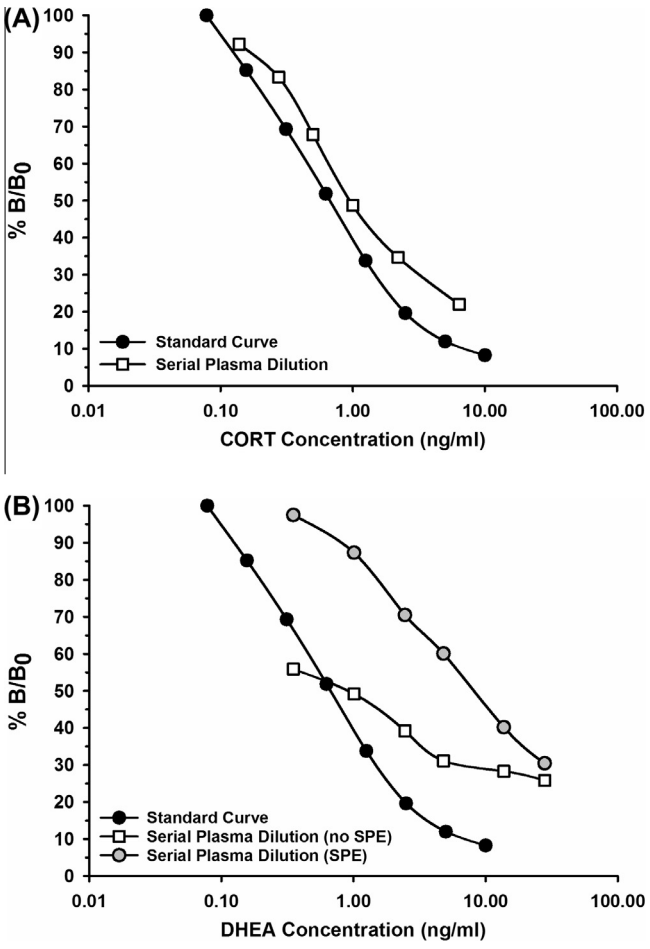

Fig. 1. Comparison of assay standard curves with serially diluted plasma samples from northern cardinals for (A) corticosterone (CORT) and (B) dehydroepiandrosterone (DHEA). DHEA in diluted plasma was compared with and without solid phase extraction (SPE) prior to assay.

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deionized water. Samples were then loaded into the columns, after which an interference elution was done using 10 ml of 40% methanol (MeOH), to remove lipids and steroids that were gluconurated or sulfated and could potentially interfere with the subsequent DHEA assay. A final elution using 5 ml of 90% MeOH was performed to extract the purified samples, which were then dried down using a speed vacuum concentrator (Savant SpeedVac #SPD131DDA, Thermo Fisher Scientific Inc., Waltham, MA, USA) set at 60 °C for 4 h. Dried extracts were stored at −20 °C until assayed.

Plasma DHEA concentrations were measured using a commercial DHEA ELISA kit (Diametra DKO124, Immunodiagnostic Systems Ltd, Milano, Italy). This kit was validated for use in cardinals again by demonstrating a parallelism between serially diluted plasma, but only after SPE, and the DHEA standard curve (Fig. 1B) and by demonstrating high recoveries from plasma of a dose of exogenous DHEA (86–89%), and recovery was eliminated by steroid stripping with dextran-coated charcoal (1–3%). This kit exhibits a very low cross-reactivity with other steroids, including DHEAS (about 0.004%). Dried extracts were reconstituted in 3 µl of absolute ethanol and 60 µl of buffer from the assay kit and the assay was run according to manufacturer's instructions. Again all plasma samples were assayed in duplicate, with initial and restraint samples from the same individuals run on the same plate. Plasma DHEA concentrations were again calculated by interpolation from the standard curves using GraphPad Prism version 4 (GraphPad Software Inc., La Jolla, CA, USA). The sensitivity of the DHEA assay ranged from 0.5 to 0.9 ng/mL and the mean intra-assay and inter-assay coefficients of variation were 7.3% and 11.9%, respectively ($N = 3$ plates, 112 samples total).

