The effect of fasting on Local Steroidogenesis in the Brown Anole, *Anolis sagrei*

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Department of Biology

Abstract

Everyday animals must confront and respond to stressors in their environment. The activation of the stress response (i.e. the "fight or flight") enables the individual to survive stressors through hormone cascades that dictate physiological changes such as energy allocation. In our model system, the brown anole lizard (*Anolis sagrei*), this cascade, which initiates in the brain, ultimately involves the release of glucocorticoids (GCs) such as corticosterone (CORT; a steroid hormone) from the adrenal glands. Along with GCs, the adrenal cortex also secretes the androgen steroid, dehydroepiandrosterone (DHEA), which has been shown to possess positive anti-stress effects. This is traditionally known as the systemic response, but recent research has focused on local steroid production by organs other than that of the adrenal. While research in local steroidogenesis has been conducted, there is no clear reason why steroids are being produced locally. In order to determine if stress could induce local production, anoles were either fasted or maintained on a normal diet to determine how the lack of food (a stressor) affects local steroidogenesis. CORT levels were only significantly higher in the intestine samples of fasted anoles compared to controls. Further, hormone levels were higher in multiple organs when compared to plasma, providing evidence for local steroidogenesis.

Introduction

Fig 1. A male brown anole, Anolis sagrei, in an aggressive display.

The saying "stress is a part of life" is true, and animals must routinely deal with stressful changes in their environment. In a physiological sense, stress is clearly defined as the presence of a potential challenge, or threat, to an animal's internal stability that evokes a physiological "stress response". In vertebrates, this stress response involves the endocrine system, and is an adaptation that allows animals to cope with environmental changes by enabling physiological and behavioral mechanisms that ultimately promote survival (Charmandari et al. 2005). During stress, heart and breathing rates increase, there is an elevated awareness and alertness, and a sudden release of energy from stored reserves (Sapolsky et al. 2000). There are two distinct phases in this stress response. The first phase involves the secretion of the catecholamine neurotransmitters, adrenaline (or epinephrine) and noradrenaline (or norepinephrine) from the adrenal medulla. These act quickly to raise heart and respiratory rates, and promote vasodilation to increase the blood flow to working muscles and other tissues that require nutrients and oxygen supply (Sapolsky et al. 2000). Within a

couple of minutes, the second phase occurs involving the secretion of steroid hormones, namely glucocorticoids (GCs) into the blood from the adrenal cortex (Charmandari et al. 2005). Corticosterone (CORT) is the primary GC in reptiles, birds and small mammals, but is functionally comparable to cortisol, which predominates in fish and most mammals. This endocrine cascade is known as the hypothalamic-pituitary-adrenal (HPA) axis (Fig. 2; Sapolsky et al. 2000, Hill et al. 2012). The HPA axis initiates with neuroendocrine cells in the paraventricular nucleus (PVN) of the hypothalamus, secreting a corticotrophin-releasing hormone (CRH), which in turn stimulates the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland, into the bloodstream. Then, ACTH will act on its respective receptors to stimulate the synthesis and secretion of GCs from cells in the adrenal cortex. These GCs can then act globally on glucocorticoid receptors (GR) or mineralocorticoidreceptors (MR) depending on the tissue being examined (Luca et al. 2013), including specific areas of the brain (e.g., the hippocampus, prefrontal cortex, and amygdala)

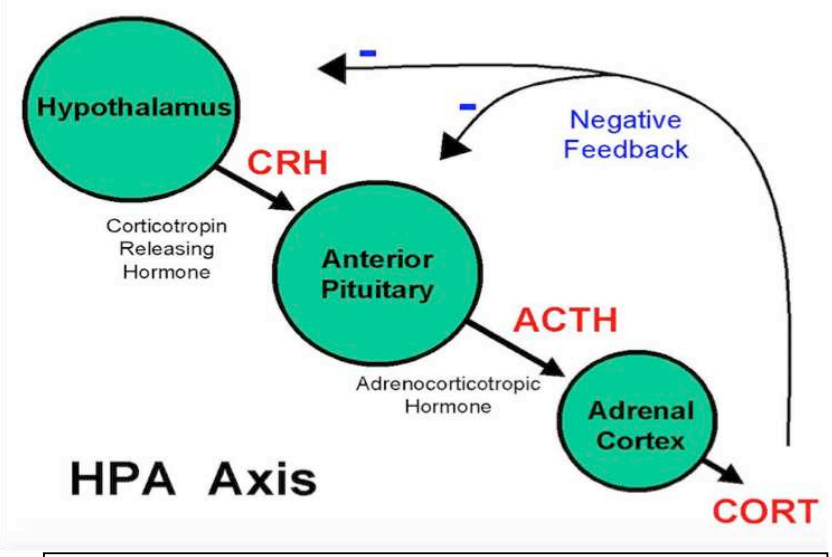

Fig 2. The hormone cascade of the hypothalamic-pituitary adrenal (HPA) axis, otherwise known as the stress axis.

which ultimately results in a widespread and coordinated physiological response that makes survival possible during stress (Liu, Yuen 2010). While the "acute stress" response is adaptive for short-term survival, society has focused on the negative consequences of stress that result from persistent and prolonged exposure to GCs (otherwise known as "chronic stress"). Chronic stress produces many negative effects on health including suppressing the immune system, interfering with reproductive physiology, affecting cardiovascular health and cognitive impairment (reviewed in McEwen 2008).

Whether an organism is mammalian, avian, or reptilian, their physiological response to stress is universal. For this reason, a variety of model organisms may be used to study this response. Although traditionally known as a systemic response with circulating glucocortocoids produced from the adrenal gland, research has began to focus on local hormone production by organs other than the adrenal. While evidence has been found for local steroiodogenesis in many model organisms, this data has not been found in reptiles. Further, the driving force behind local steroidogenesis has not been determined. For this reason, anole lizards were fasted to evaluate how a stressor such as fasting alters local production in reptiles.

The adrenal gland

The adrenal gland is the primary endocrine gland associated with stress and is composed of two main sections, an inner medulla, and outer cortex. The adrenal medulla secretes epinephrine

(adrenaline), while the cortex secretes steroid hormones such as GCs (Rosol et al. 2001). The adrenal cortex is composed of three distinct layers; 1) the outer zone, called the *zona glomerulosa*, produces aldosterone which functions in fluid and blood pressure control, 2) The central, *zona fasciculata*, is the thickest section, composing about 70% of the adrenal cortex and functions in GC production, and 3) in some species, the innermost *zona reticularis* produces some GCs and androgens (Rosol et al. 2001). It is interesting to note that despite the adrenals' small size, the gland is one of the most vascularized organs, highlighting its overall importance in the endocrine system (Vrezas 2012). Although important, the adrenal does not act alone and requires two negative feedback loops to function properly.

Adrenal Regulation

Adrenal steroidogenesis is regulated by two main endocrine feedback loops, the hypothalamic-pituitary-adrenal axis (HPA axis), and the renin-angiotensin-aldosterone system (RAAS). RAAS regulates both blood pressure and fluid homeostasis through a cascade pathway. The first substrate, angiotensinogen is cleaved by an aspartyl protease, renin, forming a decapeptide, angiotensin I. Angiotensin I is further cleaved by an angiotensin-converting enzyme (ACE) to produce the octapeptide, Angiotensin-II (Ang-II). Ang II then causes vasoconstriction by activating ang-II type 1 receptors within blood vessels and increasing arginine vasopressin release from the posterior pituitary gland (Lavoie 2003).

