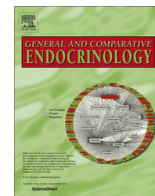




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Sources of variation in plasma corticosterone and dehydroepiandrosterone in the male northern cardinal (*Cardinalis cardinalis*): II. Effects of urbanization, food supplementation and social stress

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ABSTRACT

Perturbations in an organism's environment can induce significant shifts in hormone secretory patterns. In this context, the glucocorticoid (GC) steroids secreted by the adrenal cortex have received much attention from ecologists and behaviorists due to their role in the vertebrate stress response. Adrenal GCs, such as corticosterone (CORT), are highly responsive to instability in environmental and social conditions. However, little is understood about how adrenal dehydroepiandrosterone (DHEA) is influenced by changing conditions. We conducted field experiments to determine how circulating CORT and DHEA vary during restraint stress in the male northern cardinals (*Cardinalis cardinalis*). Specifically, we examined how four different changes in the physical (urbanization and food availability) and social (territorial conflict, distress of a mate) environment affect CORT and DHEA levels. The majority of cardinals responded to restraint stress by increasing and decreasing CORT and DHEA, respectively, however this depended on sampling context. Cardinals sampled from urban habitats had both lower initial and restraint stress CORT concentrations, but a comparable DHEA pattern to those sampled from a forest. Supplementing food to territorial males did not alter circulating initial DHEA or CORT concentrations nor did it change the response to restraint stress when compared to unsupplemented controls. Exposing cardinals to varying durations of song playback, which mimics a territorial intrusion, did not affect CORT levels, but did attenuate the DHEA response to restraint stress. Examining a larger dataset of males captured before, after or at the same time as their female mate, allowed us to address how the stress of a captured mate affected the male's CORT and DHEA response. Males showed elevated initial and restraint CORT and DHEA when their female mate was captured first. Taken together, these data demonstrate that both CORT and DHEA secretion patterns depends on environmental, and particularly current social conditions.

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1. Introduction

Environmental instability has major consequences for the survival and reproductive success of organisms and thus being capable of coordinating an appropriate response to such stress is a necessity. In vertebrates, the hypothalamic–pituitary–adrenal (HPA) axis is an endocrine cascade activated by a stressor that regulates physiology and behavior that ultimately promote survival (Wingfield and Sapolsky, 2003). During HPA activation, the adrenal gland responds to pituitary-derived adrenocorticotrophic hormone (ACTH) by secreting glucocorticoids (GCs), such as corticosterone

(CORT) in birds (Sapolsky et al., 2000). These GCs regulate the reallocation of energy reserves towards vital physiological functions, promotes survival behaviors and suppresses immune functions and reproduction in a holistic effort to cope with environmental challenges (McEwen and Wingfield, 2003). The causes of environmental instability, though highly variable, can be broadly divided into two types: 1) those involving sudden changes in the physical environment that result in shifts in the resources in an area, such as food availability or changes in habitat structure; and 2) those involving intraspecific interactions that alter an individual's social standing, such as mating conflicts. Although, both forms of instability can alter GC secretion in wild animals, they differ in the degree to which they depend on context. For example, depletion of food resources is an energetic stressor that imposes direct and consistent physiological impacts on individuals (...) However,

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stress associated with a territorial challenge depends on the condition of the intruder and the resident, the nature of the interaction, quality of the territory, and the presence or absence of a female (...). Thus physical and social environments may represent different sources of variation in GC secretion.

The adrenal gland, in addition to GCs, also secretes the androgen precursor, dehydroepiandrosterone (DHEA) in response to ACTH (Boonstra et al., 2008; Kalimi et al., 1994; Soma et al., 2014a,b), although this may vary across species (Bélanger et al., 1990, 2002; van Weerden et al., 1992). Ascertaining the biological role of DHEA has been elusive, in part because a specific receptor has not been explicitly identified (Labrie et al., 2001; but see Webb et al. (2006) and Widstrom and Dillon (2004)). Thus DHEA presumably is converted to active androgens (e.g., testosterone or T) and estrogens (e.g., estradiol or E₂) to exert its effects (Soma et al., 2014a,b). Research has also been limited due to the lack of an appropriate research model as mice and rats have essentially undetectable DHEA levels (van Weerden et al., 1992). In humans and red squirrels (*Tamiasciurus hudsonicus*), acute stress increased circulating DHEA levels (Boonstra et al., 2008; Strous et al., 2006), but no effects on DHEA concentrations were observed in the song sparrow (*Melospiza melodia*) (Newman et al., 2008; Soma and Wingfield, 2001). Functionally, rodent and *in vitro* studies have suggested DHEA counteracts GC effects on neurons (Kimonides et al., 1999; McNelis et al., 2013; Kalimi et al., 1994), and studies in song sparrows and Siberian hamsters (*Phodopus sungorus*) suggest DHEA may promote aggressive behaviors during non-breeding contexts (Soma et al., 2014a,b). Despite the growing body of literature, still little is understood concerning how instability in the physical and social environmental impacts DHEA concentrations.

