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Steroidogenesis in the Green Anole Lizard Brain and Gonad

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Abstract

Seasonally breeding animals reproduce during certain times of the year and, subsequently, behaviors, steroid hormone levels, and brain morphology change. The green anole lizard (*Anolis carolinensis*) is an excellent model to study the regulation of steroid hormone production because they have distinct hormonal and behavioral differences between sexes and seasons. As in other vertebrates, steroidogenesis in anoles is under the control of the hypothalamus-pituitary-gonadal (HPG) axis. We tested the hypothesis that natural variations in steroid hormone levels between sexes and seasons are mediated within the brain and gonad by examining four genes involved in steroidogenesis: StAR, Cyp17α1, HSD17β3, and Cyp19α1. Adult male and female lizards were wild-caught during both the breeding (BS) and non-breeding (NBS) seasons. Gonads, brains, and blood were collected and stored at -80 °C. RNA from brain and gonad was extracted, reverse transcribed into cDNA and then gene expression was measured by qPCR (normalized to β actin). We found that whole brain mRNA expression of StAR, Cyp17α1, and HSD17β3 have no differences between sex or season. Cyp19α1 mRNA expression in the brain was increased during the NBS in females, potentially revealing the presence of regulatory signaling for aromatase expression in the brain. In the anole gonad, StAR mRNA expression levels were increased in both males and females during the BS, while the expression levels of many of the other steroidogenic enzymes are increased when StAR expression is decreased, suggesting that the enzymes in the steroidogenic pathway are, in fact, potentially regulated independently of StAR. This work expands knowledge on the seasonal regulation of steroidogenesis in both the brain and gonad of a reptilian species but more work is necessary to further determine the regulatory mechanisms.

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Introduction

Steroid hormones consist of many different classes such as progestogens, corticosteroids, androgens, and estrogens (Reviewed by Luu-The 2013). Steroid hormone synthesis is comprised of a cascade of oxidative enzymes that transform cholesterol into different steroids in a process known as steroidogenesis (Rocha *et al.* 2009) (Figure 1). These hormones are then delivered into circulation and perform their action away from the site where they were produced. It is reported that gonads and other tissues, including the brain, produce sex steroid hormones (Van, 2013 ; Reviewed by Luu-The et al., 2005). Steroidogenesis that occurs in the gonads resulting in sex steroid hormone production is under the control of the hypothalamic-pituitary-gonadal (HPG) axis system.

The HPG axis is a biological pathway that results in the production of gonadal steroid hormones. The pathway starts with the hypothalamus of the brain, which releases gonadotropin releasing hormone (GnRH) to the anterior pituitary, which activates the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) into serum. LH then enters gonadal tissue to it trigger the production of steroidogenic acute regulatory protein (StAR), the rate-limiting step in steroidogenesis (Carr 1998; Reichlin 1998). The sex steroids that are produced, negatively feedback to the hypothalamus resulting in a decrease in the amount of gonadotropins secreted. Without each key piece of the process the body would not be able to produce or regulate any steroid hormones (Meethal & Atwood 2005). Many of the steps of the HPG axis system have been thoroughly studied. However, the steroidogenesis rate-limiting step and many enzyme conversions within the steroidogenesis pathway are less studied, and might reveal how steroid hormone levels are regulated at the production level.

StAR

A key step in steroidogenesis is getting cholesterol to the first steroidogenic enzyme; P450scc. P450scc is confined to the inner membrane of the mitochondria while cholesterol accumulates in the outer mitochondrial membrane. Cholesterol is hydrophobic, not allowing diffusion through the intermembrane space and, as a result, StAR is necessary to mediate the delivery of cholesterol to the inner mitochondrial membrane (Reviewed by Sierra 2004). Once StAR shuttles cholesterol to the inner mitochondrial membrane, P450scc cleaves the side chain of cholesterol, beginning steroid hormone synthesis (Kallen *et al.* 1998; Reviewed by Manna *et al.* 2009). The theca and granulosa cells in the ovary, leydig cells in the testes, and adrenal cortical cells of the adrenal glands have been found to contain StAR enzyme (Van, 2013). StAR mRNA has been also been detected by northern blot, southern blot, and PCR analysis in human ovaries, testis, and kidneys (Sugawara *et al.* 1995). Several reports demonstrate the role of StAR in steroidogenesis. In mouse leydig tumor cells, hormone stimulation by LH induced the expression of StAR, ultimately resulting in an increased synthesis of pregnenolone(Clark *et al.* 1994). Pregnenolone synthesis has also been seen to be induced by StAR via radioimmunoassay in rhesus monkey kidney cells (Sugawara *et al.* 1995). Additionally, known StAR gene mutations cause lipoid congenital adrenal hyperplasia, which is characterized by the inability to metabolize cholesterol into steroid hormones (Reviewed by Miller 1997).

The brain has been found to be an independent steroidogenic tissue, both by expressing StAR and the other necessary enzymes needed to convert cholesterol into steroid hormones (Reviewed by Sierra 2004) and by regulating the amount of cholesterol

present through *de novo* synthesis (Barres & Smith 2001). In the brain, the most active steroidogenic cells are astrocytes which produce neuroprogesterone (Zwain & Yen 1999) and StAR has been localized in these cells; therefore studying StAR expression may be a useful indicator of active neurosteriodogenesis (Karri *et al.* 2007). StAR has also been found to be widely distributed in the adult rat brain as seen by RNase protection assay, while selectively restricted to particular cell populations like glial cells, neurons, and proliferating precursors (Furukawa *et al.* 2002). The mechanism used by the brain during the rate-limiting step of steroidogenesis remains unknown, but since StAR is an essential protein in steroidogenesis, this widespread presence suggests a mechanism that can control local steroid supply in a spatio-temporal manner, potentially influencing brain functions such as neuronal survival, neurogenesis, myelination, and synaptic plasticity

Cyp17α1

Cyp17α1 is a gene that codes for cytochrome P450 17α-hydroxylase/C17-20lyase, an enzyme that plays an important role in the steroidogenesis pathway by catalyzing the synthesis of androgenic precursors (George *et al.* 2008). This enzyme converts progesterone (P) into 17-hydroxyprogesterone, as well as pregnalone into 17-hydroxypregnenolone and further converts it into dehydrepiandrosterone (DHEA) (Figure 1). These hormone precursors are further processed into sex hormones and glucocorticoids (Spatz 2004). In the human adrenal glands Cyp17α1 activity is found both in the fasciculata and reticularis zones of the cortex showing consistency with its role to catalyze both cortisol and DHEA (Reviewed by Rainey *et al.* 2002). In the gonads of the sea bass (*Dicentarchus labrax*), Cyp17α1 activity levels were was found to be sexually dimorphic, with males having higher levels than females (Blanco *et al.* 2016).