2.7. Statistical analysis

All data were tested for adherence to normality and *homoscedasticity* (i.e., equal variance) assumptions, and where necessary were log-transformed prior to further analysis. Morphometric data from this study was combined with that of another study (Wright and Fokidis, 2016) to generate a body condition index using the residuals of an ordinary least squares regression of body mass on tarsus or beak lengths. The regression of mass on tarsus length had a higher goodness of fit ($R^2 = 0.83$, $P = 0.001$) than that of mass on beak length ($R^2 = 0.67$, $P = 0.013$), and thus was used as the body condition measure in subsequent analyses. For each study, repeated measures analysis of variance (rmANOVA) was used to compare changes in CORT and DHEA from initial levels to those after restraint (the within-subjects factor) and treatments (i.e., vein type, ACTH-treatment, or month) as the between-subjects factors. The rmANOVA is robust to issues of small sizes in large part due to the within-subjects design when in conjunction with the type III sum of squares. However as these data violate assumptions of sphericity; Greenhouse-Geisser corrections that adjust the degrees of freedom were employed (Greenhouse and Geisser, 1959). Post-hoc comparisons were made using Fisher's least-significant difference (LSD) tests. Body condition, the time taken to capture the bird, time of day, date, CP width, muscle and fat scores were added to the models as random factors along with all relevant interactions. All analyses were performed using Sigma Plot version 13 (Systat Inc., San Jose, CA) with α set at 0.05. Data are presented as means ± standard error.

3. Results

3.1. Brachial and jugular circulation

Based on data from a subset of birds collected in Oct 3, 2013, restraint stress increased jugular CORT and simultaneously decreased jugular DHEA (Fig. 2). Based on these data the 30 min period for restraint was sufficient to induce a maximal change in both steroids (Fig. 2). Whether the brachial or jugular vein was sampled had a significant effect on both the CORT ($F_{2,12} = 3.62$, $P = 0.028$, Fig. 3A) and DHEA ($F_{2,12} = 3.04$, $P = 0.035$, Fig. 3B) response to capture restraint. Initial brachial samples had higher CORT levels than initial jugular samples ($P = 0.008$), however restraint-induced levels of CORT did not differ between veins ($P = 0.163$). In contrast, DHEA did not change with restraint stress

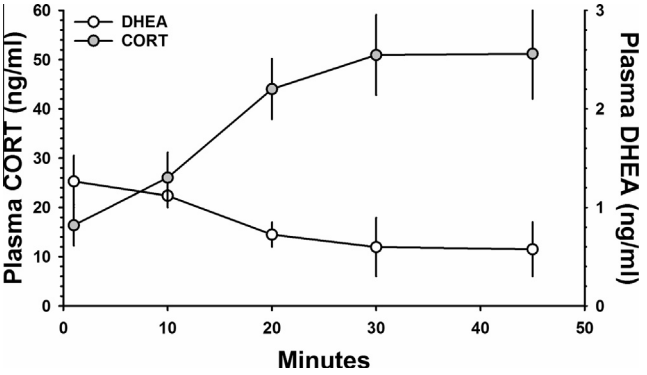

Fig. 2. Changes in corticosterone (CORT) and dehydroepiandrosterone (DHEA) in the jugular circulation of northern cardinals ($N = 5$) in response to restraint stress.