To assess environmental instability, field studies of vertebrates often compare initial (i.e., baseline) steroid levels with those after a period of handling stress. This widely-used method has garnered important information concerning the ecological roles of steroids, and in particular GCs, in wild animals (Deviche et al., 2010; Fokidis et al., 2011b; French et al., 2008; Holding et al., 2014; Pereyra and Wingfield, 2003; Pravosudov et al., 2002; Refsnider et al., 2015; Woodley et al., 2014). In recent years, how animal endocrine systems respond to urbanization has garnered significant interest, and here the CORT response to stress is paramount. As urban landscapes encroach on natural environments they present numerous novel stressors to wildlife including: human disturbance (Fernández-Juricic et al., 2005); traffic (Bautista et al., 2004); noise (Slabbekoorn and Peet, 2003); feral animal predators (Baker et al., 2008; Woods et al., 2003); introduced and invasive competitors (Shochat et al., 2010); pollutants (Janssens et al., 2001); street lighting (Longcore and Rich, 2004); and warmer temperatures (McLean et al., 2005). Several bird species have been examined in detail regarding the effects of urbanization on HPA activity, however generalizations have been difficult to make due to variation between species (Bonier, 2012). In some species, urban populations suppress their stress-induced CORT secretion (Partecke et al., 2006), presumably to avoid the costs of overstimulation in a "stressful" environment. Other species show a greater CORT response to handling in urban areas (Fokidis et al., 2009; Schoech et al., 2007), perhaps to enable them to continually cope with stressors as they encounter them. Thus species-specific CORT responses may indicate whether birds are positively and negatively affected by the instability associated with urbanization. In contrast to CORT, no study has investigated variation in DHEA levels between cities and natural animal populations. Among the benefits cited for birds in urban areas is gaining access to higher and more predictable food resources which serves to decrease environmental instability (Fokidis et al., 2011a; Heiss et al., 2009). Food supplementation of free-living birds typically lowers baseline CORT levels

and decrease the CORT response to handling stress (Clinchy et al., 2004; Schoech et al., 2007), whereas fasting imposes the opposite effects (Fokidis et al., 2013, 2012, 2011a). Fasting is also known to elicit an increase in plasma DHEA in both avian and mammalian species (Fokidis et al., 2013, Grasfeder et al., 2009), however the effects of food supplementation on DHEA in free-living animals have not been investigated.

Environmental instability associated with changing intraspecific social interactions also modulate patterns of GC secretion. Changes in circulating CORT levels during conspecific social challenges are usually tested using simulated territorial intrusion (STI) (Deviche et al., 2014, 2012; Newman and Soma, 2011). In many bird species, CORT levels remain constant during antagonistic interactions (Fokidis et al., 2011b), possibly since CORT may interfere with T secretion which may assist the individual in winning a territorial bout (Deviche et al., 2010). However, in species where T increases are not seen (e.g., those with short breeding seasons), CORT increases are observed instead because the bout is viewed as a stressor and/or because the response requires energy mobilization. Furthermore, some birds also exhibit increases in plasma DHEA levels during STI (Hau and Beebe, 2011; Hau et al., 2004). Another source of social instability is the distress of a mate (e.g., during a predatory event) often associated with distress calling vocalizations (Conover, 1994). Distress calls may serve as warnings to other birds (Conover, 1994; Hill, 1986), or may provide information about an individual's quality to the predator (Laiolo et al., 2004). Observing one's mate being captured may stimulate a stress response in the observer, particularly for species with strong pair bonds. However, the effect of pair distress on CORT and DHEA levels has received little, if any, attention from researchers, yet constitutes an important component of environmental instability.

The aim of this study was to examine how environmental instability in different contexts influences CORT and DHEA concentrations in free-living adult male northern cardinals (*Cardinalis cardinalis*) to determine the degree to which the responses of these adrenal steroids are parallel. Specifically, we compared plasma CORT and DHEA concentrations before and after restraint stress in four contexts of environmental instability: 1) between birds inhabiting urban and natural forest habitats; 2) between birds that were food supplemented with controls; 3) between birds challenged with varying durations of a STI; and 4) birds experiencing the distress of a captured mate with those that were not exposed to this stimulus. Northern cardinals are common resident songbirds in Central Florida, and both sexes actively defend territories year-round (DeVries et al., 2015, 2012, 2011; Jawor, 2007). Research on this species has focused on the regulation of year-round T secretion in both males and females, as both sexes behave similarly during territorial conflicts and both participate in parental duties thus suggesting a strong pair bond (DeVries and Jawor, 2013; DeVries et al., 2015, 2014, 2012, 2011; Jawor, 2007; Jawor et al., 2014; Jawor and MacDougall-Shackleton, 2008; Nealen and Breitwisch, 1997). In contrast to T, very little research has been done on other androgens in this species (but see Barron et al., 2012 and Owen et al., 2012). To our knowledge, no research has examined DHEA in cardinals. This study is the first to examine how the context of environmental instability influences the secretory patterns of both CORT and DHEA in a free-living species.

