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Evidence for fasting induced extra-adrenal steroidogenesis in the male brown anole, *Anolis sagrei*

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ABSTRACT

Glucocorticoids (GCs) and dehydroepiandrosterone (DHEA) are steroids secreted by the adrenal glands into circulation to effect distant target tissues and coordinate physiological processes. This classic systemic view of steroids has been challenged by evidence that other tissues can independently synthesize their own steroids. Little is known however regarding circumstances that can promote this extra-adrenal steroidogenesis. Here we tested if fasting can induce tissues to increase GC and DHEA synthesis in the brown anole lizard *Anolis sagrei*. Lizards fasted for eight days lost body mass and increased fatty acid oxidation. Fasting also increased plasma concentrations of DHEA and corticosterone, but not cortisol. Corticosterone concentration within the adrenals, heart, intestines, lungs and liver exceeded that in plasma, with the latter two increasing with fasting. Levels of DHEA in the adrenals and heart were higher than in plasma, but no significant effect of fasting was observed, expect for a noticeable increase in intestinal DHEA. Two steroidogenic genes, the steroidogenic acute regulatory (Star) protein and Cyp17a1, a cytochrome P450 enzyme, were expressed in several tissues including the liver, lungs and intestines, which were increased with fasting. Continued research should aim to test for expression of additional enzymes further along the steroidogenic pathway. Nonetheless these data document potential extra-adrenal steroidogenesis as a possible mechanism for coping with energy shortages, although much work remains to be done to determine the specific roles of locally synthesized steroids in each tissue.

1. Introduction

Steroids regulate physiological processes that occur under a wide range of energetic conditions, such as development, reproduction, and metabolism. In vertebrates, steroids are typically produced by de novo steroidogenesis through the conversion of cholesterol to the major biologically active steroids in the adrenal cortex, the gonads and the placenta (Aguilera, 1993; Chatuphonprasert et al., 2018; Flueck and Pandey, 2014). In other peripheral tissues, the specific interconversions of steroids and their precursors can take place due to local expression of steroidogenic enzymes (Andersson, 2001; Baulieu, 2000; Rosati et al., 2016, 2017, 2019; Slominski et al., 2013). Conceptually, the sites of steroid synthesis have been unified in a framework involving a shift from systemic to local steroidogenesis that has been referred to as a "Balkanization" of the endocrine system (Schmidt et al., 2008). For example, the central nervous system can shift from being primarily a steroid target, to the autonomous production of neurosteroids, either de novo or through metabolism of peripherally sourced intermediates (Baulieu, 2001, 1999; Fokidis et al., 2015). Such peripheral steroid synthesis is both well-documented and evolutionary conserved (do Rego and Vaudry, 2016; Labrie, 2015), however little is understood regarding the conditions that initiate the transition from systemic to local steroidogenesis.

The endocrine system facilitates adjustments in energy expenditure and the homeostatic control of nutrients necessary to maintain physiological processes, even during major life-history transitions. Studies have suggested links between compromised nutrition and an increase in local steroidogenesis. In mammals, fasting increases hepatic production of the androgen precursor, dehydroepiandrosterone (DHEA) by increasing transcriptional coactivators (Grasfeder et al., 2009) and Cyp17a1, the cytochrome P450 enzyme that serves as a 17α-hydroxylase and 17, 20 lyase during steroid intermediate biosynthesis (Milona et al., 2019). In zebra finches (*Taeniopygia guttata*), food restriction increased hepatic DHEA levels along with neural estradiol levels, with both at levels above plasma concentrations (Fokidis et al., 2013). The rate of steroid biosynthesis is limited by provisioning of cholesterol to the inner mitochondrial membrane by the action of the steroidogenic acute regulatory (Star) protein (Miller and Strauss, 1999), and several studies

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have shown it plays a pronounced role in both lipid and glucose metabolism and is influenced by signals associated with food intake (Cherradi et al., 2001; Qiu et al., 2017; Ramanjaneya et al., 2008).

As important metabolic hormones, glucocorticoids (GCs) plays vital roles in food intake and energy allocation, particularly in the context of stress (Dallman et al., 1995). Food restriction generally elevates circulating GCs in both field and laboratory studies. Chronic elevation of GCs in circulation can in turn decrease energy balance and lower body condition (Fokidis et al., 2012), however the labile nature of GCs in blood makes identifying a condition of chronic stress complicated (Bonier et al., 2009). The adrenal cortex remains the predominant site of GC production and depending on the species either cortisol, corticosterone, or both are secreted (reviewed in Di Lorenzo et al., 2020). Other organs may be capable of local GC synthesis, including the thymus, gut, skin, heart, intestinal mucosa, and the brain (reviewed in Taves et al., 2011). Indeed significant evidence suggests the presence of complete regulatory axes for paracrine GC synthesis, including the mediators of the hypothalamic-pituitary-adrenal (HPA) axis, such as corticotropin-releasing hormone (CRH) and adrenocorticotropin hormone (ACTH) in lymphoid tissues and skin (Andersson, 2001; Ottaviani et al., 1998; Slominski et al., 2013).

Considerable debate continues regarding the specific function of these paracrine steroid pathways and how disparate they are from the roles played by systemic hormones. Among the roles ascribed to these steroids are: protecting tissues from apoptosis (Charalampopoulos et al., 2006; Peri et al., 2009); bolstering local immunity and suppress inflammation (Ahmed et al., 2019; Taves et al., 2017); immune programming during development (Taves et al., 2016, 2014); and acting as neuromodulators and neurotransmitters (Saldanha et al., 2011, 2013). Limits on the availability of energy can drastically alter each of the roles, however little research has explicitly tested the relationship between steroidogenesis and energy. Here we tested the hypothesis that fasting increases extra-adrenal glucocorticoid and DHEA synthesis in the invasive brown anole (*Anolis sagrei*). We used changes in body mass and circulating energy substrates to develop an appropriate food restriction testing paradigm which enabled an investigation of how circulating corticosterone, cortisol and DHEA concentrations are impacted by fasting. Then we documented local (i.e., extra-adrenal) steroidogenesis using two criteria: 1) the presence of tissue concentrations of steroids that exceed the levels in plasma; and 2) the expression of two steroidogenic genes in the tissues with higher than plasma steroid levels. Extra-adrenal steroidogenesis in the context of limitations on energy procurement has both ecological and biomedical implications, and thus a comprehensive study of the effects of fasting on local steroid synthesis is warranted.

2. Materials and methods

2.1. Field capture and general husbandry

Adult male brown anoles were captured with fishing line nooses on the Rollins College campus, in Winter Park, Florida. Upon capture each subject was weighed (± 0.1 g) and measurements of the snout-vent length (SVL; (± 0.1 mm) were taken. Anoles were then transferred to a captive colony with each individual housed singly in terraria cages (30.5 × 16.5 × 29 cm) separated by partitions to avoid interaction. Each cage contained a carpet liner, a mesh hammock, a perch made from PVC, and a live plant. Colonies were maintained on a 14:10 h (light to dark) photoperiod cycle using 40-watt full spectrum light bulbs and at a constant temperature (27 °C) and relative humidity (84%). Subjects were fed two large crickets and the cage and plant was sprayed with water every two days. The health of the anoles was evaluated daily and they were weighed weekly. All animal handling and experimental procedures were approved by the Institutional Animal Care and Use Committee at Rollins College.