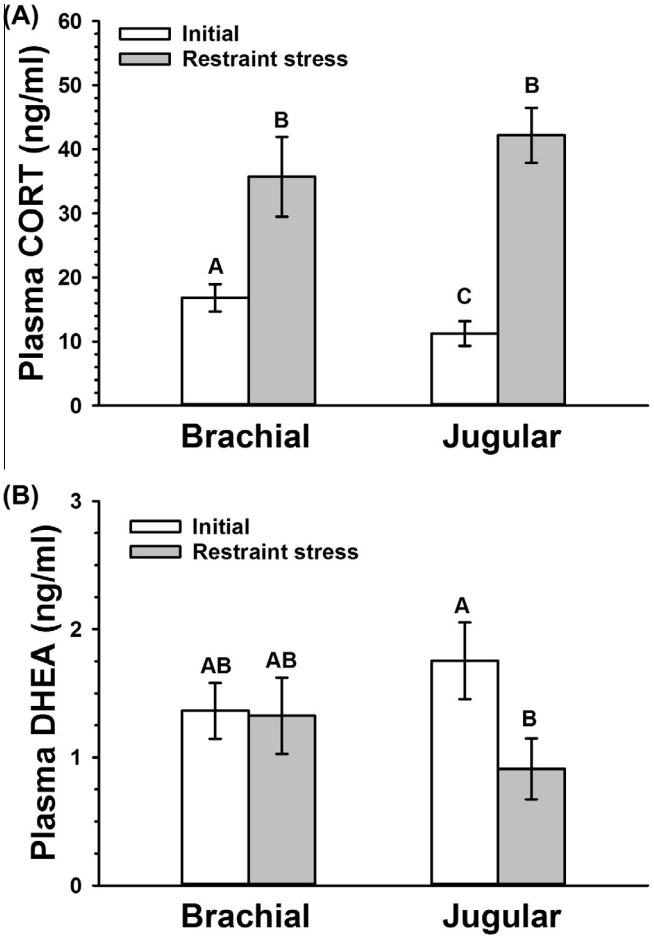

Fig. 3. Initial and restraint stress (A) corticosterone (CORT) and (B) dehydroepiandrosterone (DHEA) concentrations collected from brachial ($N = 8$) and jugular ($N = 10$) circulation of northern cardinals. All data are expressed as means ± standard errors and data points with identical letters do not differ at $P < 0.05$.

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in brachial circulation ($P = 0.673$), but did decrease with restraint in jugular blood ($P = 0.041$). However, both initial and restraint DHEA concentrations did not differ between veins. Birds with a higher body condition and fat score had higher jugular DHEA concentrations ($F_{1,13} = 2.76$, $P = 0.045$ and $F_{1,13} = 2.04$, $P = 0.033$, respectively). No other variables were associated with CORT or DHEA levels regardless of vein type (all $P \geqslant 0.094$).

3.2. Regulation by exogenous ACTH

Pre-injection CORT concentrations did not differ between treatment groups ($P = 0.283$), however exogenous ACTH injection increased CORT concentrations beyond the vehicle injected control birds ($F_{2,13} = 3.03$, $P = 0.022$, Fig. 4A). In contrast, ACTH injection did not significantly increase DHEA concentrations above those of the saline injected control birds ($F_{2,13} = 0.41$, $P = 0.370$, Fig. 4B). No differences in morphometric characteristics or capture times were present between control and ACTH injected birds (all $P \geqslant 0.106$).

3.3. Seasonal patterns

Throughout a single calendar year (i.e., 2014), adult male Cardinals exhibited seasonal (i.e., monthly) variation in both initial and restraint CORT ($F_{2,76} = 10.47$, $P < 0.0001$, Fig. 5A) and DHEA ($F_{2,76} = 12.96$, $P < 0.0001$, Fig. 5B) concentrations. For both initial and restraint, CORT levels appear to increase during the first half of the year, but then declines sharply from June to July to reach levels equivalent the start of the year (Fig. 5A). However, sample sizes from June to July were the lowest in the study (see Fig. 5A and B), and thus need to be interpreted with caution. Initial DHEA concentrations were higher than restraint levels during the five months of fall-winter (Jan, Feb, Oct, Nov, and Dec), but only in July did restraint significantly increase DHEA levels (Fig. 5B). There was also a slight significant negative relationship between initial CORT and body condition ($F_{1,75} = 1.71$, $P = 0.048$). Furthermore, CP width was also slightly positively correlated with CORT ($F_{1,75} = 2.17$, $P = 0.033$). No other variables were associated with either initial nor restraint CORT or DHEA levels across seasons (all $P \geqslant 0.094$).