2. Materials and methods

2.1. Ethics statement

Each experiment was conducted under United States Geological Survey Bird Banding Laboratory permit #23847, Florida Fish, and

Wildlife Commission scientific collecting permit #LSSC-13-00057, and with 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. Experiment 1: variation between urban and forest populations

Growing interest in the anthropogenic effects on stress physiology has prompted comparisons between human-modified habitats (e.g., cities) and natural habitats (Costantini et al., 2014; Fokidis et al., 2009; French et al., 2008; Grunst et al., 2014; Heiss et al., 2009; Payne et al., 2012; Schoech et al., 2007). Although this work has mostly focused on CORT, to date no study has investigated such spatial variation in steroid precursors, such as DHEA. To determine whether CORT and DHEA can vary with habitat-type, adult male cardinals were sampled from Dec 7–22, 2013 in both an urban-suburban location centered on the communities of Winter Park, Maitland, and North Orlando, Florida (mean population density: 1238.8 people/km²; $N = 11$ Cardinals), and from a forested natural habitat, the Hal Scott Regional Preserve a 9387 acre expanse of vast undeveloped flatwood forests and open prairie that straddle the Econlockhatchee River and located 30 miles east of Orlando ($N = 12$). Birds were captured using mist nets coupled to conspecific song playback. The time it took for the bird to respond to the playback (i.e., either show up or sing in response; hereafter *response time*), and the time it took for them to strike the net and be captured (hereafter *capture time*) was recorded for all birds.

Within 2 min of capture, an *initial* blood sample (300 μ l) was collected from the right jugular vein using a 28 gauge heparinized needle and a 3 cc insulin syringe. Birds were then kept in an opaque cloth bag for 30 min, to induce an acute stress response after which another *restraint stress* blood sample was also collected from the jugular. Previous research demonstrated that the 30 min was sufficient time to determine the CORT and DHEA response to restraint stress (Fokidis, submitted for publication). We collected additional measurements from captured birds 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). Birds were released on site with a uniquely numbered US Geological Survey aluminum leg band. 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.

2.3. Experiment 2: variation in response to food availability

Several studies have demonstrated that energy balance can alter adrenal steroid secretion and field studies have noted changes in CORT concentrations with food provisioning in songbirds (Clinchy et al., 2004; Herring et al., 2011). Further, captive studies have suggested a link between food availability and DHEA levels (Fokidis et al., 2013), but this remains untested by field studies. To test the hypothesis that food provisioning alters adrenal CORT and DHEA secretion, the territories of adult male cardinals were randomly assigned to either: 1) a food supplemented treatment group ($N = 14$) with a consistent supply of wild bird seed (Wild Bird Seed Ration #20, Pennington Seed Inc., Madison, GA, USA) or 2) to an unsupplemented control group ($N = 13$) exposed to an empty bird feeder. Prior to the experiment, resident cardinal territories were located by aggressive response to playback, which is suggestive of territoriality although we cannot exclude the possibility that neighboring birds also used the feeders. This experiment was conducted in and around the Rollins College campus in Winter

Park, Florida from 16 Oct to 21 Nov 2014. Cardinals were confirmed by direct observation to use each feeder at least two days prior to their capture. Food supplementation continued for at least four weeks until birds were captured and subjected to the same restraint stress and blood sampling as described for experiment 1. Birds were then released at the site of capture. Samples were stored at -80°C until assayed for CORT and DHEA.

2.4. Experiment 3: variation in response to simulated territorial challenge

Engaging in territorial behavior is regulated by sex steroids (namely T) and is both energetically costly and potentially stressful. Several studies in birds have shown increased T secretion in response to social challenges (Wingfield, 2012), and this may also involve increased DHEA secretion, which can be neutrally converted to E₂ to facilitate aggression (Heimovics et al., 2012; Pradhan and Soma, 2012; Pradhan et al., 2010; Schlinger et al., 2008; Soma et al., 2014a,b). Excessive confrontations over territory can induce a stress response thus changing CORT levels in circulation. To test whether territorial challenges are interpreted as physiological stressors that alter CORT and DHEA in circulation, individual male cardinals were exposed to STI for either 1) <5 min ($N = 12$); 2) 30-min ($N = 11$); or 3) 60-min ($N = 8$) from Mar 2–7, 2014 in the Hal Scott Regional Preserve and Park. Mist nets were kept open for the first 5 min of the STI. However if the bird was not captured in that time, the net was closed and then the STI continued for either 25 or 55 min, alternating between these two treatments to avoid temporal variation in sampling. After this time, nets were open to capture the birds, and upon capture birds were exposed to the restraint stress protocol and blood was sampled as described for experiment 1. Samples were stored at -80°C until assayed for CORT and DHEA.

2.5. Experiment 4: variation in response to pair distress

In many bird species, including cardinals, challenges to territory are met by both the male and female residents, which may help to reinforce the pair bond and increase the likelihood of successfully defending the territory against intruders (DeVries et al., 2015; Fedy and Stutchbury, 2005; Gill et al., 2007). Captured cardinals produce loud distress calls that frequently attract the attention of other birds (Norris and Stamm, 1965). To test whether the capture of a female mate induces a stress response in the male (hereafter *pair distress*) we combined CORT and DHEA data from birds captured here, with those from another study (Fokidis, submitted for publication). Typically, a pair of birds would respond together to each playback, and whether the male or female was captured first was noted as was the time that elapsed between capturing both birds. To encourage production of distress vocalizations, the female remained in the net until the target male was captured. To minimize confounds, only data from experiments where the jugular vein was sampled and where both members of the pair were captured were used. Birds were divided into three groups: 1) males that were captured first (Male-1st: $N = 78$), 2) males captured after their female mate was captured at ≥ 3 min before (Male-2nd: $N = 22$), and 3) males that were captured within 10 s of their mate (Male-Same: $N = 16$) to compare CORT and DHEA levels. As these compilation of samples were conducted for different studies, we included the date, location of capture, time exposed to the conspecific playback and the time taken to capture the male in the analysis to examine any confounding effects on steroid patterns.