The HPA axis interacts with the renin-angiotensin system by the activity of Ang-II. More specifically, Ang-II stimulated mRNA expression of steroid-producing enzymes such as steroidogenic acute regulatory protein, 3β-hydroxysteroid dehydrogenase, CYP11B1, and CYP11B2 (Oki 2013). Further, glucocorticoids induce ACE production in rats, both in cultured cells and *in vivo* lung tissue (Mendelsohn 1987). This direct relationship illustrates the importance of both systems and how they interact. Localized activity of ACE and GCs in the lungs and other tissues also point towards local effects of these hormones (Mendelsohn 1987).

The HPA-Axis

The hypothalamic–pituitary–adrenal axis (HPA axis) is anatomically composed of the paraventricular nucleus of the hypothalamus (PVN), the anterior pituitary gland, and the adrenal gland (Smith 2006). When responding to a stressor, dedicated neurons localized in the PVN synthesize and secrete corticotropin-releasing hormone (CRH). CRH then binds to its receptor in the anterior pituitary, stimulating the synthesis and release of adrenocorticotropic hormone (ACTH) into general circulation which then acts to stimulate the adrenal cortex to release GCs throughout systemic circulation (Wang 2004).

The HPA axis is tightly regulated by negative feedback loops from GCs in circulation (Fig 2). As GCs bind to their glucocorticoid receptor (GR), they alter transcription levels of HPA components (Smith 2006). The rise in GC levels decreases mRNA levels of an ACTH precursor

molecule, and therefore the amount of ACTH produced by the pituitary (Keller-Wood and Dallman 1984). As GC concentration increases following stress exposure, the HPA activity is inhibited. Further, the presence of GCs in an organism alter the expression of GC receptors, or GRs. A negative feedback loop between circulating GCs and their receptors is present in order to prevent tissue damage from high GC levels (Bamberger et. al. 1996).

The above description of HPA axis stimulation and inhibition are the current models for systemic hormone production. Yet, organs may in fact not depend solely on circulating hormones produced by the adrenals during stressful situations, and instead rely on local synthesis.

Extra-adrenal steroid production in response to stress

Originally, GC synthesis was only thought to occur in the adrenal glands (Kostadinova et al. 2012), however more recent research suggests GC synthesis occurs in other tissues, including the intestine, brain, skin, and heart (reviewed in Taves et al. 2011). This localized (extra-adrenal) synthesis of GCs may occur independent of adrenal hormone production, and thus is not directly related to circulating GC levels. These tissues contain the necessary steroidogenic enzymes to produce these hormones as detected by gene expression (i.e., mRNA) that suggests production of steroidogenic enzymes. (Taves et al. 2011). Local (tissue) GC production has been documented in both mammals and birds, and recently these observations raise the possibility of "localized

HPA axis in each tissue", which regulates this GC production, independent of adrenal regulation (Taves et al. 2011).

Along with GCs, the adrenal cortex also secretes the androgen steroid, dehydroepiandrosterone or DHEA (Van Voorhees et al. 2014). As with GCs, other tissues can also synthesize DHEA, although this production is much less studied. In humans, DHEA is most present in circulation in its sulfated form (DHEAS), though the relationship between DHEA and DHEAS can vary across vertebrate species (Maninger et al. 2010). In the context of chronic stress, DHEA is thought to exhibit "anti-stress" properties, specifically by protecting tissues from excessive GC exposure (Kalimi et al 1994). Unlike other hormones, no specific DHEA receptor has been identified, and thus DHEA may exert its effects by being converted to more bioactive steroids, such as testosterone or estrogen (Labrie et al. 2001). As a steroid precursor lacking a receptor, the presence of DHEA is tested by expression of the steroidogenic enzymes necessary to produce androgens. If the enzymes are present, then DHEA is being used in that tissue to form testosterone and/or estrogen.

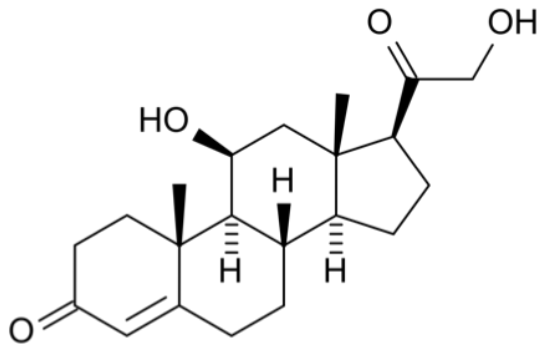

Fig 3. Chemical structure of corticosterone.

The dichotomy of having the same tissues produce both a "stress" and an "anti-stress" hormone is intriguing, and yet has received little research attention. Despite the growing

research on extra-adrenal steroid production, little is known about the factors regulating the ability to synthesize steroids locally in varying organs.

Corticosterone

In lizards, such as anoles, corticosterone (CORT) is the primary GC, comparable to the commonly known human hormone, cortisol. The structure contains three six membered rings and one 5 membered ring fused to form one planar molecule (Fig. 3). CORT is a glucocorticoid that induces glycogenesis, protein catabolism and suppression of digestion and the immune system (Yang, Wilczynski 2003). These physiological changes occur in order to mobilize energy to where it is needed and to suppress unnecessary functions. CORT also affects reproduction by suppressing androgens such as testosterone.

Role of CORT in tissues during stress

Many studies have focused on plasma CORT levels as an overall indicator for stress, with minimal regard to local organ production of this hormone. However, CORT is released by the mammalian cardiovascular system under stressful conditions such as myocardial infarction with the increased gene expression (mRNA levels) of CYP11B2, the aldosterone- producing gene. (Davies et al. 2003). Through RNase protection and GR steroid binding assays, Sheppard (2002) determined a high level of CORT present in cardiomyocytes. Thus, not only are glucocorticoids circulating throughout an organism, but they are being localized to organs such as the heart in

times of stress. This finding was further confirmed through the presence of mRNA for steroidogenic enzymes in heart muscle (Sheppard 2002). mRNA expression and the presence of both glucocorticoid and mineralocorticoid receptors in heart cells (cardiomyocytes) suggest an important role of GCs in the heart. Due to elevated GC levels in heart failure patients, it is hypothesized that changes in GC concentrations may cause the different responses during heart trauma. According to Young (2001), GCs were only detected in patients with myocardial infarction and heart failure and CYP11B2 was expressed only in failing hearts. Though other factors such as drug exposure or a stressful cellular environment may be involved, it does suggest GCs may also play an important protective role.

The role of extra-adrenal CORT

Although a relatively new area of study, local steroid production has been detected in a number of organs from human and mouse models. Evidence for extra-adrenal CORT production is present, but the physiological significance is unclear and it depends on the relative local concentration, target cell proximity, and organ-specific hormone regulation (Davies et al. 2003). Depending on the organism, CORT levels vary between organs, making it difficult to compare among species. If local CORT concentrations are low, their presence may play an important role in the specific organ from which it is being produced. Interestingly, local steroidogenesis is

independent of adrenal GC synthesis, making their comparison difficult. Among the uncertainty, one theme in local GC synthesis is the location of synthesis within immune-involved organs.

Thymus

One such organ, the thymus, is an immune gland responsible for T-lymphocyte development, and was the first organ in which extra-adrenal steroid production was detected (Vacchio et al 1994). It is interesting to note, the mouse thymus secreted the highest GC levels during fetal development and following birth when the adrenal gland is not fully active. Therefore, the thymus had to locally produce GCs, and not rely on the adrenal gland (Ashwell 2000). During this time of lymphocyte development, GCs determined the fate of thymocytes before they developed into mature T-cells. When compared to the adrenal, thymus CORT levels were high but remained lower than adrenal levels (Vacchio et. al. 1994), suggesting a lower concentration locally.