There is very little research available that examined Cyp17α1 expression in the brain. The only research I have found claims that in Sprague-Dawley rats, Hartley guinea-pigs adrenal glands and brain by immunohistochemistry (Le Goascogne *et al.* 1991), and through *in vivo* experiments in the adult mouse brain Cyp17α1 expression and activity are undetectable (Liu *et al.* 2009).

HSD17β3

17 beta-hydroxysteroid dehydrogenases (17β-HSD) are a group of enzymes that have many functions including moderating concentrations of steroids, bile and fatty acids. These enzymes are found in many vertebrates, invertebrates, and microorganisms. There are multiple forms of 17β-HSDs that exist, with very different roles. For example, type 1 catalyzes the reduction of estrone into estradiol (E2) while type 3 (HSD17β3) helps convert androstenedione to testosterone (T) (Reviewed by Mindnich *et al.* 2004). HSD17β3 specifically plays an important role in the conversion of precursor hormones into T (Figure 1). It is primarily expressed in the testes (Mindnich *et al.* 2004) detected by a pCMV expression vector (Andersson *et al.* 1995), however, it has also been found through RT-PCR in human adipose tissue (Corbould *et al.* 1998), the brain temporal lobe (Stoffel-Wagner *et al.* 1999), and primary osteoblast-like cells (Feix *et al.* 2001). HSD17β3's main function is to convert androstenedione into T within the testes (Reviewed by Mindnich *et al.* 2004) but it has also been shown that androstenedione can be converted into T within the human temporal lobe of the brain (Watzka *et al.* 1999). A dysfunction in this enzyme in humans results in neuronal diseases and reproduction disorders (Reviewed by Mindnich *et al.* 2004).

Cyp19α1

Cyp19α1 is another cytochrome P450 enzyme, better known as aromatase. It converts androstenedione into estrone and T into E2, the most active estrogen (Figure 1) (George *et al.* 2008). Cyp19α1 has been identified in the brains of many different vertebrate species. Aromatase appears to play a major role in controlling reproductive functions, especially male sexual behavior. For example, in Japanese quail (*Coturnix japonica*), male copulatory behavior is activated when T has been aromatized into E2 (Balthazart & Foidart 1993). In the male quail, behavioral effects of T can be simulated by administration of natural or synthetic estrogens but not by non-aromatizable androgens suggesting that aromatase does indeed play a crucial role in causing male reproductive behaviors (Balthazart 1997). Aromatase has also been found to facilitate female receptivity. For example, aromatizable androgens trigger female copulatory behavior in female musk shrews (*Suncus murinus*) (Rissman 1991). In the Atlantic croaker (*Micropogonias undulates*), Cyp19α1 expression has been seen to change with gonad function, in females increased levels are seen in developing gonads while decreased levels are noted at the end of vitellogenesis, supporting that estrogen plays a role in promoting and sustaining oocyte growth (Nunez & Applebaum 2006). In males Cyp19α1 expression does appear to have an overall increase in spawning animals (Reviewed by Abney 1999). Estrogens are known to interrupt cell development and function of the mammalian testis, therefore low Cyp19α1 expression in the Atlantic croaker developing testes minimizes the estrogen-dependent inhibition of androgen synthesis needed for germ development (Reviewed by Hess 2003).

HPG axis regulation

The expression of the components of the HPG axis (GnRH, LH, etc.) can be altered due to seasonal changes or sex differences, leading to changes in reproductive activity as well as circulating steroid hormones. In amphibians, such as the green frog (*P. esculentus*), males have higher brain mRNA expression levels of StAR, HSD17β3, and Cyp19α1 during their reproductive period compared to their non-reproductive period, while females only showed seasonal differences of HSD17β3 and Cyp19α1 mRNA expression in the brain using real-time quantitative RT-PCR (Santillo *et al.* 2017). In green frogs it has also been observed that enzymes of neurosteroidogenesis correlates with the seasonal changes in the circulating sex hormone levels, and as hormone levels rise so does enzyme activity (Rastogi *et al.* 2005). It has been well established that domesticated sheep are seasonally breeding, it has also been seen that their steroid hormones fluctuate seasonally (Martinet *et al.* 1993; Reviewed by Lincoln 2002). During their breeding season elevated levels of P and E2 cause the expression of female estrus behavior and have a positive feedback on production of T by the testis, in turn triggering libido expression in males (Tulley *et al.* 1983; Tilbrook & Cameron 1990). Within the central nervous system of rams it has been seen that T is converted to E2 by aromatase (Perkins & Roselli 2007). There is further evidence that DHT, a metabolite of T, is responsible for sexual behavior maintenance in rams. The brain regions of rams where male reproductive behaviors are controlled are seen to be enriched with aromatase noting that aromatase is present with the brain as well as the gonads (Roselli *et al.* 1998, 2000).

Reptiles are interesting to study as they are ancestors to both birds and mammals (Fountaine *et al.* 2005). Most lizards are seasonally breeding and are known for their

periodic cycles of active and inactive gonads (Gautam *et al.* 2013). These seasonally breeding lizards are a great model for studying seasonal expression levels of genes involved in steroidogenesis, meiosis and many other reproductive processes which are not as easily detected in other species and are desperately lacking in previous research information.

Green anole lizards

Green anole lizards (*Anolis carolinensis*) are reptiles known for seasonal behavioral and hormonal level changes (Jones *et al.* 1983; Rosen & Wade 2001). They are found in southeastern United States with their reproductive season occurring from April through July (Lovern *et al.* 2004). The green anole has a very distinct breeding season (BS) and non-breeding season (NBS) and because of this it is one of the main reptilian species of endocrine and behavioral research (Wade 2005).