2.6. 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 songbirds, and has been validated by us for use in the cardinals (Fokidis, submitted for publication). All plasma samples were assayed in duplicate, with samples from the same individuals (i.e., initial and restraint stress) 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 5.8 to 8.1 pg/mL and the mean intra-assay and inter-assay coefficients of variation were 7.3% and 11.2%, respectively ($N = 6$ plates, 392 samples total).

2.7. 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. This SPE methodology has been validated for cardinals and previously described (Fokidis, submitted for publication). Dried plasma extracts from the SPE were stored at -20°C until assayed for DHEA using a commercial DHEA ELISA kit (Diametra DKO124, Immunodiagnostic Systems Ltd, Milano, Italy). Dried extracts were reconstituted in $3\ \mu\text{l}$ of absolute ethanol and $60\ \mu\text{l}$ of buffer from the assay kit and the assay was run according to manufacturer's instructions. This ELISA kit was validated for use in northern cardinals (Fokidis, submitted for publication) and all plasma samples were assayed in duplicate, with initial and restraint stress 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 0.4 to 0.7 ng/mL and the mean intra-assay and inter-assay coefficients of variation were 7.4% and 11.1%, respectively ($N = 6$ plates, 392 samples total).

2.8. Statistical analysis

All data were first tested for adherence to assumptions of normality and homoscedasticity (i.e., equal variance), and if necessary data were log-transformed prior to further analysis. Morphometric data from this study was combined with that of another study (Fokidis, submitted for publication) 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 the highest goodness of fit ($R^2 = 0.83$, $P = 0.001$; (Fokidis, submitted for publication) and the residuals were used as a body condition metric in subsequent analyses. For each experiment, repeated measures analysis of variance (rmANOVA) was used to compare changes in CORT and DHEA from initial and after restraint stress (the within-subjects factor) and treatments (i.e., urban/forest, food supplement/control, STI duration, male capture order) 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. Finally, to assess the relationship between

CORT and DHEA, all data from this study and from (Fokidis, submitted for publication) that was collected from the jugular vein were combined tested using Pearson correlations. 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 \pm standard error.

3. Results

3.1. Level of urbanization

Urban and forest-dwelling cardinals showed differences in the CORT ($F_{2, 21} = 6.18$, $P = 0.011$, Fig. 1A), but not the DHEA stress response ($F_{2, 21} = 0.85$, $P = 0.372$, Fig. 1B). Urban cardinals had both lower initial and restraint stress concentrations of CORT compared to forest birds ($P = 0.027$ and $P = 0.041$, respectively). Initial DHEA did not differ between habitats ($P = 0.28$), and levels decreased to the same degree with restraint stress ($P = 0.46$). Urban cardinals also were in better body condition ($F_{1, 22} = 2.20$, $P = 0.031$) and had a higher muscle score ($F_{1, 22} = 3.04$, $P = 0.026$) than forest caught birds. No other variables were associated with neither initial nor restraint stress CORT or DHEA levels across seasons (all $P \geq 0.166$).

3.2. Food availability

Food supplementation of wild cardinals did not influence CORT ($F_{2, 23} = 0.480$, $P = 0.498$, Fig. 2A) compared to unsupplemented control birds. Although cardinals did significantly increase CORT

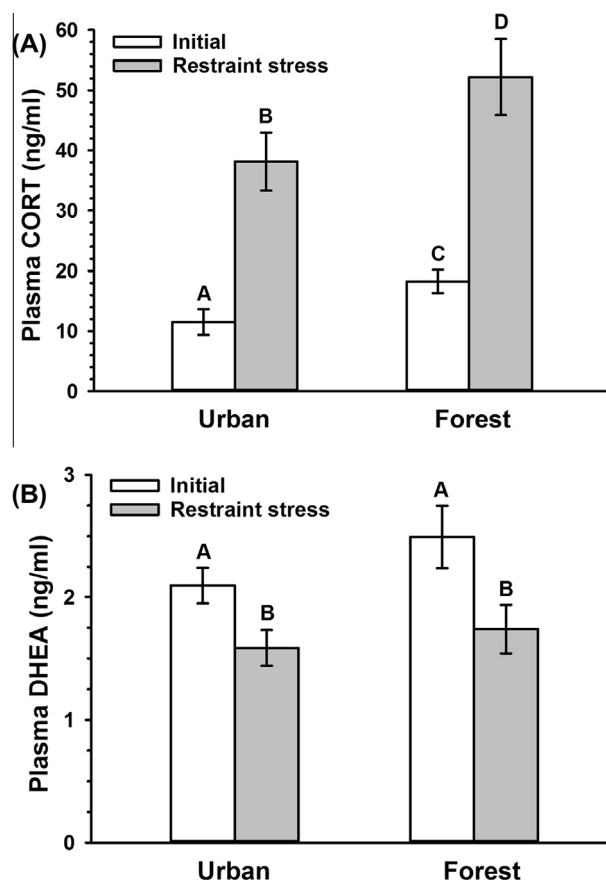


Fig. 1. Acute effects of restraint stress on (A) corticosterone (CORT) and (B) dehydroepiandrosterone (DHEA) concentrations between northern cardinals captured in both an urban location (in and around Winter Park, Florida, $N = 11$) and a natural forest habitat (Hal Scott Preserve, Florida, $N = 12$). All data are expressed as means \pm standard errors and data points with identical letters do not differ at $P < 0.05$.