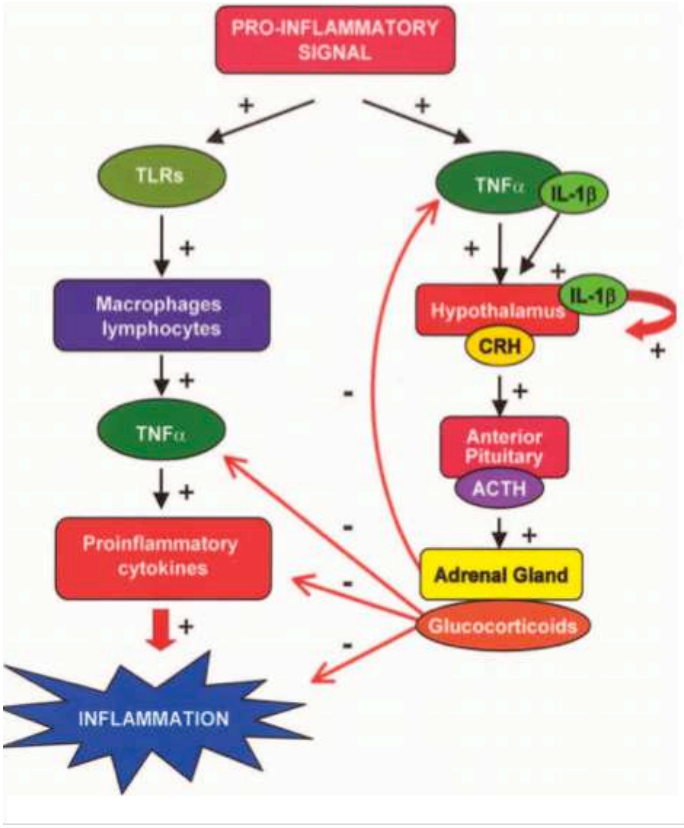

Fig 4. HPA negative feedback loop on the proinflammatory response. Adopted from

Brain

When produced locally, CORT acts as a neurosteroid in the brain by affecting neuron formation, development, myelination, and neuronal reactions to stress (Kostadinova et al 2012). The enzyme, P450 (encoded by the CYP11B1 gene) that catalyzes the final step in CORT synthesis is expressed in rat hippocampus as both mRNA and protein (Higo et al. 2011). Following adrenalectomy, significant CORT levels were still detected in the brain suggesting extra-adrenal CORT production is occurring. This could also suggest CORT is not being metabolized and instead persists. According to Higo et al. (2011), low CORT levels contribute to synaptic plasticity and neuroprotection in hippocampal neurons. Hippocampal synaptic plasticity enhances with low CORT levels by changing how the necessary glutamate receptors function (Tse et al. 2011). CORT alters the functional properties of glutamate receptors by changing the motility and function of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid subtypes of glutamate receptor (AMPAR) that are responsible for the expression of synaptic plasticity. Another receptor, N-methyl-D-aspartate receptors (NMDARs), involved in inducing synaptic plasticity, are also altered (Tse et al. 2011). Although chronic levels of high CORT are associated with negative health effects, low CORT levels may be beneficial locally.

Skin

The skin serves as a physical and chemical barrier from the environment and protects organisms from chemicals, infections, and pathogens. Following an injury to human skin, keratin-producing epidermal cells (keratinocytes), induce the release of interleukin-1 (IL1) to activate keratinocytes in order to begin the proinflammatory phase of wound healing (Vukelic et al 2011). Keratinocytes expressed CYP11B1, the enzyme that catalyzes the final conversion of deoxycorticosterone to CORT (Vukelic et al 2011). The interplay of increased CYP11B1, CORT, and GC receptor expression found in wounds may function to both prevent excessive inflammation from leading to tissue damage and initiate the proinflammatory response and (Vukelic et al. 2011). This initial response involves many steps; first toll-like receptors (TLRs) recognize pathogen-associated molecular patterns (PAMPs) that are specific to each pathogen (Hermoso, Cidlowski 2003). TLR activation then activates the innate immunity, activating B cells. Interestingly enough, this initial response is tightly regulated by the HPA axis. Proinflammatory cytokines activate the HPA, causing GC synthesis, release, and the formation of a negative feedback loop on proinflammatory molecules (Fig. 4). More specifically, GCs inhibit cytokine gene expression and of course their activity on target cells. Similar to other extra-adrenal organs, the skin also produces low CORT levels

Lungs

Similar to the skin, lungs are constantly exposed to foreign substances such as antigens, microbes, and pathogens. For this reason, they contain a high concentration of immune cells (Kostadinova et al 2012). Mouse lung tissue expressed steroidogenic enzymes necessary for CORT production (Hosteller et al. 2012). At first glance, the ability for lungs to synthesize an immunosuppressant, CORT, seems counterintuitive. Yet, excessive immune responses can lead to tissue damage, therefore, the lung must sense inflammatory responses and induce local CORT synthesis to prevent extensive lung tissue damage (Hosteller et al. 2012).

Role of extra-adrenal DHEA

Although specific functions have been elusive to discern, DHEA may play a protective role during stress. Maninger et al (2010) found increased circulating DHEAS concentrations in rhesus monkeys (*Macaca mullata*) with repeated exposure to acute stress. Administering DHEA following immobilization stress reversed the weight gain seen in chronically stressed rats (Hu 2000). Furthermore, DHEA supplementation given to Wistar rats reversed the effects of a high fat diet, which induced insulin resistance (Veras 2014). Evidence from these studies illustrate the potential for DHEA to mitigate the harmful effects of stress, however how this varies across organs has not been investigated. Extra-adrenal DHEA activity is not fully understood and it is evident that local organ production of such hormones continues to be a new topic of interest.

DHEA plays a much greater role as a systemic androgen precursor. Recent studies have focused on stress responses mediated by DHEA in organs, especially the heart. Excess GCs, possibly produced by increased HPA activation, have been linked to cardiac dysfunction such as heart hypertrophy, or thickening of the cardiac muscle (Nakamura 2004). Following DHEA treatment, hypertrophic responses reduced in cardiomyocytes, even during GC receptor stimulation.

DHEA has protective effects that are exerted on a number of organs (Pelissier et al. 2004). When the colon, small intestine, and livers of rats were treated with DHEA, it leads to decreased oxidative damage of proteins and lipids. To date, DHEA synthesis has been detected in the liver, gastrointestinal tract, and adrenal (Tashiro 2000). Following surgery, DHEA localization in the intestine was studied by immunohistochemistry to determine that parietal cells actively synthesized DHEA in gastric mucousa (Tashiro 2000).

Fasting as a stressor

Food availability is one of the most important factors impacting the physiology of an organism. The lack of food is clearly perceived as a stressor as it elevates circulating GC levels and imposes drastic effects including; suppressing reproduction and immunity, and mobilizing stored energy reserves resulting in a further effect of fasting. Field research on the red-legged kittiwake (*Rissa brevirostri)* documented that food shortages increased CORT (the predominant GC in birds and reptiles) levels alongside decreases in body mass (Kitaysky et al. 2001). Similar

studies in domestic chickens (*Gallus gallus*) have shown that increased CORT secretion lowers the levels of the sex steroids; testosterone and progesterone, thereby completely suppressing reproduction entirely (Henriksen et al. 2011). These studies have focused on circulating (i.e., systemic) CORT levels likely derived from the adrenal gland, but how food availability may alter local steroid production remains essentially unstudied.

A recent study demonstrated that acute fasting (6 hours) increased aggression and changed circulating steroid levels in male zebra finches, *Taenopygia guttata* (Fokidis et al. 2013). Specifically, the lack of food caused both secretion of CORT and DHEA to increase, as well as a simultaneous decrease in the sex steroids, testosterone and estrogen, in a number of tissues (Fokidis et al. 2013). This provided the first evidence for an effect of fasting on hormone distribution across tissues, but was limited in the number of tissues investigated and though studied in birds, it has not been tested in reptiles.