Males are larger in body size than females and have a large red throat fan called a dewlap that extends as part of their ritualized courtship or aggressive displays. These behaviors are only performed during the BS (Lovern *et al.* 2004). Adult males have high T concentrations in the BS when territorial and courtship behaviors are high, and they have low T concentrations in the NBS when territorial and courtship behaviors are low or nonexistent (Lovern *et al.* 2001; Neal & Wade 2007); suggesting that T (or its metabolites) may be activating male reproductive behaviors during the BS. In general the adult female anole lizards have plasma T levels that are 20 to 45 fold lower then adult males (Lovern *et al.* 2001). Adult female lizards also have seasonally dimorphic behaviors with increased receptivity during the BS (Reviewed by Wade 2012). A

receptive female once mated with a male will become unreceptive within minutes and will stay this way until the next ovulatory cycle (Jones *et al.* 1983). A ratio of low circulating levels of E2 to P have been seen to play a role in female receptivity and the mating-induced receptivity termination while an even E2/P ratio correlates with non-receptivity behavior (Jones *et al.* 1983). In the male brain it has been seen that administering E2 and having it be present is associated with increased motivation for male copulation behaviors (Latham & Wade 2010). Therefore, due to the highly seasonally and sexually dimorphic behaviors and hormone levels green anoles are an excellent model organism to study seasonal regulation of steroid hormone production.

Aromatase and E2 have been characterized within the brain of the green anole lizard. In whole brains from BS males and females there was higher aromatase activity in males, and in NBS animals the enzyme activity was equivalent between sexes. Comparing BS to NBS males there was significantly higher aromatase activity in the BS males (Rosen & Wade 2001). As seen in green anoles, seasonal and/or sex variances in neuronal aromatase activity has been documented in many species that range from mammals to telosts (Reddy *et al.* 1973; Callard *et al.* 1977, 1981, 1983; Roselli *et al.* 1985; Schlinger & Callard 1987; Schlinger *et al.* 1989; Silverin & Deviche 1991; Gobbetti *et al.* 1994; Soma *et al.* 1999). The regulation of aromatase is mostly due to T sensitivity where females are less sensitive to the effects of T than males as seen in studies on birds (Schumacher & Balthazart 1986; Steimer & Hutchison 1990), and on mammals (Reddy *et al.* 1973; Roselli *et al.* 1984, 1985; Steimer & Hutchison 1990; Hutchison *et al.* 1991; Romeo *et al.* 1999) which suggests T upregulates aromatase

protein activity in the brain regions associated with male reproductive behaviors (Rosen & Wade 2001).

Aromatase-positive cells have been documented by *in situ* hybridization techniques in specific regions of the green anole brain important for reproductive behavior, such that males have a greater total number of cells expressing aromatase mRNA in the preoptic area (POA) of the brain when compared to females (Cohen & Wade 2011). The density of these aromatase mRNA-expressing cells was also found to be higher during the BS when compared to the NBS. The regional synthesis of E2 from T may play a role in controlling sex-specific reproductive behaviors (Rosen & Wade 2001). It has been seen that E2 levels are associated with male motivation to copulate and reproduce (Latham & Wade 2010). Most other male sexual behaviors, including courtship displays, are found to be androgen dependent and copulation with a female will follow if that female is receptive (Winkler & Wade 1998; Rosen & Wade 2001). Little to nothing is known about the expression of aromatase within the reptilian gonads. Interestingly in this species and all other reptile species, to our knowledge there is no current research available examining Cyp17α1, HSD17β3, and StAR seasonal expression.

Experiment

The present study was designed to test the hypothesis that natural variations in steroid hormone levels between sexes and seasons are mediated within the brain and gonad by these four different genes: StAR, Cyp17α1, HSD17β3, and Cyp19α1, which we predicted to be upregulated during the BS compared to the NBS. To our knowledge, this

is the first study to quantify StAR, Cyp17α1, HSD17β3, and Cyp19α1 mRNA expression levels within green anole lizard brains and gonads. We investigated mRNA expression levels within whole brain and whole gonads in both BS and NBS males and females to determine whether the known hormonal differences that occur between these time points may be a result of an upregulation of one or more of these genes. StAR, Cyp17α1, HSD17β3, and Cyp19α1 genes play major roles within the steroidogenesis pathway and by looking at their expression levels in the green anole we can begin to examine seasonal regulation. The opportunity to study expression level differences occurring in the steroidogenesis pathway enzymes between seasons and sexes of green anole lizards may help determine the specific regulation factors involved with changes in steroid hormone levels.

Materials & Methods

Animals and tissue collection

Wild-caught adult green anole lizards were shipped from Charles Sullivan Company (TN) once during the BS in May 2015 and once during the NBS in October 2015 to Minnesota State University Mankato MN. They were sacrificed upon arrival and were dissected to obtain whole brain and gonads, which were frozen in cold methyl butane and stored at -80°C. Breeding state was confirmed by a visual inspection of each lizard's reproductive system. As follows, BS females were confirmed by checking the ovaries for large yoking follicles. NBS females were confirmed by ensuring the ovaries were small with no follicle enlargement. BS males were confirmed by checking for enlarged testes and NBS males were determined by a lack of testes enlargement.

Primer Design

Specific primer sets for β-Actin, StAR, Cy17α1, HSD17β3, and Cyp19α1 were designed from the annotated sequences in the anole genome using the National Center for Biotechnology Information (NCBI) and Ensembl software and ordered from Integrated DNA technologies (Table 1). To confirm primer specificity, PCR reactions were performed using Quick-Load *Taq* 2X Master Mix (New England Biolabs, NEB) and run on a 1.5% agarose gel to confirm band size and quality. Amplicon sequences were confirmed to match the anole gene sequences (GeneWiz). Primer concentrations were optimized for all genes (Table 1) using PCR.