2.2. Study 1: Shifts in circulating metabolites with fasting

A total of 40 subjects were assigned to one of four groups ($N = 10$ per group) that either: 1) was fed two crickets the day before sampling (FED group); or 2) experienced a 3-DAY fast; 3) a 6-DAY fast; or 4) an 8-DAY fast. Each of the four groups were randomly assigned, however some adjustments were made to ensure these groups did not statistically differ in body weight prior to commencing the experiment (ANOVA: $F_{1,19} = 0.161$, $p = 0.103$). To avoid a potential confounding effect of the time of day, the experiment was staggered with only four subjects (one from each group) being sampled per day over the course of two weeks. The order of sampling for each subject was recorded. The entire experiment was repeated a second time (two months later) with the same subjects, but each again randomly assigned to a new group. The results were then pooled together ($N = 20$ per treatment group) and the trial order was noted to include in subsequent data analysis.

To minimize potential stress effects due to researchers being present in the colony room, all sampling was done in an adjacent room. Prior to the start of the study, subjects were removed from the cage and a blood sample (25–60 μl) was collected within 1 min by rupturing the orbital sinus with a heparinized capillary tube with collection taking no longer than 30 s. Blood was stored on ice until transported back to the laboratory, where plasma was separated using centrifugation (10 min at 2500 rpm) and stored at −20 °C until assayed. Subjects were then weighed using a Pesola scale (± 0.5 g) and released back into their home cage. The process was then repeated for the next subject, with only four animals sampled per day. Subjects had blood samples collected and were weighed at both the beginning and the end of the study to determine changes with treatment.

Plasma free glycerol which indicates intrinsic lipid mobilization (Guglielmo et al., 2002), and total triglycerides were measured using a sequential spectrometric endpoint assay that uses only 5 μl of the same sample (Fokidis et al., 2012; Fokidis and Deviche, 2011; Guglielmo et al., 2002). The unbound "true" triglyceride fraction was calculated as the difference between the total triglycerides and free glycerol concentrations which indicates lipid deposition. Plasma concentrations of the ketone, β-hydroxybutyrate concentration was determined using a commercial colorimetric assay (Cayman Chemical Company; Ann Arbor, MI, USA). Samples were assayed in duplicate and sample concentrations were calculated based on interpolation from a standard curve. Assay sensitivities were 0.16 mM for the glycerol and triglyceride assay, and 0.01 mM of the β-hydroxybutyrate. The mean intra- and interassay coefficients of variation were as follows: 6.3 and 8.5% for glycerol; 5.2 and 9.3% for triglycerides; and 3.5 and 10.7% for ketones. To validate these assays we tested both parallelism between the standard curves and serially diluted samples (all $p \geq 0.194$), and the recovery of samples spiked with known concentrations (83% ± 10.0%, 87 ± 12.4%, and 91 ± 9.4% for glycerol, triglycerides, and β-hydroxybutyrate, respectively).

2.3. Study 2: Changes in circulating steroid secretion with fasting

Subjects were randomly assigned to one of two groups: 1) a FASTED that experienced an eight day fast ($N = 15$) and 2) a CONTROL group provided two large crickets every two days ($N = 15$) with no meal provided on the day prior to sampling. Groups did not differ in body weight prior to commencing the experiment (two-sample *t*-test: $t = 1.013$, df = 14, $p = 0.275$). The experiment was again staggered with four subjects sampled per day in an adjacent room to avoid stress to the colony. Blood samples were collected from the orbital sinus with a heparinized capillary tube with collection taking no longer than 30 s. Subjects had blood samples collected and were weighed at both the beginning and the end of the study to determine changes with treatment.

Commercial competitive enzyme-linked immunoassay (ELISA) kits were used to measure total corticosterone (No. K014-H5, Arbor Assays Inc.; Ann Arbor, MI, USA), total cortisol (No. K003-H5, Arbor Assays Inc.; Ann Arbor, MI, USA) and total DHEA (No. DHA31-K01, Eagle

Biosciences Inc.,; Nashua, NH, USA). These assays were highly specific to their respective hormones based on their low cross-reactivities (all binding ranges from 0.001% to 1.2%, manufacturers' specifications). Plasma samples were prediluted 6-fold prior to the corticosterone assay to insure they were within the linear range of the standard curve. Steroid assays were performed according to the manufacturer's protocol and 96-well microplates were read using a spectrophotometer at 450 nm. These assays were validated using pooled anole plasma samples by demonstrating parallelism between serially diluted samples and the standard curve; by showing a high recovery of exogenous steroid (84–90% recovery); and a very low recovery of samples stripped of steroid using dextran-coated charcoal (3–6% recovery). Final concentrations were determined by interpolating from the assay standard curve of known concentrations (19.5–10,000 pg/ml for corticosterone, 50–3200 pg/ml for cortisol; and 150–40,000 pg/ml for DHEA) using GraphPad Prism version 6 (GraphPad Software Inc., La Jolla, California, USA). The sensitivity (i.e., lowest limit of detection) of these assays calculated from two standard deviations from a zero standard were 21.5 pg/ml for corticosterone, 10.3 pg/ml for cortisol, and 14.1 pg/ml for DHEA. The mean intra-assay and inter-assay coefficients of variation were 9.4% and 15.9% for corticosterone, 7.3% and 10.3% for cortisol, and 9.2% and 17.0% for DHEA respectively.

2.4. Study 3: Changes in tissue steroid concentrations with fasting

As in study 2, subjects were randomly assigned to one either a FASTED that experienced an eight day fast ($N = 13$); and 2) a CONTROL group provided two large crickets every two days ($N = 12$). Groups did not differ in body weight (two-sample t-test: $t = -0.498$, df $= 23$, $p = 0.623$) and only with five subjects sampled per day in an adjacent room to avoid stress to the colony. Following the experiment, anoles were quickly weighed and then sacrificed within 3 mins of handling by rapid decapitation to avoid stress-related changes in steroid concentrations. A trunk blood sample was rapidly collected in heparinized capillary tubes and stored on ice until separated into plasma and formed elements (i.e., blood cells) using centrifugation. Anoles were then dissected, and the following organs were collected: the adrenals, heart, lungs, liver, stomach, small intestines, pancreas, kidney, testes, hemipenes and dorsal muscles. Any stomach or intestinal contents were removed and tissue samples immediately snap frozen using dry ice with subsets of these samples stored in RNAlater stabilization solution to optimize mRNA isolation and PCR conditions for study 4. All samples were stored at −80 °C until further processing for steroids.

2.4.1. Tissue processing and solid phase extraction

Soft tissue samples were weighed to the nearest +0.1 mg and then 50 mg was homogenized (settings: 5500 rpm for 20 s, Precellys 24 homogenizer (Bertin Technologies; Paris, France) while suspended in ice-cold 84% HPLC-grade methanol (MeOH) in a 19:1 ratio (volume-to-sample mass). For plasma and blood, the equivalent of 50 μl was also suspended in 84% MeOH. These samples were left overnight at 4 °C and after centrifuged at 3000 *g* for 10 min with the resulting supernatant being collected for solid phase extraction (SPE) to extract steroids. This SPE method has been previously described in detail (Fokidis et al., 2013; Newman et al., 2008). Briefly, the supernatant was first diluted with 10 mL of deionized water (dH_2O). Then carbon-bonded silica C18 filter column cartridges (Agilent Technologies, Santa Clara, CA, USA) were placed on a vacuum manifold and primed with 3 ml of 100% ethanol (EtOH) followed by 10-mL of dH_2O before loading the diluted sample. Then, 10 ml of 40% MeOH was used to remove fats (e.g., triglycerides, cholesterols, and fatty acids) from the sample that could interfere with the assay. Columns were then run dry, and steroids were finally eluted using 5 ml of 90% MeOH. Eluted samples were dried in a speed vacuum concentrator (ThermoFisher Scientific Inc., Pittsburgh, PA, USA) at 60 °C for 4 h. All SPE extractions included a solvent blank as a negative control. Dried extracts were then stored at −80 °C until reconstituted for the steroid quantification assays.