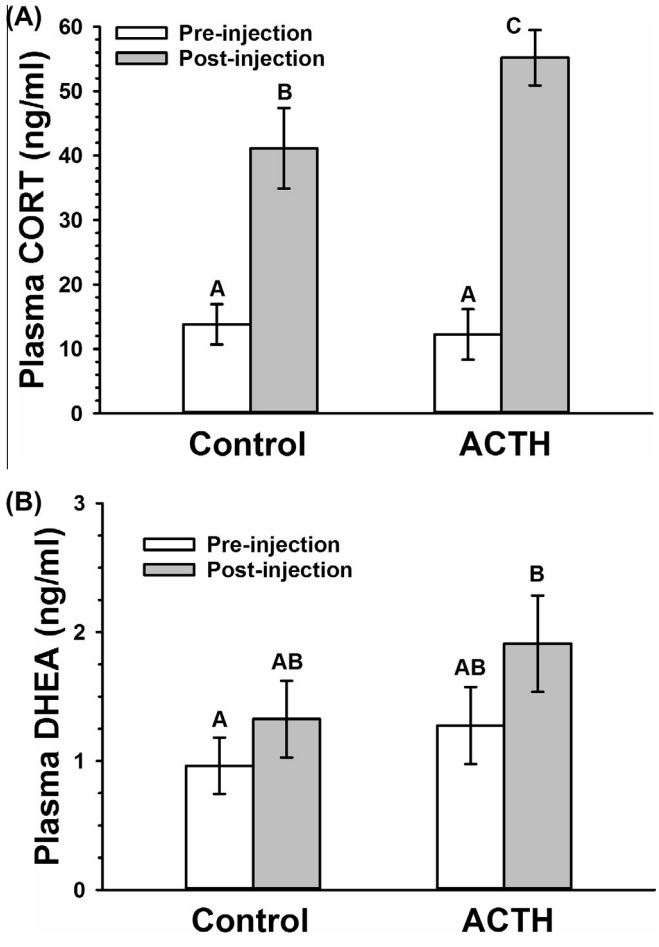

Fig. 4. Comparison of (A) corticosterone (CORT) and (B) dehydroepiandrosterone (DHEA) concentrations in response to exogenous injection with ACTH ($N = 8$) or saline vehicle ($N = 7$, control) in northern cardinals. All data are expressed as means ± standard errors and data points with identical letters do not differ at $P < 0.05$.

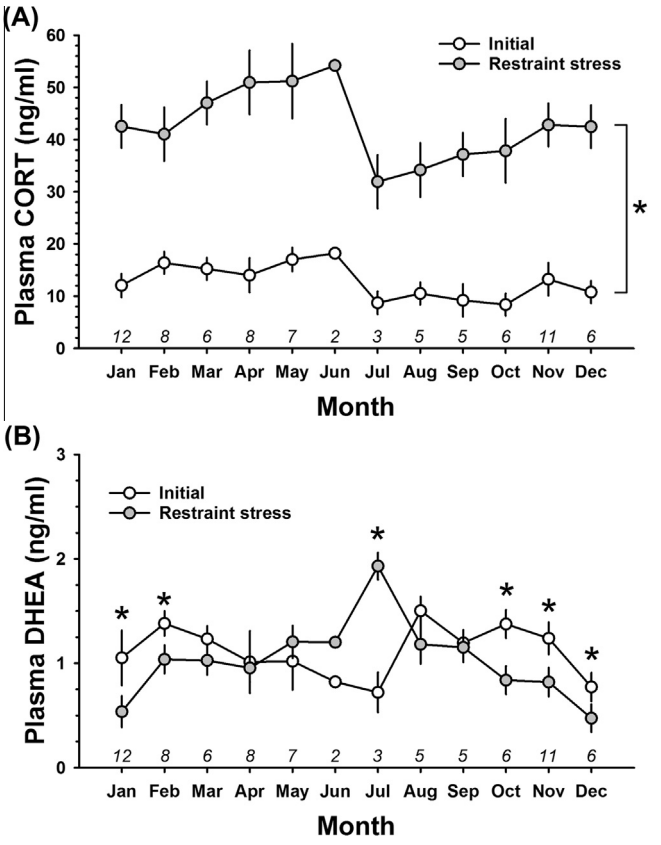

Fig. 5. Seasonal variation in initial and restraint stress concentrations of (A) corticosterone (CORT) and (B) dehydroepiandrosterone (DHEA) in northern cardinals from Central Florida USA throughout a single calendar year (2014). Numbers below data points indicate monthly sample sizes and * indicates significant differences at $P < 0.05$ between initial and stress levels for that month. All data are expressed as means ± standard errors and data points with identical letters do not differ at $P < 0.05$.

4. Discussion

I investigated how circulating concentrations of two adrenocortical steroids CORT and DHEA varied between circulation patterns, across the course of a year, and in response to both acute stress and exogenous ACTH challenge in free-living male northern cardinals. As predicted, restraint stress elevated CORT levels while simultaneously lowering DHEA concentrations, but this latter effect was only present in jugular (neutrally enhanced or depleted) than brachial (systemic) circulation. Jugular CORT levels increased in response to injection with ACTH, but no effect was apparent on

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DHEA concentration. Male cardinals sampled monthly for an entire year, demonstrated that both steroids showed significant seasonal variation. Generally, both initial and restraint levels of CORT increased with progression to breeding, but then declined sharply during the summer months. In contrast, initial DHEA concentrations were generally higher in the fall and winter, and declined during summer, with restraint levels being highest during the summer.