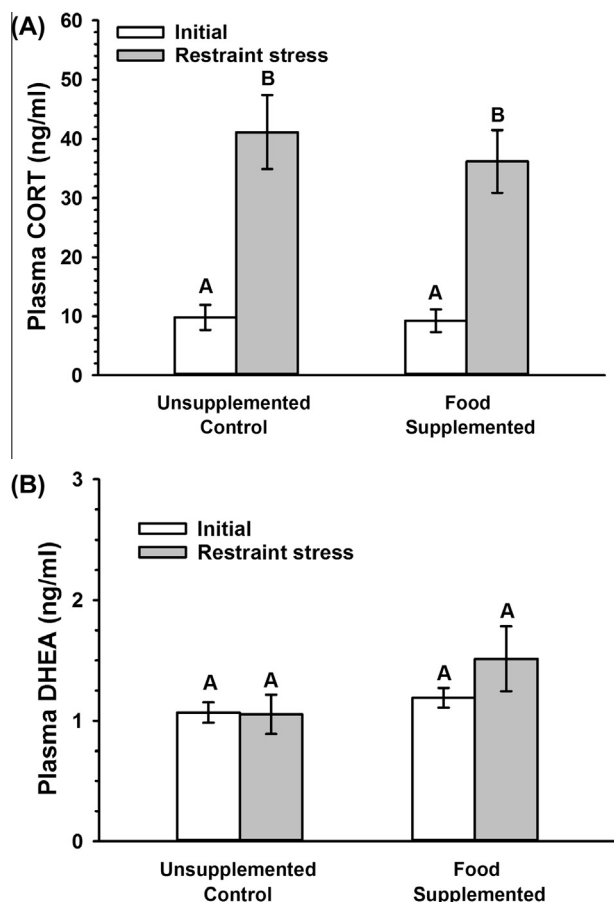


Fig. 2. Acute effects of restraint stress on (A) corticosterone (CORT) and (B) dehydroepiandrosterone (DHEA) concentrations between food supplemented ($N = 14$) and unsupplemented ($N = 13$) northern cardinals. All data are expressed as means \pm standard errors and data points with identical letters do not differ at $P < 0.05$.

during restraint stress, no differences between controls and supplemented birds in initial or restraint stress CORT concentrations were observed (all $P \geq 0.314$). In contrast, no change in DHEA concentrations were observed in response to restraint stress ($P = 0.106$), and this was not influenced by food supplementation ($F_{2, 23} = 1.72$, $P = 0.211$, Fig. 2B). No other variables differed between food supplemented and control birds (all $P \geq 0.106$), nor any relationships between these variables and CORT or DHEA levels were observed (all $P \geq 0.163$).

3.3. Simulated territorial intrusions

Exposure to varying lengths of STI did not affect the CORT response ($F_{2, 30} = 0.11$, $P = 0.739$, Fig. 3A), but did influence changes in DHEA concentrations with restraint stress ($F_{2, 30} = 0.85$, $P = 0.372$, Fig. 3B). Birds exposed to 60 min of STI had higher initial CORT concentrations than birds exposed to less than 5 min of STI ($P = 0.042$). Restraint stress resulted in a significant decrease in DHEA from initial levels in birds exposed to 5 min of STI. However, the magnitude of this change decreased with increased exposure to STI, with 60 min of STI resulting in no difference between initial and restraint stress. No other variables influenced CORT or DHEA levels in response to varying durations of STI (all $P \geq 0.091$).

3.4. Pair distress

In situations where both sexes of the pair responded to the playback recordings, the order with which the male was cap-