2.4.2. Enzyme-linked Immunoassays

As in study 2, commercial competitive enzyme-linked immunoassay (ELISA) kits were used to measure total corticosterone and total DHEA concentrations in all samples. Dried tissue extracts were first reconstituted with 5 μl of 100% EtOH, and then 115 μl of assay buffer and these were split to measure both corticosterone and DHEA. Plasma was prediluted 6-fold prior to the corticosterone assay to insure they were within the linear range of the standard curve. Steroid assays were performed according to the manufacturer's protocol and 96-well microplates were read using a spectrophotometer at 450 nm. Previous research determined steroid recoveries for each tissues (corticosterone: range 90–96%; DHEA: range 86–97%) and steroid concentrations determined by the assay were corrected for their respective recovery.

2.5. Study 4: Fasting and the presence of steroidogenic enzyme mRNA in tissues

As in study 3, subjects were again randomly assigned to either a FASTED or a CONTROL group ($N = 6$ per group). There were no differences in body weight at the onset of the study (two-sample t-test: $t = 0.620$, df $= 11$, $p = 0.218$) and only three subjects were sampled per day. Subjects were as above rapidly weighed and then sacrificed within 3 mins of handling by rapid decapitation and a trunk blood sample was rapidly collected as before and stored on ice until using centrifugation. The same organs were collected as in study 3 and stored in RNAlater stabilization solution (Thermo Fisher Scientific; Waltham, MA, USA) and kept at −80 °C until further processing.

2.5.1. RNA isolation and cDNA synthesis

Samples stored in RNAlater solution were first homogenized in Trizol (1 ml per 50 mg tissue) at in a Precellys 24 homogenizer (settings: 5500 rpm for 20 s) and then 0.2× chloroform was added. After three mins of incubation at room temperature, samples were twice centrifuged at 12,000 *g* for 15 mins at 4 °C. Isopropanol (0.5 mL/1 ml Trizol) was then added to precipitate the RNA in the sample and incubated for 10 min at room temperature and centrifuged again with the above conditions. The resulting pellet was washed with 75% ethanol and centrifuged at 7500 *g* for 5 mins and resuspended in RNase free water. RNA was treated with DNase I for 15 min at room temperature to remove residual genomic DNA, and total RNA integrity and purity were confirmed to be high quality by 1% agarose gel electrophoresis with bleach and UV-VIS spectrophotometry (Nanodrop ND-1000, Thermo Fisher Scientific Inc., Waltham, MA, USA) to determine the presence of two bands representing the rRNA (ribosomal RNA) of the 28S and 18S subunits, as per Aranda et al. (2012). Isolated RNA (1 μg/μl) was reverse transcribed into cDNA using the SuperScript IV Reverse Transcriptase kit (Invitrogen Cat. No. 18090010, Thermo Fisher Scientific Inc., Waltham, MA, USA), as per manufacturer's instructions. The quantity of cDNA was estimated using the Nanodrop system and was found to be in ample amount nearly 1000 ng/ul and cDNA was stored at −20 °C until further analysis.

2.5.2. Primer design

Primer sequence sets for Star, Cyp17a1 and Actb (Table 1) were derived from Peek and Cohen (2018) and ordered from Integrated DNA technologies Inc. (Coralville, IA, USA). Primers were analyzed for length (18–22 nucleotides) and GC content (30–80%), and primer pairs were assessed for dimerization potential using Multiple Primer Analyzer software (Thermo Fisher Scientific Inc., Waltham, MA, USA). All primers spanned multiple exons boundaries to ensure no genomic DNA was amplified and NCBI blast search of primers sequences confirmed a 100% homology to their respective predicted gene sequences on the mapped green anole genome, thus suggesting a low risk for non-specific amplification.

Table 1
Identification of primer sequences for the target genes: steroidogenic acute regulatory protein (Star) and steroid 17-alpha-hydroxylase/17,20 lyase (Cyp17a1) and the housekeeping gene: Actb. Gene information is derived for *Anolis carolinensis* (Kostadinova et al., 2014) with the qPCR conditions necessary for use in *Anolis sagrei* provided.

Gene	NCBI Gene ID#	Primer Sequences (5′ to 3′)	Amplicon Size (bp)	Primer Conc. (μg/μl)	Melting Temp. (°C)
Star	100554580	F: CACTCGCTGGAGATCCCTACC	112	0.5	58
		R: TCCACCTGCGTCTGGG			51
Cyp17a1	100553634	F: GGGAACCGAATCTACAGCCC	86	0.25	56
		R: ATCTTGGCTAGCGCTTCTCC			52
Actb	100337533	F: GACGAGGCGCAGAGTAAAAG	131	0.5	54
		R: TCAGGGGCAACTCTCAACTC			54

2.5.3. qPCR

Primer concentrations were optimized using cDNA and Quick-Load Taq 2× master mix (New England BioLabs, Ipswich, MA, USA) as per manufacturer's instructions with standard PCR conditions of 95 °C for 30s followed by 30 cycles of 95 °C for 30s, 60 °C for 30s, 68 °C for 1 min, and 4 °C holding temperature. The lowest primer concentration that generated a single band in a 1% agarose gel electrophoresis was used for subsequent analyses (Table 1).

Expression of mRNA was analyzed using quantitative real-time PCR (qPCR; Step One Plus, Applied Biosystems Instruments, Foster City, CA, USA) and reactions were conducted using Power SYBR Green PCR Master Mix (2×) with either: 90 ng cDNA (1:20 ratio for Star); 45 ng cDNA (1:40 ratio for Cyp17a1); and 45 ng cDNA (1:40 ratio for Actb). No-template and transcriptase controls in sterile RNase free water were added as negative controls. All samples were run in triplicate, with either Star or Cyp17a1 assayed alongside Actb on each 96 well plate. The qPCR conditions included an initial denaturation step of 95 °C (1 min), followed by reverse transcription at 42 °C (30 mins), and then 40 cycles of 95 °C for 30s, 50 °C for 30s, 68 °C for 4 min, and finally a final annealing and extension of 68 °C for 10 min with 4 °C holding temperature. A melt curve was also conducted for each run with steps at 95 °C (15 s), 65 °C (1 min), and 95 °C (15 s). Amplicons of cDNA were analyzed on a 2% *w/v* agarose gel in 1× TAE (100 V for 40 min) to confirm amplification of the correct product and assess presence of primer dimers. Step-One Plus Expression Suite Software (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to determine Ct values which were normalized to Actb expression, which was invariable across the treatments groups with no amplification observed in the negative controls.