RNA isolation and cDNA synthesis

Gonads from each lizard were weighed and the gonads and brains were homogenized in QIAzol and chloroform (Qiagen). RNA was extracted using the RNeasy Lipid Tissue Mini Kit (Qiagen) as per manufacturer's instruction, with an on-column DNase I (Qiagen) treatment at 25°C for 15 minutes to eliminate genomic DNA. The total RNA integrity and purity were determined by gel electrophoresis and spectrophotometry measures taken at 260/280 nm. Isolated RNA was stored at -80 °C, and 0.05 µg/µl RNA was reverse transcribed into cDNA using the ProtoScript 2 First-Strand cDNA Synthesis Kit (NEB), as per manufacturer's instructions. cDNA and was stored at -20 °C until use.

qPCR

qPCR was conducted using PowerUp SYBR Green PCR master mix (Thermo Scientific) per manufacturer's instructions. RNA control gels were run to confirm the absence of genomic DNA. PCR efficiency curves were run for each gene, using 10-fold

dilutions of cDNA (100 to 0.001 ng/µl). Expression for each gene was normalized to β-actin mRNA to determine expression level changes.

Each qPCR experiment contained 0.5 ng/µL total cDNA, SYBR green master mix, and the appropriate concentration of forward and reverse primers (Table 1). Each sample was run in triplicate, and two genes (one target gene and β-actin) were run on each plate. Each plate also contained three replicates of a negative template control (RNase-free water instead of cDNA template) for both the gene of interest and housekeeping gene (β-actin). Two plates per gene were run for each tissue type and, in order to normalize between plate runs (as appropriate), one sample was run on both plates.

The expression of individual gene targets was analyzed using the Step One Plus (Applied Biosystems) real-time PCR machine. The thermocycler program included an initial denaturation step at 95 °C (10 min) followed by 40 cycles of 95 °C (15 sec) and a combined annealing/extension step of 65 °C (1 min). An additional annealing step of 60 °C (30 sec) was added to each cycle for HSD17β3. A melt curve was also conducted for every run with steps at 95 °C (15 sec), 65 °C (1 min), and 95 °C (15 sec).

Data analysis

The qPCR efficiencies were calculated by using the critical threshold values (CT) and the linearly correlated logarithmic value of the amount of cDNA. The slope of this line was used to calculate the PCR efficiency for each gene, $E = 10^{[-1/\text{slope}]}$ (Rasmussen 2001) (Table 1). The PCR efficiencies of each gene should be approximately 2.0 for a consistent doubling of DNA at each cycle (Pfaffl 2001).

We used the Step One Plus system software (Applied Biosystems) to calculate the CT values and reaction efficiencies from individual well fluorescence readings during the reaction. We calculated the expression of each target gene of interest relative to β-actin using the equation: relative target gene expression = $100 \times [(E_{\beta\text{-Actin}}{}^{CT\beta\text{-actin}})/(E_{target}{}^{CTtarget})]$ (Burmeister *et al.* 2007). All statistical analysis was conducted using SPSS software. The data was analyzed using two-way ANOVAs to examine the effects of run, sex and season on gene expression, and Tukey B post hoc analysis was used to examine differences across groups. The data was also tested for an effect of run (due to samples being run in two separate experiments per gene) using t-tests. If an effect of run was detected (i.e. the two experiments were systematically different), then each plate was normalized to an internal control sample (run on both plates) by subtracting the value for that sample from each sample on the plate. Then the normalized data was used to recalculate relative expression prior to further statistical analysis. We detected an effect of run for both Cyp19α1 and HSD17β3 results from the brain and used this normalization procedure to compare samples across runs.

Results

Breeding state confirmation

Breeding state was confirmed visually, as well as by weighing the gonads. There was a statistically significant difference in gonad weight between BS and NBS in both male and female anoles ($F_{1,20} = 10.99$, $p = 0.003$) (Figure 2). Gonad weight was significantly correlated with StAR mRNA expression ($p = 0.003$).

β-Actin CT values

CT values for β-actin were not different across sex, season, or tissue (all $F_{1,20} < 3.54$, $p > 0.075$), therefore, the transcript expression level data are normalized to β-actin.

StAR expression

In the gonad, we detected a significant increase in StAR mRNA expression levels in the BS compared to the NBS ($F_{1,12} = 7.828$, $p = 0.016$; Figure 3). There was also a sex difference in expression levels such that males had higher expression than females ($F_{1,12} = 5.704$, $p = 0.034$; Figure 3). There was no interaction detected in the gonad ($F_{1,12} = 1.178$, $p = 0.299$; Figure 3). There were no differences detected in StAR mRNA expression levels in the green anole brains brain between seasons and sex, and no interaction ($F_{1,16} < 0.756$, $p > 0.398$; Figure 3).

Cyp17α1 expression

There was a significant seasonal difference in gonad Cyp17α1 relative expression levels with increased expression during the NBS ($F_{1,16} = 10.590$, $p = 0.005$; Figure 4). In addition, there was also a significant difference in Cyp17α1 expression levels between males and females ($F_{1,16} = 9.581$, $p = 0.007$; Figure 4) with males displaying higher expression levels. There was no interaction in the gonads ($F_{1,16} = 0.002$, $p = 0.963$; Figure 4). Cyp17α1 expression in the brain showed no significant difference in expression levels between season or sex, and no interaction ($F_{1,16} < 1.699$, $p > 0.211$; Figure 4).

HSD17β3 expression

There was a significant difference in HSD17β3 relative expression in anole gonads between BS and NBS animals, with increased expression during the NBS ($F_{1,16}$ = 12.537, p = 0.003; Figure 5). Similarly, between sexes, males had higher expression levels than females ($F_{1,16}$ = 28.562, p > 0.001; Figure 5). There was also an interaction ($F_{1,16}$ = 6.197, p = 0.024; Figure 5) such that, during both the BS (t(5) = - 4.612, p = 0.006) and NBS (t(5) = - 6.221, p = 0.002), males had higher expression in the gonads than females.

In the brain, we detected an effect of run (t(16.50) = 3.88, p = 0.001), and normalized the data before further statistical analysis. Normalized brain HSD17β3 relative expression levels showed no difference between season or sex, and no interaction ($F_{1,16}$ < 2.274, p > 0.151; Figure 5).