The brown anole as a model organism in endocrinology

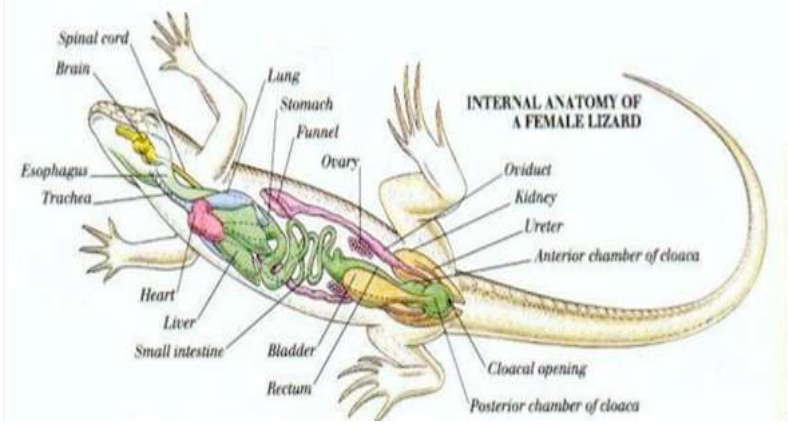

Fig 5. Gross anatomy of a female lizard.

Brown anoles (*Anolis sagrei)* are an abundant, but invasive lizard species in the southeastern region of the U.S, including Florida (Wade and Cohen 2012). Both the brown (*A. sagrei)* and the similar native green (*A. carolinesis*) anoles have been extensively used as models for neuroendocrinology and for linking steroid hormones and behavior. Anoles have multiple behaviors, one of which is exploratory behavior, which is associated with mild stress (Greemberg 2002). These visible behavioral patterns are altered and influenced by stress hormones, making the anole a simple model organism to study.

A substantial amount is understood concerning the anatomy (Fig. 5), behavior, and physiology of the *Anolis*. This study focuses on the abundant brown anole, which is more easily available and larger than the green anoles, making them a more convenient species for research.

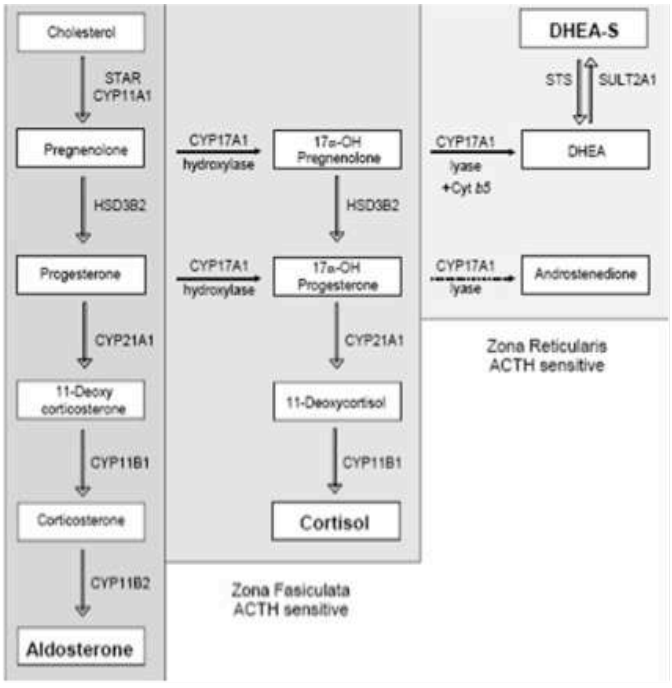

Fig 6. Steroidogenesis pathway of CORT, DHEA from cholesterol. The gene names that express the necessary enzymes are also included above each arrow. Scheme

The synthesis of steroids involves a complex metabolic pathway that involves many steroid precursors and steroidogenic enzymes (Fig. 6). Steroid hormones are a critical regulator of adult anole behavior

(Wade 2012), and in particular, anoles exhibit sexual dimorphism in both anatomy and behavior that is regulated by steroids (Lovern et al. 2004; Husak et al 2009). In several anole species including *A. sagrei*, male anoles responded to a gonadotropin-releasing hormone (GnRH) challenge (the central regulator of reproduction) by decreasing circulating testosterone levels while simultaneously elevating CORT levels in the field (Husak et al. 2009). This suggests that a robust stress response is maintained during reproduction even when artificially stimulated by the predominant regulator of testosterone (i.e., GnRH) (Husak et al. 2009). Similar research demonstrated that injection with arginine vasotocin (AVT) also increased circulating CORT suggesting a maintained robust stress response even in captivity (Dunham and Wilczynski 2014). The influence of food on hormone levels in anoles, has only been investigated in a single study by Lovern et al. (2008). Here, female anoles on an *ad libitum* diet (without restraint) were in better body condition, and had higher plasma testosterone concentrations than a fasting group (Lovern et al. 2008). A study conducted by Tokraz et al. (1998) on Anolis lizards showed that as testosterone spiked during the breeding season, CORT levels decreased. This provides a trend suggesting that the stress response inhibits reproduction in the lizard, as in most species. Despite the substantial endocrine research on anoles, these studies focused on circulating steroid levels and not the ability of the organs to produce the steroids themselves. Again, while the above

studies have set some foundation for local steroidogenesis, there have been no studies to this point on reptiles.

While there are some hypotheses on the local stress response, there are few studies that explain how or why local production occurs. If in fact, chronic fasting could induce a systemic stress response, there should also be evidence of increased local steroid production when compared to a control group. If local production occurs, it is hypothesized that hormone levels within organs should be higher than circulating, or plasma levels.

Materials & Methods

Collection and captivity

Adult male anoles were captured on and around the Rollins College campus using a fishing pole noosing apparatus. Following capture, each anole was weighted (±0.1g) and their snout-vent length (SVL; (±0.1mm) and tail length (±0.1mm) were measured. The lizards were housed in cages (12 x 6.5 x 11.5 inches) on botanical carts with four 40-watt full spectrum light bulbs per shelf. Each cage contained a carpet bottom; a mesh hammock, a PVC perch, and a live plant (*Pothos sp.)*. A warm (27° C) and humid (94% relative humidity) environment was maintained by spraying the cage and plant with water once daily. A photoperiod of 14-hour light to 10-hour dark was maintained. The health of the anoles was evaluated daily and all

experimental procedures were approved by the Institutional Animal Care and Use Committee at Rollins College.

The fasting experiment

Before initiating the study, anoles were allowed to acclimate to captivity for a week. Anoles were then randomly assigned (determined by a random number generator) to one of two treatment groups: 1) a group that was fasted for one week (experimental; N=13) and 2) a control group given two large crickets every 2 days (N=12). The groups were equally weighted to ensure equal mass averages between the groups before the experiment began ($t= -0.498$, $df= 23$, $p=0.623$). To avoid the effects of the time of data collection the experiment was staggered, with only five lizards being studied per day over the course of a week.

Tissue Sample Preparation

Following the experiment (1.5 weeks), anoles were sacrificed by rapid decapitation and a trunk blood sample was rapidly collected in heparinized capillary tubes and stored on ice. Anoles were then dissected and several organs were collected (Table 1). Tissues were immediately snap frozen in dry ice and stored at -80^{o}C until further processing. Prior to tissue preparation, tissues were weighed to the nearest $\pm$ 0.1mg and then homogenized using zirconia beads in a mixture of 84% methanol (by vol.) and placed in an automatic bead ruptor. Samples were left overnight at

4^{o}C and then centrifuged with the resulting supernatant being collected for solid phase extraction (SPE).