2.6. 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. Repeated measures analysis of variance (rmANOVA) was used to assess changes in body mass, metabolites, and circulating steroids in response to the fasting treatments (the *within-subjects'* factors). The treatment (i.e., days of fasting) is the *between-subjects* main effect, and both the sampling order (i.e., date and time of sampling) and trial number were included as *covariates*. Post-hoc comparisons were made using Fisher's least-significant difference (LSD) tests. The relevant interrelationships between the proportional changes in body mass, metabolites and steroids were analyzed using Pearson correlations.

Tissue steroid concentrations and mRNA gene expression (*dependent variables*) were assessed using multivariate analysis of variance (MANOVA) with both tissue type and treatment (fasting vs. control) as independent factors, and with sampling order and body mass included as *covariates* in the statistical model, as well as the interaction of tissue type*treatment. Several tissues did not produce either detectable steroids or mRNA of sufficient integrity to allow successful qPCR amplification, and thus some data cells were missing. To permit further analysis with these data we used the listwise exclusion function on the MANOVA, to avoid biasing the variance. The effect of fasting on specific tissues was assessed using Fisher's least-significant difference (LSD) post-hoc tests. Comparisons between mean plasma steroid concentrations and those in other tissues were conducted using two-sample *t*-tests. The interrelationships between the change in body mass and mRNA or steroids were analyzed in different tissues using Pearson correlations. To assess and report the effect size of the MANOVA analysis, we used the partial eta squared (η^2p), with a value greater than 0.24 to indicate a large effect size. All analyses were performed using Sigma Plot version 13 (Systat Inc., San Jose, CA) with alpha level set at 0.05. Response variables with before and after data are presented as lines plots with raw data, and all other data are reported as means ± standard error.

3. Results

3.1. Fasting paradigm

Although body mass decreased with fasting overall ($F_{1,39} = 4.17$, $P = 0.006$, $\eta^2p = 0.481$), it was only significant following 8 days of fasting (Fig. 1A). In contrast, there was no difference between fasted and control subjects for both circulating triglycerides ($F_{1,39} = 0.38$, $P = 0.540$, $\eta^2p = 0.09$; Fig. 1B) and glycerol ($F_{1,39} = 0.92$, $P = 0.811$, $\eta^2p = 0.002$; Fig. 1C), although there was a noticeable non-significant decrease in triglycerides after the 8-day fast (Fig. 1B). Levels of β-hydroxybutyrate increased with fasting after 6-days ($F_{1,39} = 9.31$, $P \leq 0.001$, $\eta^2p = 0.952$; Fig. 1D). Sampling order had a significant effect on triglyceride levels, with samples collected earlier being higher ($F_{1,39} = 3.65$, $P = 0.032$, $\eta^2p = 0.414$), however sampling order and trial number did not influence any other variables (all $p \geq 0.51$). Correlational analysis reveal a significant negative relationships between the change in body mass and the change in β-hydroxybutyrate ($r = 0.47$; $N = 80$, $P = 0.013$), and between concentrations of triglycerides and β-hydroxybutyrate ($r = 0.39$; $N = 80$, $P = 0.042$). Other variables were not correlated (all $P \geq 0.13$). Based on this data, we selected the 8-day fast as our food restriction testing paradigm (i.e., FASTED treatment) for subsequent studies.

3.2. Plasma steroids and fasting

As expected, fasting increased circulating corticosterone concentrations ($F_{1,29} = 5.11$, $P \leq 0.001$, $\eta^2p = 0.304$), whereas control subjects did not significantly alter levels during this time (Fig. 2A). Levels of cortisol were an order of magnitude lower than those of corticosterone and was highly variable (Fig. 2B). Fasted subjects did not differ from controls in cortisol concentrations in circulation ($F_{1,29} = 1.03$, $P = 0.631$, $\eta^2p = 0.002$). Fasted subjects had higher circulating DHEA levels compared to fed controls ($F_{1,29} = 4.15$, $P = 0.003$, $\eta^2p = 0.207$; Fig. 2C), however the increase in DHEA concentrations within the fasted subjects was not significant (Fig. 2C). Neither sampling order nor body mass influenced plasma steroid concentrations (all $P \geq 0.093$).

3.3. Tissue steroids

Tissue type had a significant effect on corticosterone concentrations ($F_{1,20} = 11.61$, $P \leq 0.001$, $\eta^2p = 0.763$), with the highest levels predictably in the adrenal gland (Fig. 3A). Corticosterone was detectable in