Cyp19α1 expression

Gonad Cyp19α1 relative gene expression showed no difference between season or sex ($F_{1,16}$ < 2.765, p > 0.116; Figure 6); however, there was a significant interaction detected ($F_{1,16}$ = 12.192, p = 0.003; Figure 6) such that during the BS, males had higher expression than females (t(5) = -2.934, p = 0.032), with no difference in the NBS (t(5) = 2.052, p = 0.095).

In the brain, we detected an effect of run (t(11.80) = 2.85, p = 0.015), and normalized the data before further statistical analysis. Normalized brain Cyp19α1 relative expression levels show no significant differences between sexes and no interaction ($F_{1,16}$ < 2.056, p > 0.171; Figure 6). There was, however, a difference between seasons, with a

significant increase in expression levels during the NBS ($F_{1,16}$ = 30.127, p = 0.001; Figure 6).

Discussion

The present study was designed to test the hypothesis that natural variations in steroid hormone levels between sexes and seasons are mediated within the brain and gonad by these four genes involved in steroidogenesis: StAR, Cyp17α1, HSD17β3, and Cyp19α1 and that they would be upregulated within the BS compared to the NBS. Steroidogenesis is a multistep process, which ultimately converts cholesterol into steroid hormones in a tissue specific matter (Hattangady *et al.* 2012; Li *et al.* 2017). The entire steroidogenic process is under acute and chronic regulation controlled by each tissue-specific tropic hormone (Lehoux *et al.* 1998). For example FSH controls P and E2 synthesis in the granulosa cells of the ovary while LH regulates P synthesis in the granulosa-luteal cells of the ovary, androgen production in the theca-interstitial ovarian cells, and T synthesis in the Leydig cells of the testes (Hu *et al.* 2010). The steroid products have been seen to potentially contribute to a negative feedback effect on steroidogenesis (Li *et al.* 2017). Knowing changes occur in the steroidogenesis pathway, we are able to study the gene expression level differences between the seasons and sexes of green anole lizards potentially determining the specific regulation factors involved with these changes. We found sex and seasonal differences in the regulation of these genes in both the brain and gonads (summarized in Table 2).

StAR

StAR gene expression in the green anole gonad is seasonally and sexually dimorphic. We found that StAR mRNA expression levels in the gonad were higher during the BS compared to the NBS, showing it may be a potential cause in the major circulating hormonal changes that occur between the seasons. StAR production during the BS was highest in males. Similarly in lizards, human StAR mRNA has been found to be present and specifically expressed in the testis and ovaries (Sugawara *et al.* 1995). It is interesting to note that we found StAR expression in the gonads where it has also been seen in humans. Although we specifically looked at mRNA expression itself instead of protein expression, it is interesting to note that StAR protein expression has also been found in the adult mouse ovary, testis and adrenal glands (Clark *et al.* 1995) leading us to believe that StAR mRNA may also be present. This was in contrast to immunoblot analyses reported by (Clark *et al.* 1995), where the StAR protein levels were found to be higher in the adult mouse ovary and adrenal glands of which contain a higher number of steroidogenic cells when compared to the numbers of leydig cells of the testis.

There was, overall, an increased expression of StAR during the BS in the gonads of both sexes, suggesting that steroidogenesis may be increased to produce higher circulating steroid hormone levels in breeding animals. To our knowledge there has been no previous work in any species, investigating StAR expression levels in the gonads, or expression differences between seasons. We also found expression levels to be higher in male lizard gonads than females suggesting that there may be a much larger increase in expression of StAR mRNA to compensate in general for the lower number of steroidogenic Leydig cells making up only 2% of the adult testis compared to the higher frequency of cells in the ovaries (Clark *et al.* 1995). Alternatively this increase in

expression could mean that males need more overall steroid hormone production in the breeding season than females.

In the green anole brain we were able to detect StAR mRNA, however, there were no significant differences in expression levels between the sex or season. The first attempts of StAR localization in human and mouse tissues failed to detect a presence in the brain (Clark *et al.* 1995; Sugawara *et al.* 1995). It was then predicted that if StAR was found in the brain it would be present at lower levels, given the reduced nature of neurosteroid synthesis when compared to the adrenal glands and gonads (Stocco 2000). It has since been seen through RT-PCR and *in situ* hybridization techniques that StAR transcripts are indeed present in the rat brain at two to three orders of magnitude less than what was found in the adrenal gland (Furukawa *et al.* 2002). Similarly, we detected low expression of StAR in the lizard brain and found that anole whole brain StAR expression levels are relatively stable and unchanging when compared between sexes and different seasons. It is possible that steroidogenesis is not altered seasonally in the brain or, alternatively, that steroidogenesis may be regulated in a region-specific manner. It is possible as well that mRNA may not being translated within the brain, or any of the hormone level changes that are occurring are from gonadal hormone interactions with the brain. We used RNA extracted from whole brains for this study, which would make it difficult to detect subtle regional differences.

Cyp17α1

Cyp17α1 gene expression levels were the highest in the green anole male testis during the NBS. To our knowledge, there is little to no research on Cyp17α1 seasonal

expression levels in the gonads. Due to lower steroid hormone production in the NBS (possibly due to decreased StAR levels), it is possible that a lack of inhibition of Cyp17α1 could cause this increase expression in the NBS. We also found that anole testes had higher overall expression levels than ovaries, regardless of season. Similarly, male adult zebra fish (*Danio rerio*) and frog (*Rana rugosa*) gonads were found to have a higher amount of Cyp17α1 expression levels when compared to females when using a specific branched DNA assay (Hinfray *et al.* 2011; Suda *et al.* 2011). These results suggest that Cyp17α1 expression is seen to be more important for male reproduction in lizards.

Our results revealed that Cyp17α1 mRNA was expressed in the brain of the green anole, with no difference in expression levels between the sexes or seasons. Similarly, Cyp17α1 relative mRNA expression levels in the brain of adult zebra fish were not different between males and females (Hinfray *et al.* 2011). In adult frogs Cyp17α1 mRNA expression was not found in the brain at all (Suda *et al.* 2011). Our results suggest that Cyp17α1 mRNA is expressed in the brain, but is not regulated differently among the sexes in whole brains in this species. Little work is available on Cyp17α1 in the brain, and more work is needed to understand the role of this enzyme in neurosteroidogenesis.