Solid phase extraction

A widely used technique, SPE, enables the extraction of nonpolar compounds from a highly polar solution, such as steroids from aqueous tissue samples, using special carbon-bonded silica (C18) chromatographic filters (Newman et al. 2008; Fokidis et al. 2013). The process involves six steps for extracting and purifying steroids from the surrounding sample. Briefly, these steps are: 1) *solvation*; which uses an organic solvent to saturated a filtering material; 2) *equilibration*: using water to provide a gradient that concentrates the steroids; 3) *sample loading*; adding the tissue samples; 4) *interference elution*; using an organic solvent to remove impurities and substances that may interfere with further steroid analysis; 5) *sample elution*; removing the purified steroid sample; 6) *sample drying*; using a vacuum concentrator.

Here, SPE was performed with 5 mL C18 column cartridges (Agilent Technologies, Santa Clara, CA, USA). The columns were first primed with 3 mL of 100% ethanol (solvation) and then 10 mL of distilled water were added (equilibration). The homogenized tissue samples were diluted with 10 mL of water and loaded after priming (sample loading). Following addition of the samples, 10 mL of 40% methanol was loaded and eluted until the columns ran dry at maximum vacuum pressure for five minutes (interference elution) The final purified solutions

were collected with 5 mL of 90% methanol (sample elution) and dried in a speed vacuum concentrator (Thermo Fisher Scientific Inc., Pittsburgh, Pennsylvania, USA) at 40 °C for 4 hours (sample drying). This technique has already been validated for use in Dr. Fokidis' laboratory and it has demonstrated to yield a high recovery (83-96%) of steroid from various tissues. The dried samples were then stored at -80 C until reconstituted for the steroid quantification assays.

Enzyme-linked Immunoassay (ELISA)

Competitive ELISA kits were used to measure both CORT (Arbor Assays Inc., Ann Arbor, Michigan, USA) and DHEA (Eagle Biosciences Inc., Nashua, New Hampshire, USA) from tissue samples. Dried samples were reconstituted with 5 µL of 100% ethanol and 115 µL of the kit's assay buffer with 50 µL each used for both the CORT and DHEA assays. Prior to the CORT assay, plasma samples were prediluted 10-fold to insure they were within the linear range of the standard curve. The respective assay kits protocol was followed and the 96 well microplates were read using a spectrophotometer at 450 nm for both CORT and DHEA. Final concentrations were determined by interpolating from the assay standard curve of known concentrations (0-10,000 pg/mL for CORT and 0-60 pg/mL for DHEA) using GraphPad Prism version 6 (GraphPad Software Inc., La Jolla, California, USA).

Data Analysis

All data were first tested for both normality (Kolmogorov-Smirnov test) and equal variance (Levene's test). If normally distributed then a parametric two-tailed t-test was performed, between control and fasted steroid levels for each tissue, or between plasma and tissue levels. If the data violated assumptions of normality and equal variance then a non-parametric Mann-Whitney Rank Sum Test (U-test) was used to determine significance. Although analysis of variance (ANOVA) was considered, not all organs were collected and steroids successfully quantified for each individual, resulting in missing data that limited the use of this approach. Statistical analyses were performed using Sigma Plot version 13 (Systat Inc., San Jose, California, USA) with α set at 0.05. All graphical data here are presented as means ± standard error.

Results

This study sought to observe how stressors such as fasting could alter steroid hormone levels. In order to determine if fasting could induce a systemic stress response, CORT and DHEA levels were compared between control and fasted groups. To determine the presence of local steroidogeneis, steroid levels within each organ were compared to circulating plasma.

Fasting causes significant weight loss in anoles compared to fed controls

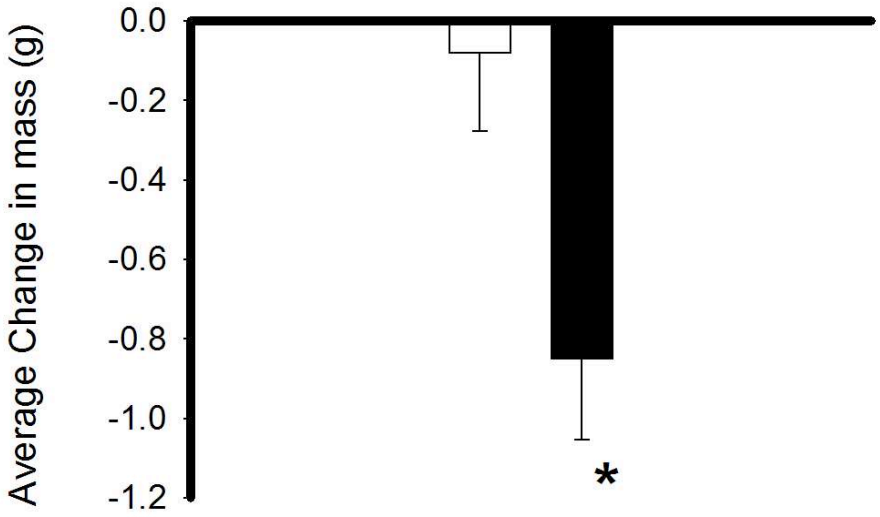

Fig 7. Change in body mass between control (white) and fasted anoles (black).

The control group had an average change in mass of -0.077g while the fasted group had an average change of -0.85 g, which was statistically significant ($t=3.02$, $df= 23$, $P=0.006$; Fig. 7). Thus, the fasting protocol was sufficient to induce a physiological stress in the treatment group.

Table 1. Abbreviations for organs collected from brown anoles.

Abbreviation	Organ	Control (N)	Fasted (N)
PL	Plasma	12	13
ADR	Adrenal	9	7
BLD	Blood	6	7
BRN	Brain	12	13
HRT	Heart	12	13
LNG	Lung	11	13
LV	Liver	11	13
STO	Stomach	12	13
INT	Intestine	11	13
PAN	Pancreas	5	5
KID	Kidney	12	13
TES	Testes	12	13
HEP	Hemipenes	10	9
MUS	Muscle	12	13

Fasting did not impact tissue CORT or DHEA concentrations compared to controls.

While the fasting experiment worked to induce a significant mass change, there was little to no difference between CORT (Fig. 8) and DHEA (Fig. 9) levels among control or fasted groups. Only in the intestine did fasting increase CORT levels above controls (U=36, n=13, P=0.043). Conversely, fasted anoles had lower plasma DHEA levels than controls (t=2.1, df=23, P=0.047). No other differences were observed in response to the fasting treatment (all P > 0.043).

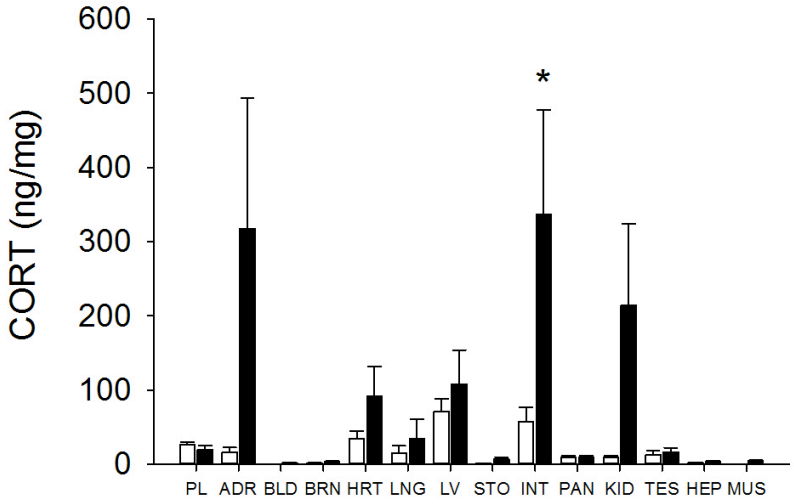

Fig 8. Average CORT concentrations between control (white) and fasted (black) anoles. An asterisk denotes a statistical significance between the groups and the abbreviations are as noted in table 1.