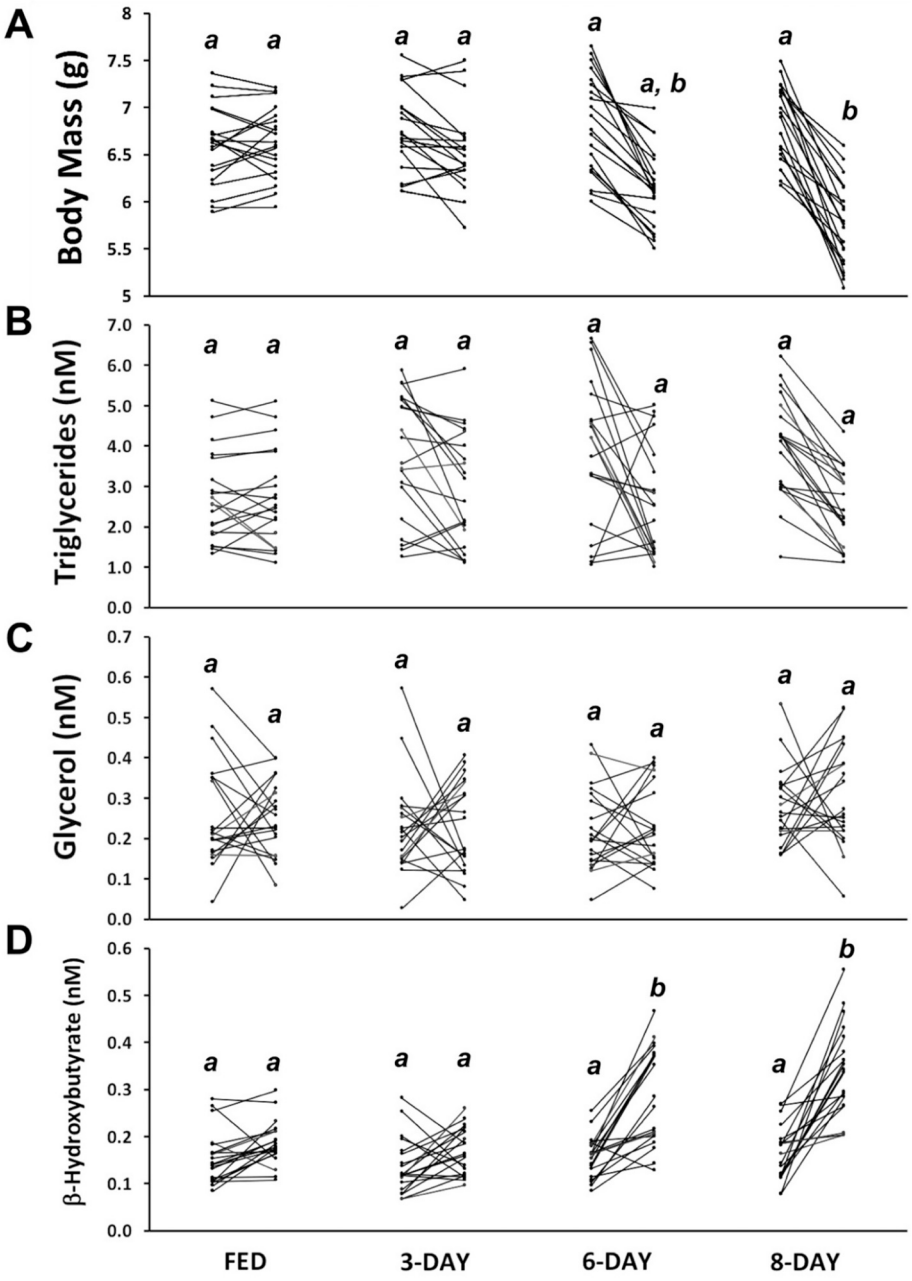

Fig. 1. Before and after **A**) body masses and plasma concentrations of energy substrates: **B**) triglycerides; **C**) glycerol; and **D**) β-hydroxybutyrate, in *Anolis sagrei* exposed to varying durations of food restriction treatments from a 1 day (FED) to an 8 day fast. Shared letters indicate no significant differences at $P \geq 0.05$ as determined by LSD post-hoc tests between groups.

all tissues, but not in all individual samples both the control and fasted subjects respectively in blood ($N = 4, 3$); heart ($N = 8, 7$); stomach ($N = 3, 5$); pancreas ($N = 6, 4$); hemipenes ($N = 5, 9$); and muscle ($N = 4, 5$). Plasma corticosterone concentrations were significantly lower than in the adrenals ($t = 7.39$, df $= 24$, $P \leq 0.001$); heart ($t = 3.11$, df $= 14$, $P = 0.008$), lungs ($t = 3.06$, df $= 24$, $P = 0.010$), liver ($t = 3.70$, df $= 24$, $P \leq 0.001$), and the intestine ($t = 4.10$, df $= 24$, $P \leq 0.001$). Fasting affected tissue corticosterone concentrations ($F_{1,24} = 3.23$, $P \leq 0.001$, $\eta^2p = 0.416$), and this varied across tissues ($F_{2,13} = 7.05$, $P \leq 0.001$, $\eta^2p = 0.15$) particularly in plasma ($P = 0.45$), lung ($P = 0.026$), liver ($P = 0.40$) and the kidney ($P = 0.031$).

Concentrations of DHEA varied across tissue types ($F_{1,21} = 4.37$, $P \leq 0.001$, $\eta^2p = 0.311$, Fig. 3B), however as with corticosterone, it was variably detectable and measurable in control and fasted samples, respectively for blood ($N = 5, 4$); stomach ($N = 4, 6$); pancreas ($N = 1, 3$); and muscle ($N = 5, 7$). The highest DHEA levels were again in adrenal tissue, but high concentrations were also observed in the heart and intestine (Fig. 3B). However, only the adrenals ($t = 10.15$, df $= 24$, $P \leq 0.001$) and heart ($t = 3.88$, df $= 24$, $P = 0.046$) had DHEA concentrations that exceeded plasma. Interestingly, in contrast to the widespread effects observed for corticosterone, fasting only increased DHEA concentration in the intestine above the levels found in control subjects ($P = 0.002$), however the overall effect of fasting on DHEA was not significant ($F_{1,21} = 0.67$, $P = 0.392$, $\eta^2p = 0.004$). In addition, tissue steroid concentrations were not significantly affected by initial body mass ($F_{1,21} = 0.11$, $P = 0.737$, $\eta^2p = 0.006$) and sampling order ($F_{1,21} = 0.26$, $P = 0.520$, $\eta^2p = 0.007$).

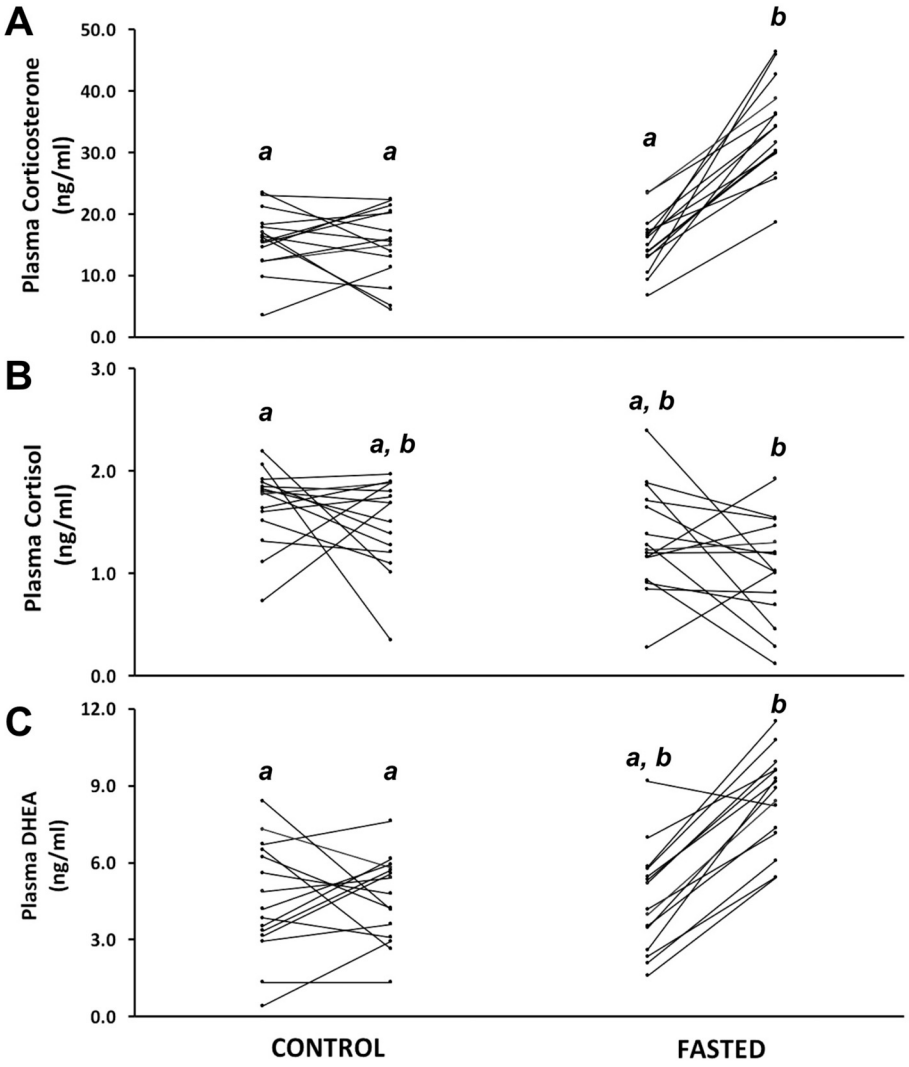

Fig. 2. Changes in plasma concentrations of **A**) corticosterone, **B**) cortisol, and **C**) DHEA in *Anolis sagrei* exposed to either a control treatment or an 8-day fasted period. Shared letters indicate no significant differences at P ≥ 0.05 as determined by LSD post-hoc tests between groups.

3.4. Gene expression

For all tissues, except plasma and blood cells, mRNA isolation and cDNA synthesis were successful. However, amplification of Star and/or Cyp17a1 (Fig. 4A) was only successful in seven tissues: adrenal, brain, intestine, liver, lungs, testes, and hemipenes. Furthermore, Actb was also successfully amplified in the pancreas, heart, and muscle (Table S1).