These data demonstrate a generally negative relationship between CORT and DHEA, but also emphasizes the importance of considering both the time of year and the vein being sampled in future research.

4.1. Regulation of CORT and DHEA during stress

As expected, restraint stress elevated CORT levels above initial concentrations, with a maximal response being observed between 30 and 40 min after capture. This is comparable to previous studies and suggests that the timing of the acute stress CORT profile is relatively consistent across vertebrates (Astheimer et al., 1994; Boonstra et al., 2008; Fokidis and Deviche, 2011). The levels of CORT reported here, are comparable to circulating concentrations reported in other studies of this species (DeVries et al., 2011; Barron et al., 2012; Owen et al., 2012; DeVries and Jawor, 2013; Wright and Fokidis, 2016). In contrast to CORT, DHEA declined with acute restraint stress, with the lowest levels reached again between 30 and 40 min of capture. Interestingly, this DHEA decrease with restraint stress was only detected when jugular blood was sampled, with no change observed in the brachial blood. Differences in hormone concentrations, including DHEA, between jugular and brachial circulations have been reported before for song sparrows (Newman et al., 2008). To further investigate the decline in DHEA with stress, subsequent studies focused on jugular samples, as opposed to brachial, which limited our ability to make direct references about adrenal DHEA synthesis and secretion, since levels may be altered by the brain through synthesis and uptake.

The traditional interpretation of this observation is the vein differences lie in the brain's uptake or secretion of the respective steroid, resulting in changes in the steroid milieu (Schmidt et al., 2008; Taves et al., 2011), thus the lower CORT concentration in jugular blood compared to brachial circulation suggests the brain uptakes CORT. This is consistent with the many known functions of GCs within the brain, including its major roles in fear memory recognition and consolidation (Rodrigues et al., 2009), appetite regulation (Crespi et al., 2004; Liu et al., 2014), neuroplasticity (Newman et al., 2010; Sterner and Kalynchuk, 2010) and its effects on hippocampal neurogenesis (Gourley et al., 2008; Srinivasan et al., 2013). The lower initial CORT concentrations in jugular plasma suggests that CORT uptake by the brain is occurring under basal metabolic conditions, but during restraint stress neural uptake is either masked by rising adrenal CORT concentrations, or decreases resulting in no difference between veins.

In contrast, the function of neural DHEA is less understood. Exogenous DHEA administration can stimulate hippocampal neurogenesis and migration in rats and birds (Goncharov and Katsya, 2013; Kimonides et al., 1999) and has increased the volumes of avian song control nuclei to levels consistent with seasonal reproduction (Soma et al., 2002). These effects of DHEA on the brain largely oppose those of CORT, leading to consideration of this androgen precursor as an "anti-stress" hormone (Kimonides et al., 1999; McNelis et al., 2013; Kalimi et al., 1994). Here, jugular DHEA decreased in response to restraint stress, even as jugular CORT levels increased. One explanation is that DHEA may be used within the brain thus lowering jugular levels, even as brachial levels do not change. This is in line with a view of neural DHEA counteracting CORT to protect the brain, whereas non-neural tissues receive a steady supply of DHEA during stress. Furthermore, another interpretation is that lowered DHEA in an acute stress situation would allow CORT to function, with an anti-stress function only being vital during chronic stress. Further study investigating DHEA profiles during chronic stress may be warranted, as are studies addressing relative rates of DHEA uptake by the brain between vein types.