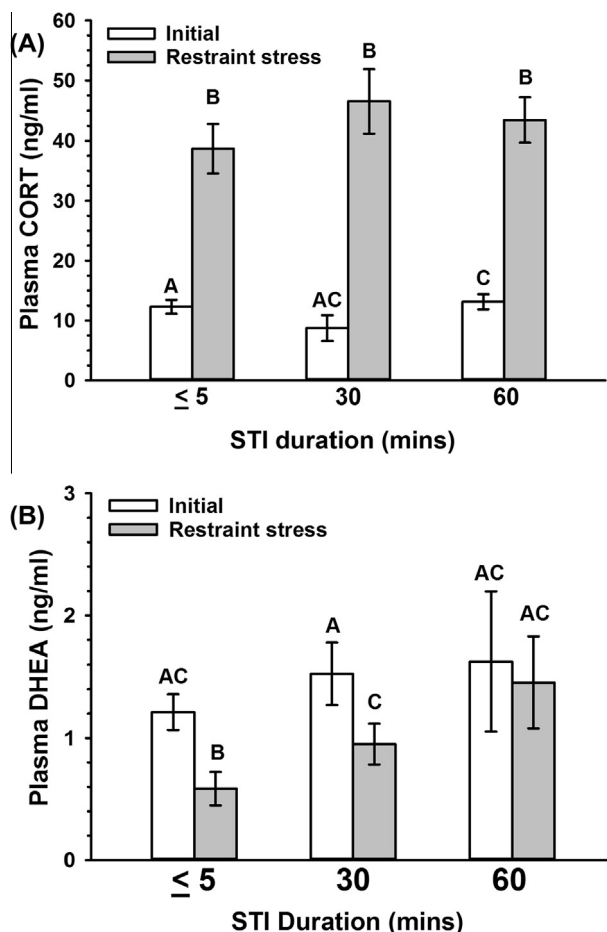


Fig. 3. Differences in initial and restraint stress concentrations in northern cardinal plasma of (A) corticosterone (CORT), and (B) dehydroepiandrosterone (DHEA), in response to simulated territorial intrusions lasting one of three durations: 1) < 5 min ($N = 12$), 2) 30 min ($N = 11$), or 3) 60 min ($N = 8$). All data are expressed as means \pm standard errors and data points with identical letters do not differ at $P < 0.05$.

tured, relative to the female, had a significant effect on the restraint stress changes in CORT ($F_{2, 23} = 5.170$, $P \leq 0.001$, Fig. 4A) and DHEA ($F_{2, 23} = 7.003$, $P \leq 0.001$, Fig. 4B). Male cardinals exposed to pair distress (i.e., were captured after their female was captured) had higher initial and restraint stress CORT concentrations ($P = 0.013$ and $P = 0.033$, respectively), compared to the males that were either captured first (initial: $P = 0.037$, restraint stress $P = 0.042$) or captured at the same time as their female resident (initial: $P = 0.029$, restraint stress $P = 0.037$). Similarly male birds that were captured after their female, had higher initial DHEA concentrations, that also did not decline with restraint stress (initial: $P = 0.009$ and restraint stress: $P \leq 0.001$). This is unlike males that did not experience distress compared to the males that were either captured first (initial: $P = 0.033$, restraint stress $P = 0.007$) or captured at the same time as their female resident (initial: $P = 0.047$, restraint stress $P \leq 0.001$). No other variables influenced CORT or DHEA levels in response to order of capture (all $P \geq 0.350$).

3.5. Relationship between CORT and DHEA concentrations

Compiling all the data collected on jugular steroid concentrations, a Pearson's correlation revealed a significant negative correlation between CORT and DHEA when considering both initial and restraint stress levels ($r = -0.281$, $N = 236$, $P \leq 0.001$, Fig. 5). How-

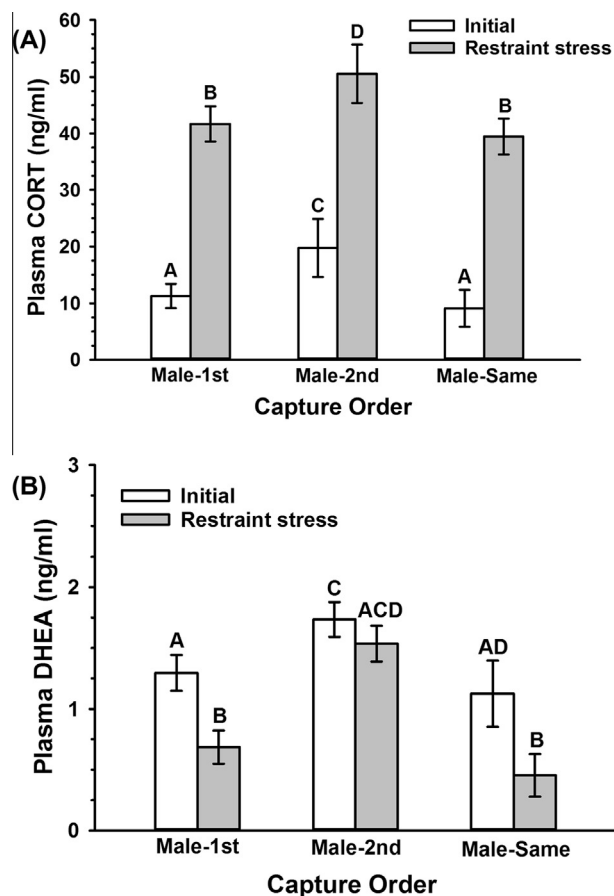


Fig. 4. Differences in initial and restraint stress concentrations in northern cardinal plasma of (A) corticosterone (CORT), and (B) dehydroepiandrosterone (DHEA), in response to the capture order of the male in the pair. Three groups are categorized as follows: males captured before the female (Male-1st: $N = 78$), males captured after at least 3 min prior to the female (Male-2nd: $N = 22$), and males captured within 10 s of the female's capture (Male-Same: $N = 16$). All data are expressed as means \pm standard errors and data points with identical letters do not differ at $P < 0.05$.

ever when considering initial and restraint stress levels separately, no significant correlations were detected (initial: $r = 0.081$, $N = 118$, $P = 0.386$, restraint stress $r = 0.0301$, $N = 118$, $P = 0.746$, Fig. 5).