Expression of Cyp17a1 mRNA, normalized to Actb, varied with tissue type ($F_{1,8} = 6.11$, $P = 0.038$, $\eta^2p = 0.27$) and was detected in both control and fasted samples, respectively for adrenals ($N = 6, 6$); brain ($N = 5, 4$); intestine ($N = 5, 6$); liver ($N = 4, 5$); and testes ($N = 6, 6$). We detected a significant increase in normalized Cyp17a1 mRNA expression with fasting compared to controls ($F_{1,8} = 3.05$, $P = 0.043$, $\eta^2p = 0.19$), with post-hoc analysis revealing the effect in the adrenal (Fig. 4B) and the intestine (Fig. 4C), but not in brain ($P = 0.67$), liver ($P = 0.523$) or testes ($P = 0.473$). Adrenal Cyp17a1 expression was negatively correlated with the change in body mass ($r = 0.31$; $N = 12$, $P = 0.040$), but intestinal Cyp17a1 expression was not correlated with mass ($r = 0.20$; $N = 11$, $P = 0.582$). No effect of sampling order on mRNA for any tissue was detected ($F_{1,8} = 1.01$, $P = 0.141$, $\eta^2p = 0.005$).

The expression of Star mRNA varied across tissue types ($F_{1,10} = 5.13$, $P \leq 0.001$, $\eta^2p = 0.24$), and was detected in control and fasted samples, respectively, from the adrenals ($N = 6, 6$); brain ($N = 3, 3$); lungs ($N = 0, 4$); hemipenes ($N = 1, 2$); liver ($N = 5, 6$); and testes ($N = 6, 6$). Fasting also had a significant overall effect on Star gene expression as compared to fed control subjects $F_{1,10} = 3.05$, $P = 0.015$, $\eta^2p = 0.18$). Unlike Cyp17a1, the expression of Star in the adrenal did not differ between fasted and control subjects (Fig. 4D). Similarly, Star expression also did not differ between groups in the brain ($P = 0.637$), hemipenes ($P = 0.904$), and testes ($P = 0.268$). However, fasted subjects expressed higher mRNA levels in the liver than controls (Fig. 4E). Only 4 of 9 lung samples were successfully amplified by qPCR, however these were all derived from fasted subjects, with no amplification in controls (Fig. 4F). The normalized mRNA levels for Star expression was not influenced by changes in body mass ($F_{1,10} = 0.15$, $P = 0.856$, $\eta^2p = 0.002$), nor sampling order ($F_{1,10} = 0.073$, $P = 0.911$, $\eta^2p = 0.001$). No significant correlations between Star mRNA and the change in body mass was observed for any tissue (all $P \geq 0.481$).

4. Discussion

There is considerable evidence for extra-adrenal steroid synthesis leading to the premise that certain conditions may favor a shift from circulating hormones to local steroidogenesis. However, very few studies have identified the conditions that spur local steroidogenesis. We used an 8-day fast in brown anoles to detect increases in plasma corticosterone and DHEA, but not cortisol. Fasting also increased steroids levels above plasma concentrations in many tissues as well as increased

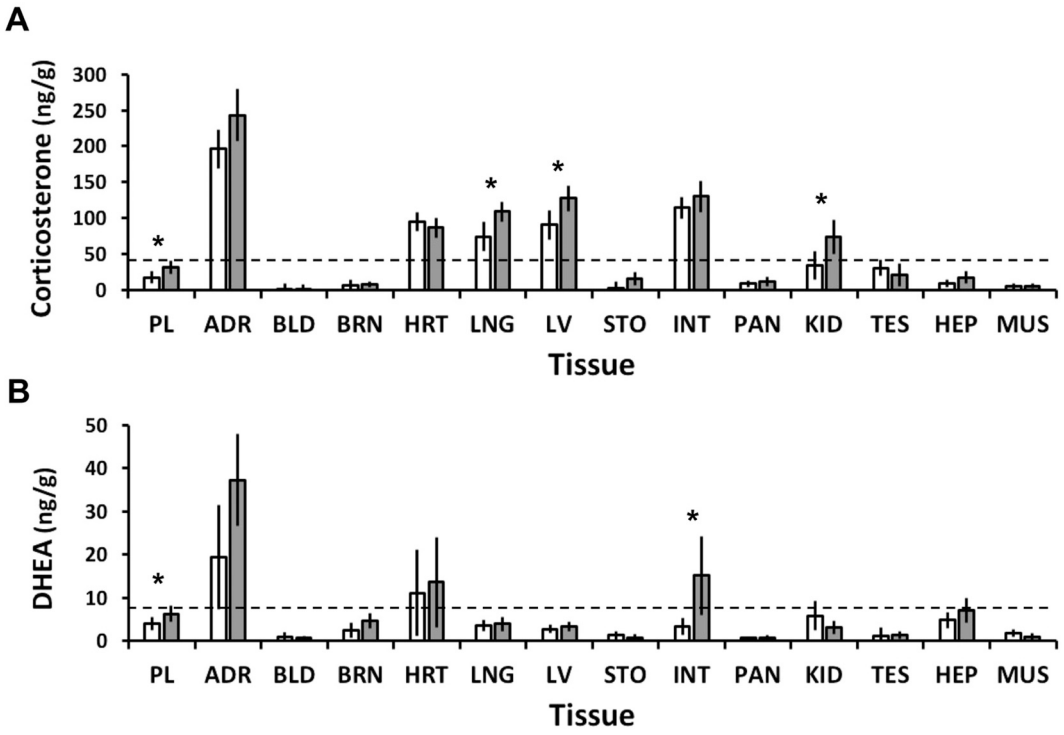

Fig. 3. Concentrations of **A**) corticosterone and **B**) DHEA from plasma and other tissues collected from fed control (white bars) and 8-day fasted (gray bars) *Anolis sagrei* lizards. Tissue types include: plasma (PL); adrenals (ADR); red blood cells (BLD); brain (BRN); heart; (HRT); lungs (LNG), liver (LV); stomach (STO); small intestines (INT); pancreas (PAN); kidney (KID); testes (TES); hemipenes (HEP); and muscles (MUS). Dashed lines indicate the maximum circulating concentrations of the respective steroid in *A. sagrei*. Bars are presented as means with ± standard error bars and * indicates significant differences at $P > 0.05$ between controls and fasted groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Star and/or Cyp17a1 gene expression in some of these tissues. These data provide evidence for fasting resulting in a significant, albeit complex effect on extra-adrenal steroidogenesis.

4.1. Metabolic effects of fasting

Subjects predictably lost body mass and increased concentrations of β-hydroxybutyrate in plasma, which suggests fatty acid oxidation is occurring, possibly due to depleted sources of glucose (Newman and Verdin, 2017). These results are consistent with other reptile studies (de Souza et al., 2004; Price et al., 2013), yet β-hydroxybutyrate may not directly correlate with mobilization of reptilian fat bodies (Price, 2017). As the primary vertebrate fat storage molecule, the plasma triglyceride concentrations normally wane as dietary input declines (Price, 2017), since they are hydrolyzed into glycerol to maintain hepatic gluconeogenesis (Guglielmo et al., 2002; Wang et al., 2019). Here, triglycerides appeared to decline but this was not significant, and no effect on glycerol was observed. Such mixed results have been reported in snakes, where it may be related to specific activity patterns (Butler et al., 2016; Neuman-Lee et al., 2015). During fasting, vertebrates mobilize carbohydrate stores followed by fat reserves, and then proteins and nucleic acids, in order to sustain glucose levels (Belkhou et al., 1991; Castellini and Rea, 1992; Cherel et al., 1988). We did not measure glucose in this study as it is tightly regulated, yet it has been known to decrease with fasting (Oliveira et al., 2013). Thus, the decline in body mass likely reflects increased fat oxidation in the liver, and not necessarily catabolism of triglycerides in fat bodies.