HSD17β3

Our results showed that HSD17β3 mRNA expression levels in the gonads are higher in male compared to female lizards. Similarly, in humans, HSD17β3 appears to be more prevalently expressed in the testis (Geissler *et al.* 1994). This is in agreement with HSD17β3's function to synthesize T and that T production in the gonads is more crucial

for males. Our results also revealed a seasonal effect such that HSD17β3 is much more prevalent in the testis during the NBS compared to the BS. Similar to Cyp17α1, it is possible that HSD17β3 expression maybe increased during the NBS due to a loss of negative feedback from very low T synthesis (again, possibly due to low StAR levels and the lack of precursors being synthesized). Furthermore we found a sex and season interaction, which revealed that, during the BS, females had relatively no HSD17β3 expression while in the NBS they had a small amount of HSD17β3 expression. For both seasons the HSD17β3 expression was far higher in males than in females. The interaction suggests that HSD17β3 mRNA expression is much more prevalent for males.

HSD17β3 expression levels in the green anole brain showed that this enzyme is expressed; however we did not detect differences in expression levels between groups. Similarly, in the human temporal lobe, HSD17β3 expression has been detected by real-time quantitative RT-PCR throughout the different stages of life, with no differences found (Watzka *et al.* 1999). HSD17β3 is known to catalyze the ending stages of androgen and estrogen biosynthesis, therefore playing a crucial role in local steroid biosynthesis in the brain (Watzka *et al.* 1999). HSD17β3 expression levels may not be sex or seasonally dimorphic because it may play a role in moderating concentration levels of local steroid hormones in the brain. Alternatively, if there are subtle differences between various brain regions, we could not detect this with the methods used. More work is needed to investigate this possibility.

Cyp19α1

Cyp19α1 gene expression levels in the gonad showed no main effects of season or sex, although an interaction showed female expression levels were higher in the NBS and male expression levels were higher in the BS. Similarly, Cyp19α1 expression levels in adult zebra fish gonads were higher in males than females when using a specific branched DNA assay (Hinfray *et al.* 2011), and, in the Atlantic croaker, high levels of aromatase expression were found using real-time quantitative RT-PCR in the testes during spawning (Nunez & Applebaum 2006), indicating a potential role of aromatase and E2 in male reproduction that should be investigated further. Although expression levels were higher during the NBS in females, it is unlikely that more E2 is synthesized, due to the lack of precursor hormones as a result of low StAR expression levels. Likely, the increase expression of Cyp19α1 during the NBS could be a result of a lack of inhibition due to low circulating steroid hormone levels.

Cyp19α1 gene expression in the brain was higher during the NBS, with no difference in expression levels between the sexes. In adult zebra fish brains, Cyp19α1 expression also showed no sexual dimorphism (Hinfray *et al.* 2011). Looking at brain aromatase protein activity in BS anole lizards levels were greater in males than females and, between males, it was shown to be increased during the BS compared to the NBS (Rosen & Wade 2001). In contrast, our mRNA expression levels revealed that Cyp19α1 mRNA expression levels in the brain were significantly higher during the NBS compared to the BS (Table 2). As there are not precursor hormones for this enzyme to act on, this data suggests that this enzyme might have an absence of an inhibitory signal during the NBS. Our results could also be a result of the fact that aromatase expressing cells have

been found to vary per brain region (Cohen & Wade 2011), and we may not have been able to detect these subtle changes as we examined expression at the whole brain level.

Conclusions

Our experiment examined the seasonal regulation of steroidogenesis at the enzyme level in the green anole lizard .We found that whole brain mRNA expression of StAR, Cyp17α1, and HSD17β3 have no differences between sex or season. Therefore, the machinery for steroidogenesis is expressed within the brain at a similar rate between sexes and seasons in the green anole lizard. Cyp19α1 brain mRNA expression was increased during the NBS in females, potentially revealing the presence of regulatory signaling for aromatase expression in the brain.

In the anole gonad, as expected, StAR mRNA expression levels were increased in both males and females during the BS, suggesting that this is the main regulatory control step for gonadal steroidogenesis of those tested. However, we also found that the expression levels of many of the other steroidogenic enzymes are increased when StAR expression is decreased, suggesting that the enzymes in the steroidogenic pathway are, in fact, regulated independently of StAR, although the mechanism for this regulation has not yet been determined. Future work is needed to determine the type and level of this regulation, as well as what role, if any, it has in regulating steroidogenesis.

Figures and Tables

Table 1. Target genes specified with their NCBI identification numbers, primer sequences, amplicon size, primer concentrations, and qPCR efficiencies.

Gene	NCBI ID#	Primer Sequence	Amplicon Size (bp)	Primer Concentration µg/µl	Efficiency
StAR	100554580	F: CACTCGCTGGAGATCCCTACC R: TCCACCTGCGTCTGGG	112	0.05	2.0742
Cyp17α1	100553634	F:GGGAACCGAATCTACAGCCC R:ATCTTGGCTAGCGCTTCTCC	86	0.5	1.8961
HSD17β3	100561157	F:GTGGGGGTAAGGACAGTCAC R:CAGAAGGGCATTTTTGGCCC	71	0.2	2.6035
Cyp19α1	100565397	F:CCAGGTCTTGTGCGGATGAT R:GTAGCCACACTTGGTGGTCA	90	0.5	2.0356
β-Actin	AF199487.1	F: GACGAGGCGCAGAGTAAAAG R: TCAGGGGCAACTCTCAACTC	131	0.5	1.9595

Table 2. Summary of the relative expression results of StAR, Cyp17α1, HSD17β3, and Cyp19α1 in the anole brain and gonad.