4. Discussion

We investigated how environmental instability altered circulating levels of two adrenal steroids, CORT and DHEA in male Northern cardinals males, before and after restraint stress. Instability induced by urbanization had little effect on DHEA, but urban cardinals had lower initial and restraint CORT concentrations compared to forest birds. Decreasing environmental instability through food supplementation did not affect steroid concentrations. In investigating instability induced by social interactions, males exposed to longer durations of a STI (up to an hour) showed little change in CORT. However, decreases in DHEA with restraint stress, were negated after one hour of exposure to STI. In another test of social instability, males exposed to pair distress from the capture of their mate had higher initial and restraint stress CORT levels, and higher initial DHEA than birds that were not exposed to pair distress. Taken together, these data demonstrate that CORT and DHEA concentrations in cardinals respond separately to environmental instability, and both are particularly impacted by instability due to changing social conditions.

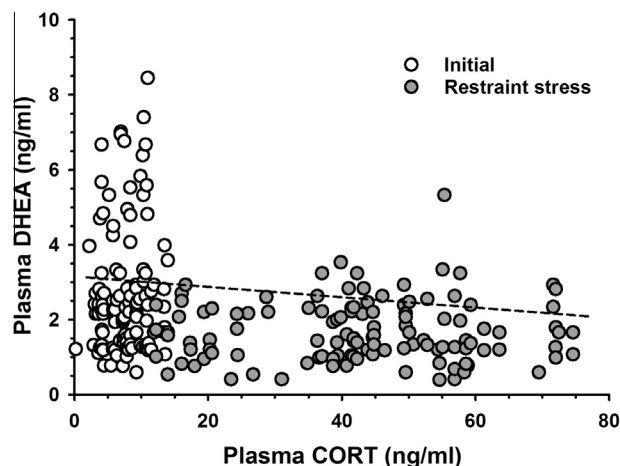


Fig. 5. Pearson's correlations between jugular plasma concentrations of corticosterone (CORT), and dehydroepiandrosterone (DHEA) both at initial and after 30 min of restraint stress for northern cardinals ($N = 118$ birds or 236 plasma samples).

4.1. Instability in the physical environment

Northern cardinals are arguably the most common songbirds in both natural and anthropogenic habitats in central Florida, and their numbers have steadily increased over the past few decades (Breeding Bird Survey 1966–2012, United States Geological Survey data). This suggests cardinals are either unaffected by or they gain benefits from the urban development in the region. In support of this hypothesis, both body condition and muscle score was higher for urban birds in our study, however this contradicts a previous study of cardinals in central Ohio, where both condition and male plumage coloration was lowered in city birds (Jones et al., 2010). Previous research has demonstrated that animals living in urban areas often modulate their stress response, presumably to cope with novel city stressors (Davies et al., 2013; Fokidis et al., 2009; Foltz et al., 2015; Grunst et al., 2014; Heiss et al., 2009; Lucas and French, 2012; Partecke et al., 2006; Payne et al., 2012; Schoech et al., 2007). Here, urban cardinals had lower initial CORT levels and reduced stress CORT levels than forest counterpart. One interpretation is that urban areas are “less stressful” compared to forested habitats, although note that the magnitude of the stress response was comparable between habitats. This may be explained by a general lower presence of predators and parasites as well as richer food resources in cities which would benefit some species (Fokidis et al., 2008; Fokidis, 2011; Foltz et al., 2015; Ripple et al., 2013). In contrast, plasma DHEA concentrations did not differ between urban and forest cardinals. Thus CORT and DHEA secretion is uncoupled, suggesting they may be regulated by different secretogues. Supporting this hypothesis, injections of ACTH increased CORT, but not DHEA in cardinals (Fokidis, submitted for publication). Future studies should aim to test CORT and DHEA responses to various exogenous secretogues to characterize the regulatory pathways that control DHEA secretion.

Research on CORT secretion in urban birds has generally demonstrated both species and city-specific results. In Phoenix, Arizona, urban birds generally maintained larger, more consistent CORT responses throughout the year, in contrast to desert counterparts that suppressed CORT secretion during the breeding season (Fokidis et al., 2009). Common blackbirds (*Turdus merula*) inhabiting German cities had suppressed baseline and stress-induced CORT levels, compared to forest populations and these differences were maintained in captivity (Partecke et al., 2006). This suggests a genetic or early developmental basis for difference between these populations (Partecke et al., 2006). These findings contrast those

from studies in the curve-billed thrasher (*Toxostoma curvirostre*) which showed food availability negated differences in CORT between desert and urban populations (Fokidis et al., 2011a). Similarly a study in Florida scrub jays (*Aphelocoma coerulescens*), a species that occupies similar natural habitats to the cardinal, showed lowered baseline CORT levels and reduced stress responses in suburban birds, compared with those in native habitats (Schoech et al., 2007). This was attributed to food provisioning in the form of bird feeders, that ultimately lowered instability in suburban areas, which decreased CORT concentrations (Schoech, 2009; Schoech et al., 2008, 2007). This pattern in scrub jays is reminiscent of differences observed here in cardinals, however here food supplementation did not alter CORT or DHEA. Thus cardinals are either not food-limited, or adrenal steroid secretion is not effected by food availability. However, the potential for effects of limited sample sizes cannot be completely excluded. One possible explanation is that our study was conducted in an urban environment, and thus birds may not have been food limited (for example, bird feeders may have been present near control birds, but out of our sight). Therefore, food supplementation would have had no effect on CORT or DHEA concentrations. As further support for this hypothesis, no differences in body condition, mass, and fat and muscle scores were observed between food supplemented birds and controls. Future studies should aim to replicate this study in native forest habitats, to determine if energetic state influences CORT and DHEA secretion in free-living birds as it does in captive studies.