4.2. Steroids in circulation

During fasting, GCs mobilize reserves, but simultaneously exacerbate the existing energy shortage and thus fasting is a physiological stressor. The increase in plasma corticosterone in fasted anoles is consistent with our understanding of GCs as catabolic hormones (Sapolsky et al., 2000; Dallman et al., 2003). Cortisol was detectable at levels an order of magnitude below corticosterone but was not affected by fasting. This supports cortisol as the non-dominant GC in *Anolis*, however very few studies have reported cortisol in reptiles. Our cortisol assay has low cross-reactivity for corticosterone, and vice versa (1.2% and 0.38% respectively, Arbor Assays Inc) and synthesis of either GC requires the same enzymes, but the early attachment of a hydroxyl group on pregnenolone requires Cyp17a1 (Akhtar et al., 2011), which we detected in the *Anolis* adrenals. Thus, both GC types are present in *Anolis*. Functionally, cortisol has a higher potency than corticosterone for both the mineralocorticoid and glucocorticoid receptors (Sapolsky et al., 2000). Even in corticosterone-dominant species, many organs exhibit high cortisol concentrations (Ovejero et al., 2013; Taves et al., 2016, 2011) and the ratio of these two GCs can vary under different physiological conditions (Gong et al., 2015; Ovejero et al., 2013). Since non-dominant GCs can still interact with receptors, they may be involved in paracrine functions in a tissue specific manner. The androgen DHEA is thought to exert physiological effects via its conversion to more potent androgens (Labrie and Labrie, 2013; Miller, 2002), despite weak binding to some receptors (Webb et al., 2006), although see Widstrom and Dillon (2004). Interestingly DHEA may reverse some negative effects of GC activation (Hu et al., 2000) leading to its characterization as an "anti-stress" hormone. Increased plasma DHEA here may reflect such protection of tissues from the simultaneous corticosterone release. However, DHEA is also an androgen precursor, and thus its presence in circulation may simply reflect increased testosterone production, considering anoles were in breeding condition. This increase in testicular secretion of testosterone could also support the higher aggression in fasted animals. Alternatively, studies have shown that DHEA may facilitate antagonistic behavior in males during periods when gonadal testosterone is low, by being converted to bioactive androgens and estrogens in the brain (Soma et al., 2015). Unfortunately, limited plasma volumes prevented us from

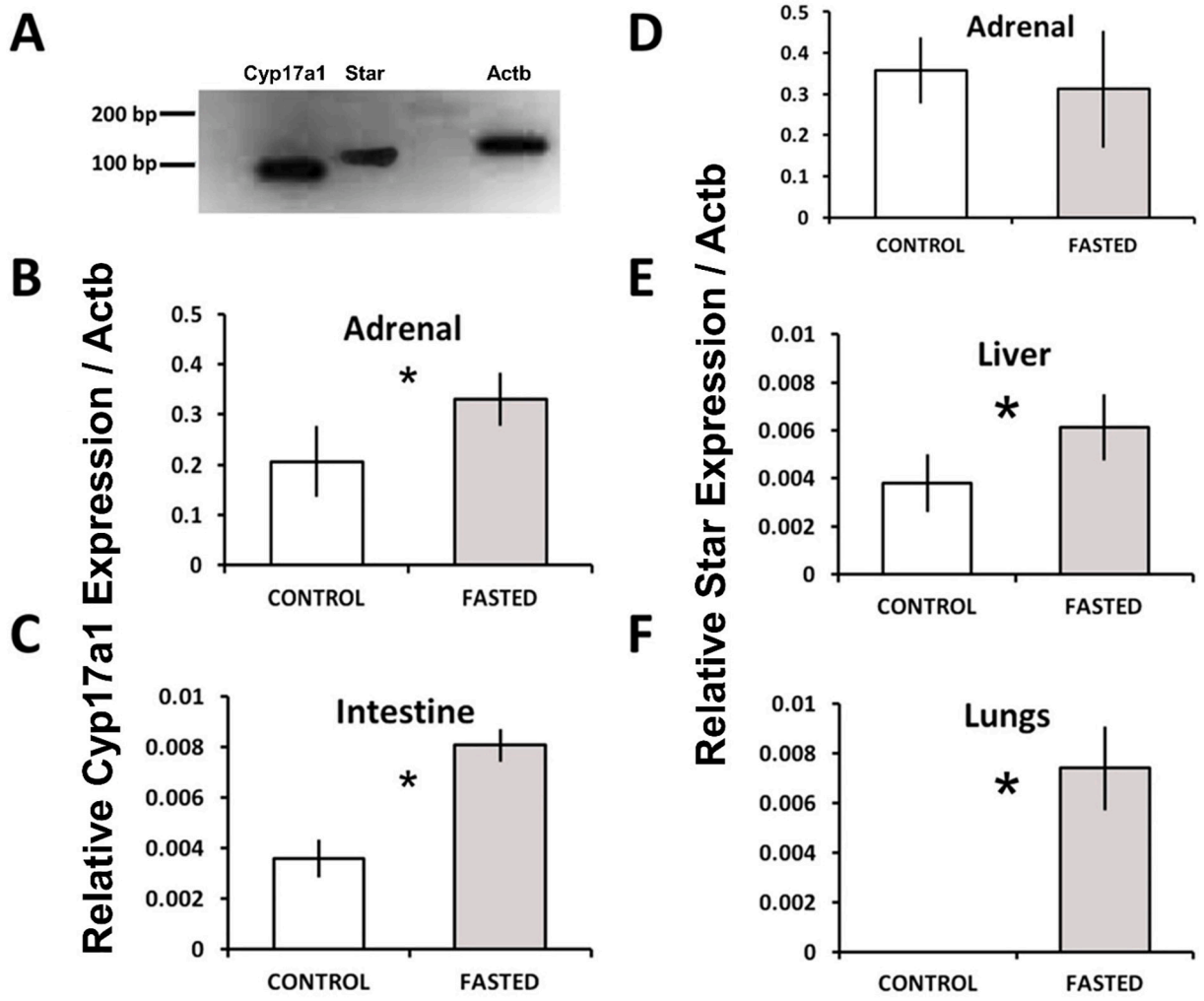

Fig. 4. Expression of genes for steroidogenic enztymes in *Anolis sagrei* using qPCR. **A**) Electrophoretic band sizes in base pairs (bp) for target genes: steroidogenic acute regulatory (Star) protein and steroid 17-alpha-hydroxylase/17,20 lyase (Cyp17a1) and the housekeeping gene: Actb, obtained from adrenal tissue. Gene expression for Cyp17a1 enzyme in **B**) adrenals and **C**) intestinal tissue and for Star protein in **D**) adrenals, **E**) liver and **F**) lung, derived from fed control (white bars) and 8-day fasted (gray bars) *A. sagrei* lizards. Both genes are presented in arbitrary units normalized to Actb expression. Bars are presented as means with ± standard error bars and * indicates significant differences at $P > 0.05$ between controls and fasted groups.

measuring sex steroid concentrations in this study, however fasting and chronic stress generally suppresses the secretion of reproductive hormones (…..). However, this has not been explored in anoles and the response of sex steroids during fasting warrants further study. Energy shortages can exacerbate social instability (Fokidis et al., 2013), and thus DHEA may serve to promote defense of food resources, however more research is warranted. Fasting also promotes hepatic DHEA secretion in rats (Grasfeder et al., 2009) which further supports a link between extra-adrenal steroidogenesis and energy shortage.