Both CORT and DHEA achieved maximal and minimal levels, respectively within 30–40 min, which suggests a concerted regulation of these adrenal steroids. In humans, androgen and corticoid production is spatially compartmentalized primarily within the cortical cells of the zona fasciculata and zona reticularis, responsible for CORT and DHEA synthesis, respectively (Ferrari and Mantero, 2005; Miller, 2009). Adrenal CORT secretion is primarily regulated by adrenocortical cell stimulation by ACTH (Sapolsky et al., 2000; Taves et al., 2011), but here exogenous ACTH failed to produce significant changes in DHEA in the cardinal, unlike reports from studies in humans (Kümpfel et al., 1999; Radant et al., 2009) and squirrels (Boonstra et al., 2008). Furthermore, research in lab mice and rats failed to provide evidence for adrenal DHEA secretion in general (van Weerden et al., 1992). In hamsters, ACTH can stimulate DHEA secretion, but only in animals on short-day conditions (Rendon et al., 2015). Our understanding of DHEA action has been hampered by limited access to small animal models for study, however here birds may play an important role in identifying DHEA functions. Unlike humans, birds do not appear to have DHEAS in circulation (Soma et al., 2008), suggesting there may not be a need for a large reservoir of available prohormone. To our knowledge, this is the first study to examine DHEA concentrations in response to ACTH injection in a bird. In this study, ACTH administration did significantly increase CORT concentrations suggesting that the dose was sufficient to stimulate adrenocortical cells, however the increase was barely above the control, suggesting that the ACTH dose used in this study did not maximize CORT secretion. The dose used here successfully increased CORT well above controls in other songbird species (Romero et al., 1998; Romero, 2006; Fokidis and Deviche, 2011). This may also explain why ACTH did not stimulate DHEA secretion, and thus future studies may explore a range of ACTH doses.

Unlike in mammals, the avian adrenal gland is not clearly defined with cortical (or interrenal) tissue intermingling with chromaffin cells with the former arranged in numerous cords composed of a double row of cells (Aire, 1980). Whether specific avian adrenal cells secrete DHEA has not been determined, but the role of the adrenal glands in DHEA synthesis appears species specific even within the mammalian clade. Future studies should utilize a combination of *in vivo* and *in vitro* studies to identify the role of the avian adrenal gland in DHEA secretion.

4.2. Seasonal CORT and DHEA patterns in the cardinal

Seasonal patterns of CORT secretion in birds have been widely studied in various species (Romero, 2006; Romero and Wingfield, 1998; Romero et al., 2008). Fundamental to our understanding of GCs, is their role as energy-mobilizing hormones, and thus as energetic demands vary, CORT often follows suite. As with previous studies, initial CORT was generally highest from late-winter to summer (Feb to June), which for cardinals in Florida coincides with the pre-breeding and breeding seasons, as compared to the late summer-fall (July to Oct) (Halkin and Linville, 1999; Kale et al., 2003). Male cardinals as with most songbirds form socially monogamous pair bonds to assist in food delivery to offspring and actively mate guard and defend a territory against conspecifics (Ritchison et al., 1994) which all increase energetic demands on the bird. The increase in initial CORT concentrations during breeding is

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thought to reflect CORT roles in promoting the mobilization of fat and protein stores for gluconeogenesis (Holberton and Wingfield, 2003; Sapolsky et al., 2000). Stress CORT levels were also higher during this time, suggesting that birds retained an active stress response during breeding, and that higher initial levels did not correspond to a tradeoff in the ability to further increase CORT during restraint. Following breeding, a sharp decline in restraint CORT concentrations is observed from June to July, and initial levels are lowest from July to Oct, which roughly coincides with the molt period (Halkin and Linville, 1999; Kale et al., 2003). The attenuation of the CORT response to restraint stress may preserve the protein investment into feather replacement during molt restraint in birds (DesRochers et al., 2009; Romero et al., 2005). This may explain the lowered CORT response to restraint stress by cardinals during the molting period observed in this study as well as the limited CORT responsiveness to ACTH injection, as the study was conducted in Sept. The drastic decrease from June to July in both initial and restraint CORT though may be a product of the small sample sizes during this time, as cardinals are more difficult to capture. One explanation for this difficulty may be a decrease in energy expenditure during the summer as temperatures are hottest in central Florida. Nonetheless, CORT levels remain lower during the late summer and fall and gradually increase for the remainder of the year.