4.2. Instability in the social environment

Another source of environmental instability are social interactions between conspecifics which can alter both GC and androgen secretory patterns, in both short-term and chronic time scales (Deviche et al., 2014; Scotti et al., 2008; Wingfield, 2012). Playback recordings meant to simulate a conspecific intruder on a bird's territory have been previously used to induce a "challenge" that often corresponds to a physiological response in the resident bird, including increased levels of T and CORT for some species (Deviche et al., 2014; Hau and Beebe, 2011). Exposing birds to STI did not change CORT concentrations between five minutes to an hour, and other studies have also failed to show CORT or T responses to STI (Fokidis et al., 2011b), although in some cases CORT increases with prolonged exposure (Deviche et al., 2012). In the cardinal, initial CORT concentrations appears not to influence T levels, even with exogenous stimulation of gonadal androgen secretion (DeVries et al., 2011). In contrast, increased STI exposure did not alter initial DHEA levels, however DHEA levels after restraint stress increased with STI exposure. This data is consistent with observations in both the song sparrow and the tropical spotted antbird (*Hylophylax naevioides*), which like the cardinal are year-round territorial species (Hau et al., 2004; Heimovics et al., 2013; Wikelski et al., 1999). Interestingly, although DHEA decreased with restraint stress in this study, after an hour of STI exposure, there was no DHEA response to restraint stress. One interpretation is an overall increase in DHEA synthesis and secretion allows sustained levels of DHEA in circulation. Currently no research has investigated how duration of STI exposure affects steroidogenic enzymes in the adrenals. However, exposure to STI does increase activity of the enzyme 3β -hydroxysteroid dehydrogenase (3β -HSD) which converts DHEA to androstenedione using the cofactor NAD^+ within the brain (Schlinger et al., 2008). Circulating steroid precursors, including DHEA, can cross the blood-brain barrier to be subsequently converted to androgens and estrogens that promote the aggressive response of a resident bird to an intruder, particularly when testosterone is low during the nonbreeding season (Soma et al., 2014a,b). This hypothesis is in line with the data from this experiment showing an increase in DHEA with STI

exposure, if levels reflect increased adrenal or neural DHEA secretion that offsets what might be depleted by the brain. Increased DHEA availability may help to explain the year-round territoriality in the cardinal. However, increased depletion of adrenal DHEA by the brain to produce active sex steroids, would result in a decrease in circulating DHEA levels. Further study comparing both brachial and jugular responses to duration of STI is warranted to aid interpretation.

Studies examining non-breeding androgen levels in cardinals have shown that T levels in both sexes vary little throughout the year and initial T at levels during the non-breeding period are only slightly lower than during breeding (DeVries et al., 2015, 2011; Jawor, 2007; Jawor et al., 2014). Further, administering 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). As our 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. More research is clearly needed to address the regulation of non-breeding territorial behavior in the cardinal.

Beyond territorial intrusion, this study also investigated how social instability associated with distress the pair bond impacted male CORT and DHEA secretion. Clearly, when the female of a pair was captured first, the male now responding to the STI alone, showed an increase in initial CORT and DHEA as well as their subsequent responses to restraint stress. Very little research has addressed hormonal changes during situations of pair distress, however studies of "monogamous" birds and mammals has shown that switching mates, pair bond dissolution, or mate separation can elevate CORT levels (LaPlante et al., 2014; Ouyang et al., 2014; Schweitzer et al., 2014). In contrast, androgens such as T are thought to suppress pair bonding behaviors (Adkins-Regan, 2008; Carter and Keverne, 2010; Prior and Soma, 2015; Prior et al., 2016), however here DHEA increased in males that were captured after their mate. No published studies have examined the relationship between DHEA and pair bonding, however a recent study in zebra finches (*Taeniopygia guttata*) did not show a relationship between DHEA concentrations and the percent of time a pair spent affiliating in both sexes (Prior et al., 2016). Increasing DHEA during pair distress may serve several functions including promoting aggression to allow the male to defend a female, to induce male-reproductive behavior in preparation for a possible re-pairing opportunity, or to protect the organism from excessive GC exposure. Although females were captured in our study, we did not include them here, as their samples were used for a different project, thus limiting our ability to make sex-biased claims about behavior. However, future studies should seek to compare and characterize male and female responses across a range of social contexts and attempt to manipulate DHEA concentrations to determine direct effects on specific pair bonding behaviors.

5. Conclusion

This study concludes that DHEA and CORT, although both originating primarily from adrenocortical tissue, exhibit distinct patterns of secretion that is contextual with respect to instability in the physical and social environment. During restraint stress, plasma CORT increases as DHEA typically decreases. This suggests the potential for differential regulation of these steroids, and their opposing patterns contradict the notion of DHEA as an "anti-stress" hormone. However this general pattern is also influenced by urbanization, food availability, and during social instability associated with distress in the pair bond or territorial challenge. Many of the observations from this study have been previously

reported for CORT, but not for DHEA. With a limited availability of traditional rodent models for research into DHEA actions, avian species provide significant opportunities to examine how environmental change impacts DHEA secretory patterns. Continued research focused on elucidating patterns between and within species, may provide fundamental groundwork for understanding the evolutionary pressures that shape the secretory patterns of adrenal steroids.

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