	StAR		**Cyp17α1**		**HSD17β3**		**Cyp19α1**	
	Brain	Gonad	Brain	Gonad	Brain	Gonad	Brain	Gonad
Sex	None	Males ↑	None	Males ↑	None	Male ↑	None	None
Season	None	BS ↑	None	NBS ↑	None	NBS ↑	NBS ↑	None
Sex x Season	None	None	None	None	None	M NBS↑ M BS ↑	None	F NBS↑ M BS ↑

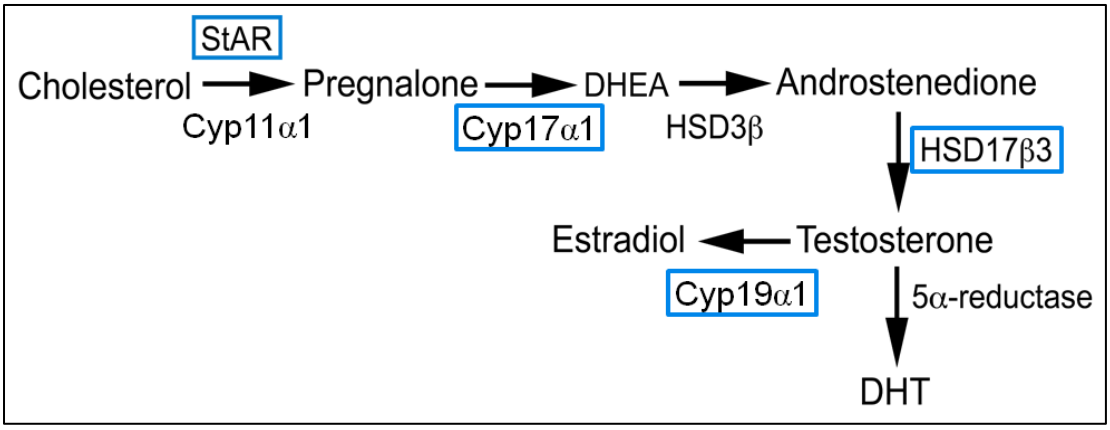

Figure 1. The abbreviated steroidogenesis pathway. Cyp11α1, cholesterol (cytochrome P450scc)- side-chain cleavage enzyme; Cyp17α1, cytochrome P450 17A1, also known as steroid 17α-monooxygenase and 17α-hydroxylase; Cyp19α1, cytochrome P450 19A1, also known as aromatase and estrogen synthase; DHEA, Dehydroepiandrosterone, also known as androstenolone is an endogenous steroid hormone; DHT, Dihydrotestosterone, also known as 5α-dihydrotestostreone is an endogenous androgen steroid hormone; Hsd3β, 3β-Hydroxysteroid dehydrogenase; HSD17β3, 17β-Hydroxysteroid dehydrogenase 3; StAR, Steroidogenic acute regulatory protein, also known as STARD1 a transport protein

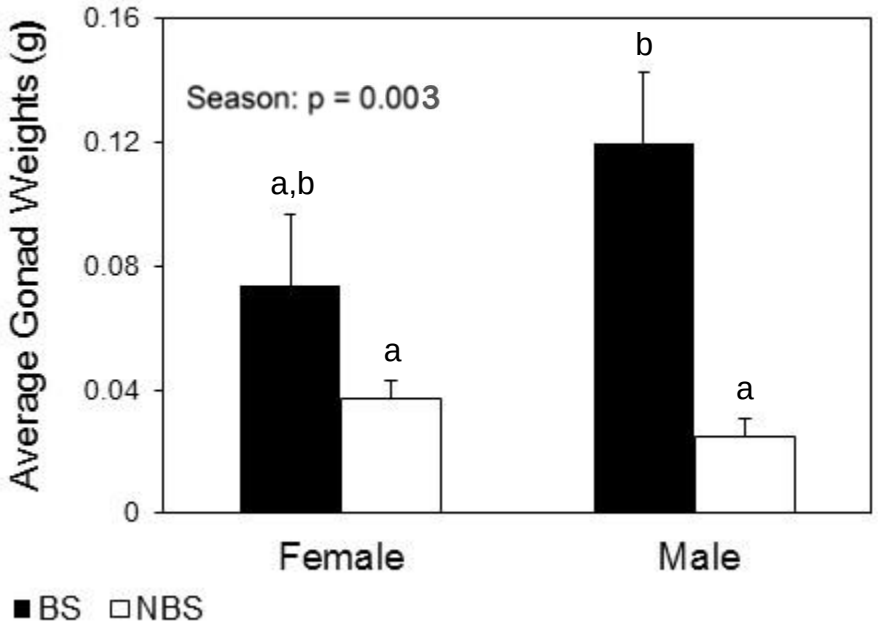

Figure 2. Average whole gonad weight of male and female green anole lizards. Breeding animals are depicted in black bars and non-breeding animals are depicted in white bars. Gonads were higher in the BS compared to the NBS. n = 6. Letters above bars denote statistical differences.