Unlike CORT, few studies in wild animals have sampled monthly to gain a complete annual cycle of circulating DHEA, despite increased pressure to gain fuller understanding of how physiological mechanisms vary across the entire seasonal cycles (Marra et al., 2015). Here, initial DHEA concentrations were generally the highest during the fall-winter months, and lowest in summer. Furthermore, restraint stress decreased DHEA levels significantly only during the fall-winter months. The higher DHEA levels during the fall-winter may be indicative of a protective function compatible with feather replacement during molt. During molt, protein investment into feathers may be compromised by excessive exposure to CORT (DesRochers et al., 2009; Romero et al., 2005) and elevated DHEA levels may potentially counteract this, therefore preserving feather development. However, in this study, CORT concentrations were lowest during the fall-winter. Furthermore, in song sparrows DHEA levels were lowest during the molt period (Newman et al., 2008; Soma and Wingfield, 2001), despite living in the more temperate, and presumably more energy-limited Pacific Northwest. Although overall CORT levels declined, cardinals still maintained a robust CORT response to restraint stress during this period. During restraint stress, cardinals also decreased their DHEA levels but this effect was only significant outside of the breeding condition (Mar-Sept), whereas maintaining high DHEA levels as CORT rises during stress would be expected if DHEA is acting as an anti-stress hormone. Together these observations do not support the hypothesis that preserving feather replacement by counteracting CORT is a primary function of DHEA during the fall-winter and suggests they can simultaneously invest in both feather growth and CORT secretion without an apparent tradeoff.

Studies of circulating DHEA in free-living birds have largely focused on its role as an androgen precursor that may drive aggressive behavior in a non-breeding territorial challenge. This is thought to involve the neural conversion of DHEA to testosterone and/or estradiol (Heimovics et al., 2013; Soma et al., 2015). In the song sparrow where much of this work has been conducted, blocking aromatase decreased non-breeding aggression, which could then be restored with estradiol treatment (Soma et al., 2000). Further research in this species has shown seasonal modulation of neural 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD) activity, the enzyme responsible for metabolizing DHEA to active androgens, specifically with higher activity during the winter months (Pradhan and Soma, 2012; Schlinger et al., 2008). Furthermore, territorial challenges induced using STI increases 3β-HSD activity (Pradhan et al., 2010; Pradhan and Soma, 2012). These data may represent a greater capacity for DHEA conversion during the non-breeding season to support the active territorial aggression observed in this species. The cardinal is also a sedentary species that displays aggression towards conspecifics throughout the year. Thus in the cardinal, DHEA may also regulate non-breeding aggression. Winter aggression in hamsters is thought to be regulated partly by melatonin, possibly though a mechanism involving DHEA (Rendon et al., 2015; Soma et al., 2015). Previous research in cardinals has shown that both male and female cardinals (both of which are involved in territorial defense) exhibit little annual variation in circulating T (DeVries et al., 2015, 2011; Jawor, 2007). Exogenous gonadotropin-releasing hormone (GnRH) elevates T levels but only during the latter months of the non-breeding period (Jan-Mar) and this was observed for both males and females (DeVries et al., 2011). The ramifications of this are that year-round testosterone concentrations may be sufficient to sustain cardinal aggression across the annual cycle. As this experiment was conducted in March, the ability of cardinals to secrete T might have been intact, and circulating DHEA may have been a byproduct of T secretion by the regressed testes. Social instability, such as during territorial disputes does not appear to alter T secretion in cardinals (DeVries et al., 2011; Jawor, 2007), although prolonged exposure to STI attenuates the decrease in DHEA levels observed with restraint stress (Wright and Fokidis, 2016). Thus DHEA may play a subtle role in territorial aggression that is independent or even complementary to T, however further study of free-living cardinals is necessary to elucidate this role.

5. Conclusion

Clearly this study demonstrates that DHEA and CORT, although both presumably originating primarily from adrenocortical tissue, they each exhibit distinct patterns of secretion. In response to acute restraint stress, CORT increases and DHEA often decreases (though sometimes increase) particularly when sampling the jugular vein, which supports the uptake of DHEA by the brain, possibly for further metabolism to bioactive steroids. This decrease in DHEA with stress contradicts its potential role as an anti-stress hormone, counteracting CORT effects. Administering exogenous ACTH replicates the CORT response to stress, but does not affect DHEA levels, suggesting differences in the regulation of these steroids. Seasonal variation in these steroids reveals a marked distinction between the breeding and non-breeding periods for both steroids. Although such patterns are well established for CORT, very little is known about the factors that influence DHEA secretion. These data demonstrate a relatively consistent CORT response to stress, but a largely independent response of DHEA depending on both the manner of sample collection and time during the annual cycle.

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