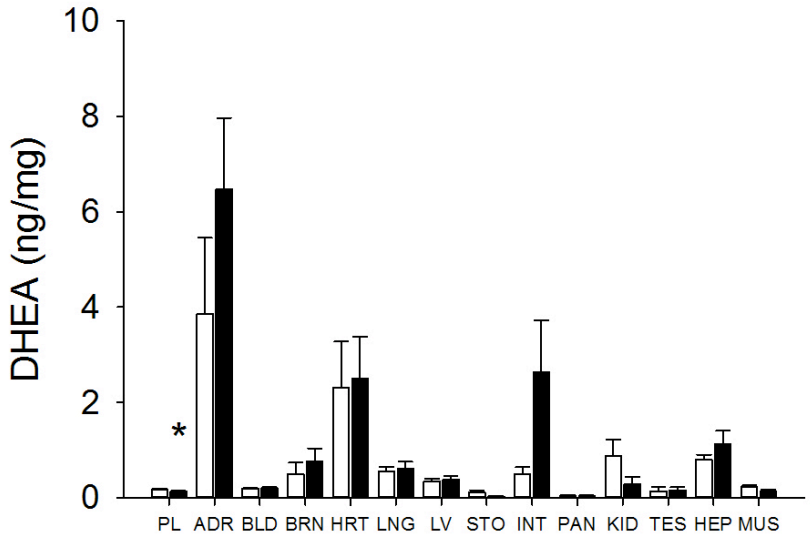

Fig 9. Average DHEA concentrations between control (white) and fasted (black) anoles. An asterisk denotes a statistical significance between the groups

Evidence for hepatic, intestinal and renal synthesis of CORT in anoles

Comparing organ steroid levels to those in circulating plasma could provide evidence of local steroid synthesis (i.e., higher hormone levels in the organ compared to plasma) or evidence of steroid utilization (i.e., higher levels in plasma compared to organs). Here, both control and fasted groups were evaluated separately. In controls, several organs had CORT levels that were statistically lower than that found in circulating plasma (Fig. 10). These tissues including: blood ($t = 4.598$, $df= 17$, $P=0.000256$); brain ($U=12$, $n=12$, $P< 0.001$); lung ($U=21$, $n=12$, $P=0.006$); stomach ($U-4$, $n=12$, $P<0.001$); pancreas ($t = 2.405$, $df=15$, $P=0.03$); kidney ($t = 3.510$, $df=22$, $P=0.002$); testes ($U=36$, $n=12$, $P=0.04$); hemipenes ($U=8$, $n=12$, $P<0.001$) and muscle ($U=12$, $n=12$, $P<0.001$), In contrast, only the intestine ($U=39$, $n=14$, $P=0.012$) and liver ($U=28$, $n=14$, $P=0.008$) had levels higher than circulating plasma.

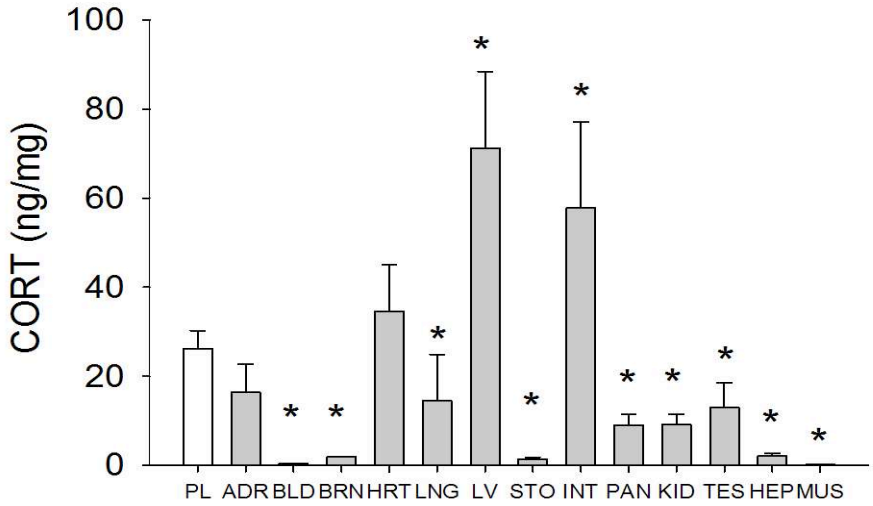

Fig 10. Local CORT production among control organs in comparison to circulating plasma (represented in white). An asterisk denotes significance from plasma.

Plasma CORT levels were significantly higher (Fig. 11) than in blood (U=11, n=13, P=0.006), brain (U=28, n=11, P=0.035), and stomach (U=41, n=13, P=0.027). In contrast, plasma CORT levels were lower (Fig. 11) than in liver (U=42, n=13, P=0.031), intestine (U=7, n= 13, P<.001), and kidneys (U=44, n=13, P=0.04).

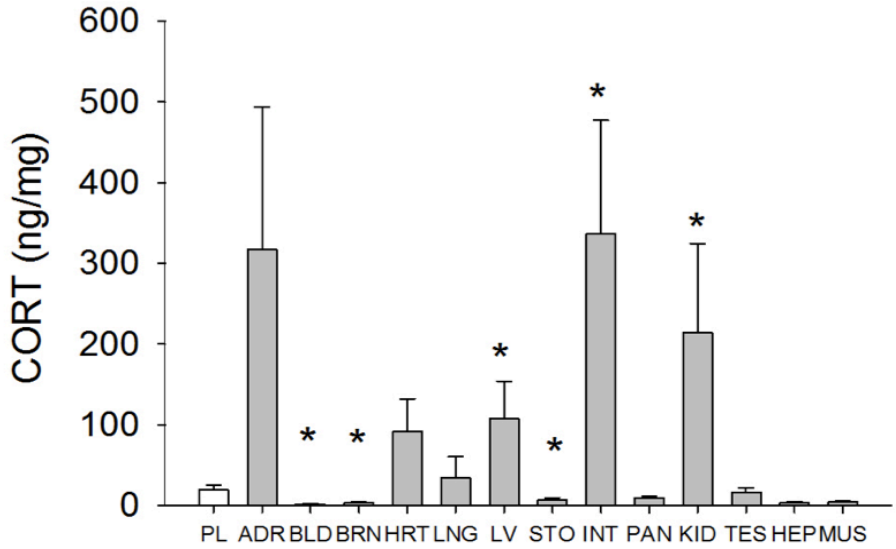

Fig 11. Local CORT production among fasted organs in comparison to circulating plasma (represented in white). An asterisk denotes significance from plasma.

Evidence for widespread tissue DHEA synthesis in anoles

For control anoles, compared to plasma, several organs had statistically higher DHEA concentrations (Fig. 12) including: adrenal (U=1, n=12, P< 0.001), heart (U=22, n=12, P=0.004), lung (U=15, n=12, P= 0.002), liver (U=26, n=12, P=0.015), and the hemipenes (U=0, n=12, P < 0.001)**.** In contrast, plasma DHEA levels were lower in pancreas (t = -4.777, df=15, P < 0.001) and the testes (U=23, n= 12, P=0.005)**.**

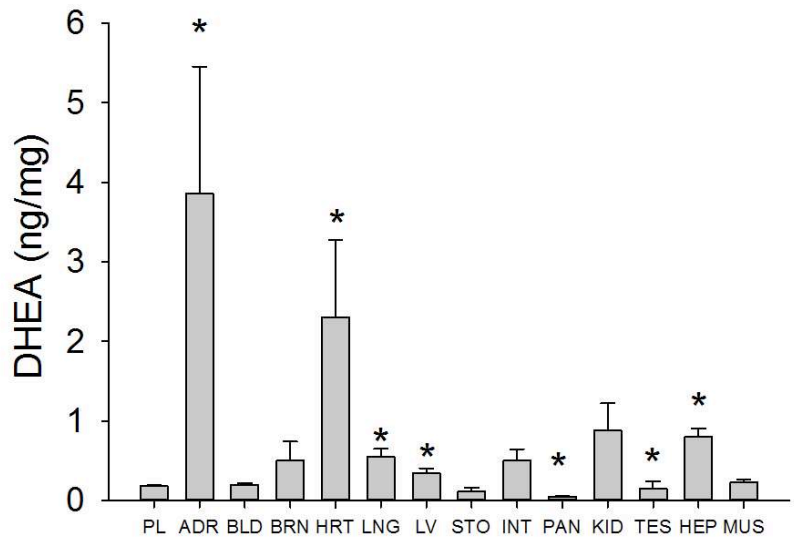

Fig 12. Local DHEA production among control organs in comparison to circulating plasma. An asterisk denotes significance from plasma.