4.3. Evidence for extra-adrenal steroidogenesis

Local steroidogenesis can occur either de novo from the uptake and conversion of cholesterol, or through metabolism of peripherally sourced steroid precursors (Hu et al., 2000). Steroidogenesis requires all necessary enzymes, cofactors, steroid precursors, intermediate metabolites, and any local machinery required to initiate, regulate, and inhibit the rate of steroid production. This information is essentially unknown for reptiles, and instead we identified tissues with steroid concentrations that exceeded plasma, as potential sites of local steroidogenesis and investigated them for the presence of two genes responsible for initiating and limiting steroidogenesis. Predictably corticosterone was highest in the adrenal, but concentrations were also observed in the heart, lung, liver, and intestine at levels above plasma. Similarly, DHEA was highest in the adrenal tissue but was also found at higher than plasma concentrations in the heart. A technical concern with tissue steroids is blood contamination of organs, however Taves et al. (2010) reported saline perfusion did not alter brain steroid concentrations. Furthermore, vascularized tissues (e.g., lung, liver) had high corticosterone, but low DHEA levels, yet these should be comparable if blood contamination was responsible.

Both Star and Cyp17a1 were detected in adrenals, brain, liver and testes, and these are consistent with studies in rats and humans (see review in Taves et al., 2011). Additionally, Star was detected in lung and hemipenes, and Cyp17a1 was found in the intestine. The presence of these two genes suggests the possibility of de novo steroid synthesis, however we could not successfully amplify other genes that are more directly involved in corticosterone production. When actb was detectable but neither Star and Cyp17a1 was observed we concluded this is evidence that steroidogenesis does not occur, as was the case with the pancreas. We also could not rule out steroidogenesis in the stomach or kidneys based on our data, however this unlikely based on the low steroid concentrations from these tissues. Future studies must test for additional steroidogenic genes. Intracellular cholesterol transport by

Star is the first step in steroidogenesis, and this is followed by its conversion to pregnenolone by the side-chain cleavage enzyme, P450scc encoded by the Cyp11a1 gene (Terry et al., 2010). Thus, the co-expression of Cyp11a1 and Star renders a tissue steroidogenic, and in support, observations of Cyp11a1 knockout mice suffer from steroid deficiency (Hu et al., 2002). Although we did not measure Cyp11a1 expression is co-expressed with Star (Lee et al., 2016). Corticosterone concentration did not appear directly related to Star expression as the brain, testes and the hemipenes all had low corticosterone levels, and perhaps gene expression in these organs is related to synthesis of reproductive hormones. The adrenals, testes, brain and the liver were the only organs to express both genes, but only the liver had corticosterone levels above plasma. As the mammalian liver expresses the enzyme, 11β-hydroxysteroid dehydrogenase, which converts inactive metabolites (e.g., cortisone) into active GCs (Raza et al., 2010), it is conceivable that the liver is capable of GC steroidogenesis.

Expression of Cyp17a1 was detected in the liver, but hepatic DHEA concentrations were lower than in plasma and adrenals, suggesting the latter as the main source for circulating DHEA, however the heart and intestine also had high levels. Interestingly, the heart exhibited high corticosterone and DHEA levels, but neither enzyme was detectable. Thus, steroid metabolism in the heart is likely not de novo despite the necessary metabolic machinery having been identified in the mammalian heart (Young et al., 2001), including the capacity for mineralocorticoid synthesis (but see Gomez-Sanchez and Gomez-Sanchez, 2001). One explanation for the lack of steroidogenic genes in cardiac muscle of *A. sagrei* may be the evolutionary divergence of mammals and reptiles about 310 to 275, with cardiac steroidogenesis performing necessary functions in homeothermic mammals, such as promoting cardiac hypertrophy (Ohtani et al., 2009), but not heterothermic reptiles.

4.4. Fasting and a functional role for locally produced steroids

Despite evidence for extra-adrenal corticosterone and DHEA synthesis in *A. sagrei*, their functions and how they differ from adrenal steroids remains unanswered. Local steroidogenesis may permit organisms to maintain high GC concentrations at specific tissues or times where they are particularly beneficial (Taves et al., 2017). For example, high local GC synthesis in the thymus, intestine and skin may restrain potentially harmful immune responses, all while circulating GCs maintain metabolic functions at an organismal level (Taves et al., 2011). Such autonomous regulation may counteract deleterious GC effects (i.e., allostatic load) since organ GCs can increase, while negative feedback depresses systemic GC concentrations (Taves et al., 2011). Local corticosterone may act as an intracellular signaling molecule that modulates tissue activity during fasting. The widespread tissue GC increases seen with fasting may involve GCs guarding tissues from damage or permitting tissue processes to continue under stress. Despite extra-adrenal steroidogenesis being well-reported, the tissue functions of these hormones are unexplored, but tissue responses to systemic GCs may offer some insight. For example, GCs effects on the lungs include improving airway conduction, surfactant secretion, reducing bronchial inflammation and regulating immunity, and local GCs can perpetuate these functions during stress (Gerber, 2015). Intestinal GCs may maintain epithelial mucosa and provide an anti-inflammatory immune barrier against infection, which may increase with fasting (Kostadinova et al., 2014). Mounting immune challenges invests significant energy, and during food shortages, upregulating local GCs may offset the costs of maintain high circulating GC levels, and this provides opportunities for further study. Contrary to GCs, local DHEA did not significantly increase with fasting, although a trend was seen in the intestines. In other studies, hepatic DHEA increased with fasting (Fokidis et al., 2013; Grasfeder et al., 2009). Here only the heart had significant DHEA levels above plasma, but whether this was derived from de novo synthesis or precursors is not known. Studies are very few, but clinical research suggests DHEA and its sulphated form, DHEA-S, can protect cardiovascular activity during high GC levels (Mannic et al., 2015).

5. Conclusion

This study adds a reptile to growing research in mammalian and avian systems describing extra-adrenal steroidogenesis. As an established endocrine model, *Anolis* lizards are known for links between social behaviors and steroids with much research on common steroids having been done. Using high-throughput methods, such as liquid chromatography tandem mass spectrometry, and investigating additional steroidogenic genes we can elucidate a complete steroidogenesis profile during fasting (see Prior et al., 2016). This can then permit experiments using adrenalectomy and pharmacological approaches to determine if these tissues have autonomous HPA axes and begin to understand their functional roles during food shortages.

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Author contribution statement

RH, AS and HBF designed the experiments. RH, AS and HBF performed the experiments. RH, and HBF analyzed the data. HBF wrote the paper and was responsible for research supervision, coordination and strategy.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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