In fasted anoles, plasma DHEA was significantly lower (Fig. 13) than in: brain (U=40, n=13, P=0.024); heart (U=7, n=13, P< 0.001); lung (U=24, n=13, P=0.002); liver (U=40, n=13, P=0.024); intestine (U=12, n=13, P< 0.001); and hemipenes (U=4, n=13, P < 0.001). Concentrations of DHEA in the stomach (U=32, n=13, P=0.008) and pancreas (t=2.860, df=16, P=0.01) were significantly lower than in plasma (Fig. 13).

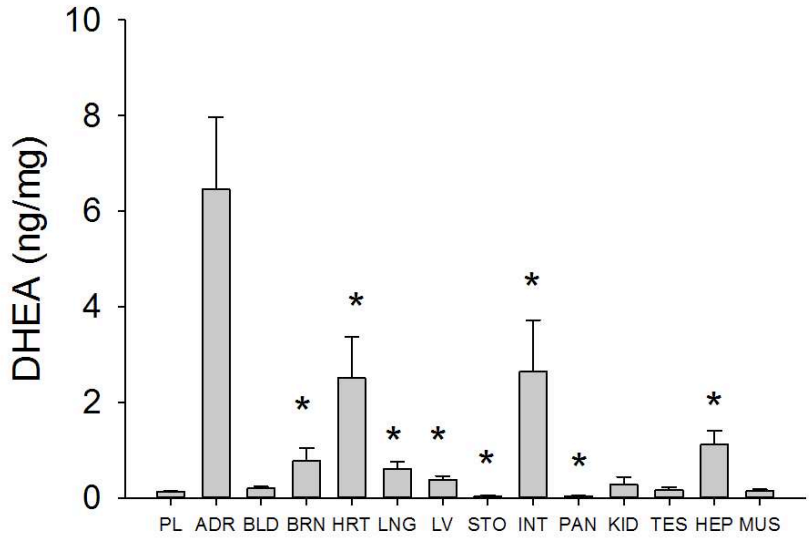

Fig 13. Local DHEA production among fasted organs in comparison to circulating plasma. An asterisk denotes significance from plasma.

Discussion

The systemic stress response involving widespread circulation of CORT produced by the adrenal cortex is well understood. However, minimal research has focused on local steroidogenesis, or the ability of non-steroidogenic organs to produce their own steroids. In this study, I sought to determine whether fasting (a chronic stressor) could promote local steroidogeneiss in anole lizards. In response to fasting, I expected organ concentrations of CORT and DHEA to exceed that of circulating plasma, which could suggest local steroid production. Several organs demonstrated higher steroid levels than those present in circulation (i.e. plasma), which provides evidence that local steroid synthesis is occurring, however there was no profound effect of fasting. As only the second study to evaluate local CORT and DHEA, it is important to further research this field to determine how and why local stroidiogeensis occurs.

Local CORT production

Digestive organs

The intestine was the only organ where CORT increased with fasting and regardless of treatment, the highest CORT levels were observed. Lowette et al. (2014) reported that fasting increased intestinal CORT, as in this study. To confirm this, they reported increased mRNA expression of 11β-hydroxysteroid dehydrogenase Type 1 (11β-HSD1), a CORT-converting enzyme in enteric (intestinal) sensory neurons.

These data suggest that intestinal CORT synthesis is extensive with local CORT playing an important role possibly through the enteric nervous system (ENS), the set of neurons embedded in the intestinal wall. CORT also reduced electrical stimulation induced by calcium, suggesting a neuromodulatory role in the GI tract during fasting (Lowette et al., 2014). The same study noted decreased mixing within the intestine with high CORT levels from fasting. Local CORT may be inducing these physiological changes (reducing the intestinal neuronal response and mixing) in order to inhibit unnecessary processes during a stressor.

While the intestine remained consistent with higher CORT levels, other digestive organs did not exhibit a similar pattern. Both the pancreas and stomach had lower hormone levels than plasma. The low pancreatic levels of CORT could be caused by increased pancreatic uptake in order to maintain physiological processes such as glucose metabolism. Interestingly, CORT affects how the body processes insulin and during stress, CORT promotes the release of glucose into circulation from energy reserves to be used for metabolism (Sapolsky et al. 2000). While insulin is necessary to regulate glucose levels, CORT does interact with the peptide through a poorly understood molecular mechanism, by making insulin less efficient at glucose uptake (Beleen et al. 2014). At the molecular level, somatostatin (SST; a hormone that inhibits growth hormone), CORT, and ghrelin all influence glucose homeostasis within the pancreas (Beleen et

al. 2014). Yet, under severe metabolic conditions, such as possibly fasting, the expression of these regulators may be compromised. While CORT was found in the pancreas, this deregulation could explain why such a low concentration was observed in the control and fasted groups. Therefore, chronic stress not only impairs the HPA axis but also how CORT receptors function (Beleen et al. 2014). Since CORT receptors may not be fully active under stress, it may be possible that less CORT is being detected in the pancreas. This could explain why such low levels of CORT were measured in the pancreas, even if the hormone was being produced.

Elevated CORT levels will shutdown unnecessary processes during a stress response. Therefore, I predicted that the stomach would produce CORT in response to fasting, to limit protein digestion, yet, regardless of treatment, gastric CORT levels remained low. As local CORT levels were lower than in circulating plasma, this may suggest the stomach uses CORT directly from circulation, as opposed to local production. In response to fasting, Filaretova et al. (2004) found that GCs had a gastro-protective role. In fact, with CORT supplementation, the decrease in blood flow velocity and mucous production was further prevented (Filaretova et al. 2004). Another study illustrated how food deprivation decreased the activity of digestive enzymes such as trypsin and lipase (Bolasina et al. 2005). As, enzyme activity is highly sensitive to nutritional conditions, CORT could be interacting with digestive enzymes to inhibit or lessen their activity.

In the liver, I reported higher organ CORT levels than in plasma from both fasted and control lizards, although the difference is greater in the latter. The liver performs metabolic functions such as glycogen storage, red blood cell decomposition, detoxification, and these processes are readily affected by stressors (Djordjevic et al., 2010). For example, an increase in GCs stimulates liver gluconeogenesis for use as an energy source during stressful conditions (Djordjevic et al., 2010). Furthermore, the liver plays an important role in detoxification and antioxidant enzyme activity is also regulated by GCs (Djordjevic et al., 2010). According to Vazquez-Medina et al. (2010), prolonged fasting conditions increases the production of reactive oxygen species (ROS), which leads to oxidative damage and inflammation. For this reason, an up regulation of enzymes responsible for breaking down ROS would be beneficial. In a combined stress study that used isolation and acute immobilization stress on Wistar rats, both CORT and liver antioxidant enzyme levels increased (Djordjevic et al., 2010). This increase suggests that GCs may signal the liver to produce more enzymes for detoxification of ROS during stress, and could therefore explain the elevated CORT levels observed in the liver in this study.

Kidney

Compared to controls, kidney CORT levels were higher in the fasted group, and both were significant above levels found in plasma. Interestingly the kidney in male anoles also performs a sexual role in supplying a nutrient medium that acts as an energy source for sperm (Cuellar et al. 1972). Based on this relationship with reproduction, one could predict higher renal activity and nutrient production during the breeding season. This study was conducted in summer at the peak of the anole breeding season in Florida (Wade 2011), thus the high CORT levels observed in the fasted kidneys may indicate an inhibition of the kidney's reproductive function in male anoles. Yet, at this time, little is known about the kidney's role in reproduction, this is solely a prediction.

Heart

Although no statistically significant differences were observed, CORT levels in cardiac muscle tended to be higher in the fasted group and generally higher than in plasma. Interestingly, the heart is composed of proteins that can be either built up (anabolism) or broken down (catabolism) depending on hormone balance (Tischler 1981). Hormones, such as the GCs present during fasting can lead to catabolic effects in cardiac muscle proteins, and fasting can induce atrophy and protein catabolism in rabbit hearts (Samarel et al. 1987). Fasting inhibits the production of new cardiac muscle protein and caused an increase in the percentage of degraded protein (Samarel et al. 1987). The high CORT levels in this study may have interfered with

protein synthesis. This hypothesis would then need to be confirmed by protein analysis to determine the impact of high CORT levels had on cardiac tissue.

Blood cells

Plasma consistently expressed much higher CORT concentrations than the blood, confirming that GCs circulate in the plasma portion and not within the white and red blood cells. Nonetheless CORT may be playing a role in red blood cells. Vertebrates cope with physiological changes (such as fasting) through immune system responses to immune challenges (Graham et al. 2012). According to Dhabhar et al. (2012), chronic stress induces a quick redistribution of immune cells throughout the blood. This stress-induced leukocyte redistribution is a fundamental response that directs leukocytes to their target organs (Dhabhar et al. 2012), with an increase in the mobilization of neutrophils, lymphoctyes, and helper T cells (Dhabhar et al. 2012). Thus while CORT levels remain low in the blood, the hormone could be used quickly to induce such immune changes. It is also important to note that this immune redistribution has been evolutionarily conserved across the vertebrates, including reptiles, and this suggests a strong adaptive advantage.

Brain

In response to stress, anoles should have elevated CORT levels in the brain which leads to a decrease in dopamine within the brain (Greenberg 2002). In these anoles, both orientation and

locomotor responses were depressed, which were similar characteristics observed in this study. Although CORT acting within the brain has been widely accepted, this study demonstrated low CORT levels regardless of the treatment. A study assessing chronic stress in rats reported that CORT affected the brain regions associated with motivation and reward, which are regulated by dopamine (Lucas et al. 2003). Further, Lucas et al. (2003) also reported low CORT levels that that correlate with reduced behavioral activity and longer latencies in reacting to mildly threatening stimuli (i.e., less anxious). Although both treatment groups contained low CORT, the fasted group was much less resistant to handling at the end of the fasting experiment. This deviation from normal anole behavior to resist handling could obviously well be due caused by a lack of energy from fasting or due to the physiological brain changes discussed.

Local DHEA production

DHEA, an androgen precursor, may have anti-stress properties against GCs during the stress response. While most comparisons of DHEA levels between fasted and control groups were not significantly different, multiple organs did contain significant concentrations above that observed in plasma. Regardless of treatment group, the adrenal, heart, and intestine continued to have higher DHEA levels than plasma supporting the hypothesis of local steroid production in these organs. This is the first study to report the presence of non-plasma DHEA and to document

local steroid production in a reptile species. While DHEA levels were measurable in the anole, it is important to note the lower concentrations when compared to other species. In anoles, DHEA concentrations ranged from 0 to7 ng/mg, whereas songbirds contained upwards of 15 ng/ng (Fokidis et al. 2013). Furthermore, DHEA levels increased dramatically in fasted male zebra finches, while fasting did not have a similar effect in this study. This difference could be due to the needs of each species; while birds might need the protection DHEA offers, anoles may be more resistant to such stressors.

Testes and Hemipenes

The testes did not show any variance of DHEA concentration in neither control nor fasted groups. The testes could either be using DHEA quickly for testosterone production or may not have a large role in sex hormone production. However the hemipenes, the copulatory organ in reptiles, contained a higher DHEA content, regardless of treatment, when compared to plasma. As a reproductive organ, the local production of an androgen precursor such as DHEA would makes sense however there is little to no research on the hormonal regulation of hemipenes. In another lizard species, snow skinks (*Niveoscincus microlepidotus),* researchers found that both the adrenal gland and gonads produced testosterone and testosterone levels peaked with maximal hemipene growth (Girling et al. 2006), suggesting a role for sex hormones in hemipenes development. Again, while evidence for local T production by the hemipenes has not been

confirmed, the presence of DHEA in this study may provide evidence for local androgen production. Lacking a receptor, DHEA must be converted to an active product such as testosterone or estrogen (Kalimi et al. 1994). It would be beneficial to run testosterone (T) assays in the future for both reproductive and non-reproductive organs to determine whether DHEA levels positively correlate with T levels, which could indicate that DHEA is used as a substrate in the specified organs.

Intestine

The intestine contained significantly elevated DHEA levels while fasting, just below that of the adrenal. As with CORT in the intestine, DHEA can have antioxidant effects, exerting a protective role by reducing tissue susceptibility to oxidation of both lipids and protein (Pelissier et al. 2004). When male rats were treated with DHEA, intestinal tissue responded with enhanced antioxidant capacity and an increased mucous production (Pelissier et al. 2004). In fact, the excess production of acidic mucous has been found to be the major protectant of intestinal epithelia (Pelissier et al. 2004). Here, DHEA could have these protective effects in the intestine, which would explain the higher level of DHEA produced by the organ.

Brain

The brain contained elevated DHEA levels in the fasted group, suggesting a protective role of the hormone in brain tissue. Again, oxidative stress can cause lipid peroxidation and degenerative changes in the hippocampus, is the region of the brain most targeted by GCs. Bastianetto et al. (1999) studied the hippocampus and found that DHEA may play a neuroprotective role by directly acting as an antioxidant to neutralize free radicals induced by chronic stressors. Again, it seems that one of the most important anti-stress roles of DHEA is through its antioxidant properties.

Future Direction

Future research should aim to increase sample size to account for individual differences among the anoles, and further to measure chronic versus acute stress, the fasting experiment could be staggered. Observing hormone levels through varying time points would determine the time at which fasting becomes a chronic stressor. If fasting does exert a stressor, performing the experiment at this time should result in a significant difference among control and fasted hormone levels. Future studies may also involve evaluating the expression of steroidogenic enzymes in the organs produced CORT and DHEA locally in this study. This data, along with complimentary tests (i.e. testosterone and glucose assays), could provide a more thorough explanation on local steroidogenesis and how it occurs.

Conclusion

Although the one-week fasting experiment was sufficient to decrease body mass suggesting it induced chronic stress on anoles, neither CORT nor DHEA levels varied between fasted and control groups**.** While the fasting experiment did not result in significant differences between the treatment groups, there was in fact evidence for local steroidogenesis for almost all organs in male anoles. Moreover, this was the first study to report DHEA production in reptiles. Overall, the digestive and reproductive systems showed the greatest hormone changes and due to their sensitivity to stressors, there's no coincidence for this trend. Although only the second study to research local steroidogenesis and the first to measure DHEA in a reptile, the results were significant in showing how a stressor could affect local production among organs. These findings may be used in future research to determine if steroidogenic enzymes are expressed in the same tissues with elevated GC levels, in order to prove local steroidogenesis occurs within organs.

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