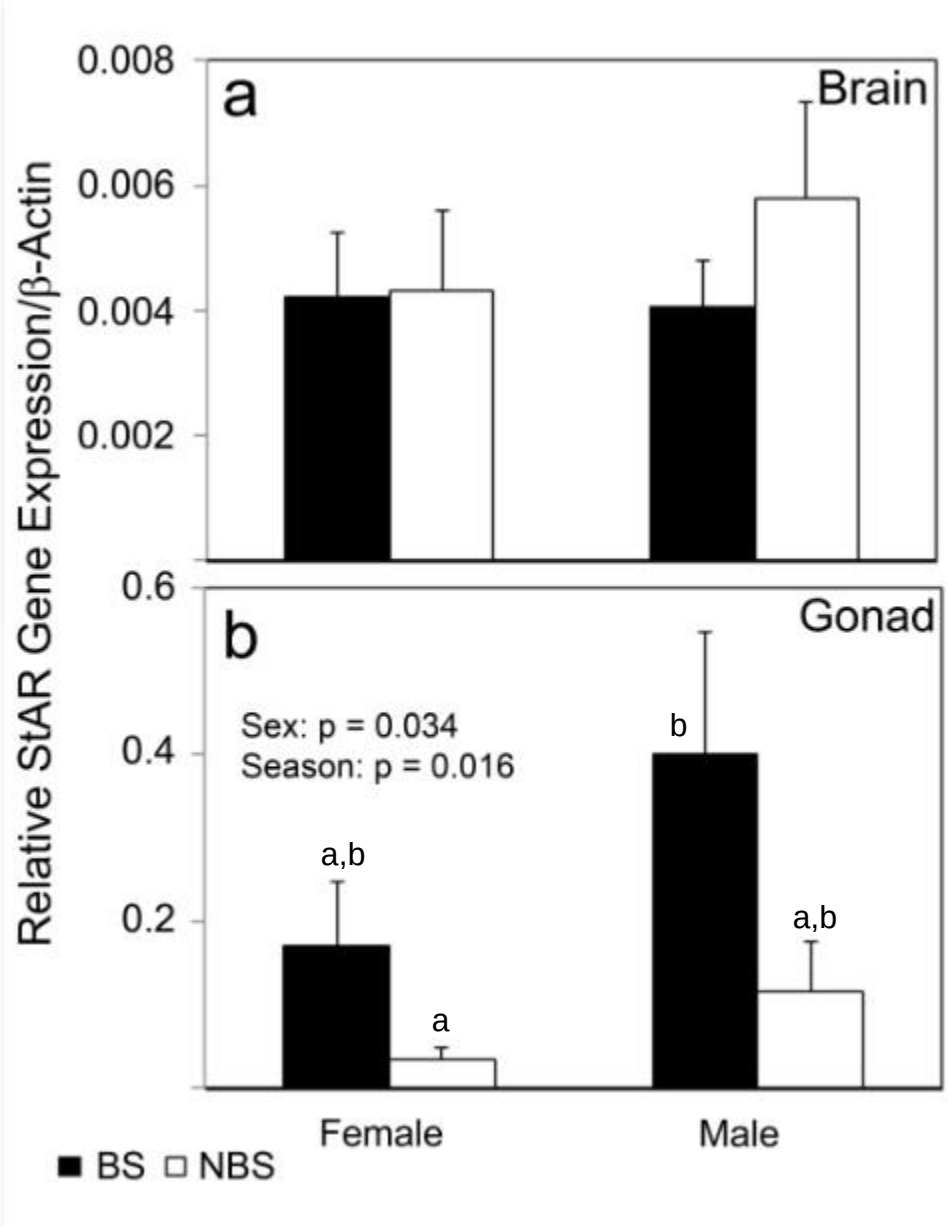

Figure 3. Relative StAR gene expression in the green anole lizard brain and gonads normalized to β-Actin. Breeding animals are depicted in black bars and non-breeding animals are depicted in white bars. (a) StAR expression in the brain was not different between sex or season. (b) StAR expression in the gonad was increased in males compared to females and it was increased in BS compared to NBS. n = 6. Letters above bars denote statistical differences.

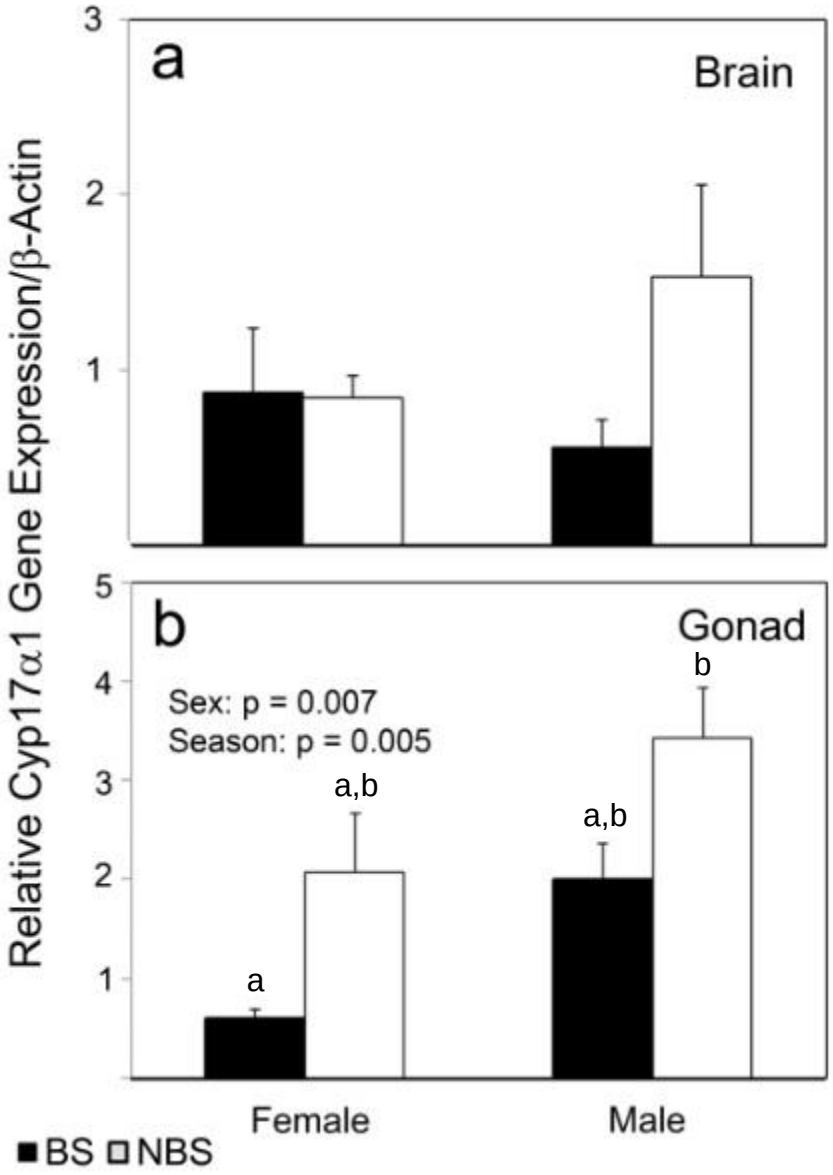

Figure 4. Relative Cyp17α1 gene expression in the green anole lizard brain and gonads normalized to β-Actin. Breeding animals are depicted in black bars and non-breeding animals are depicted in white bars. (a) Cyp17α1 expression in the brain was not different between sexes or season. (b) Cyp17α1 expression in the gonad has increased expression in males compared to females and it has increased expression in NBS compared to BS. n = 6. Letters above bars denote statistical differences.

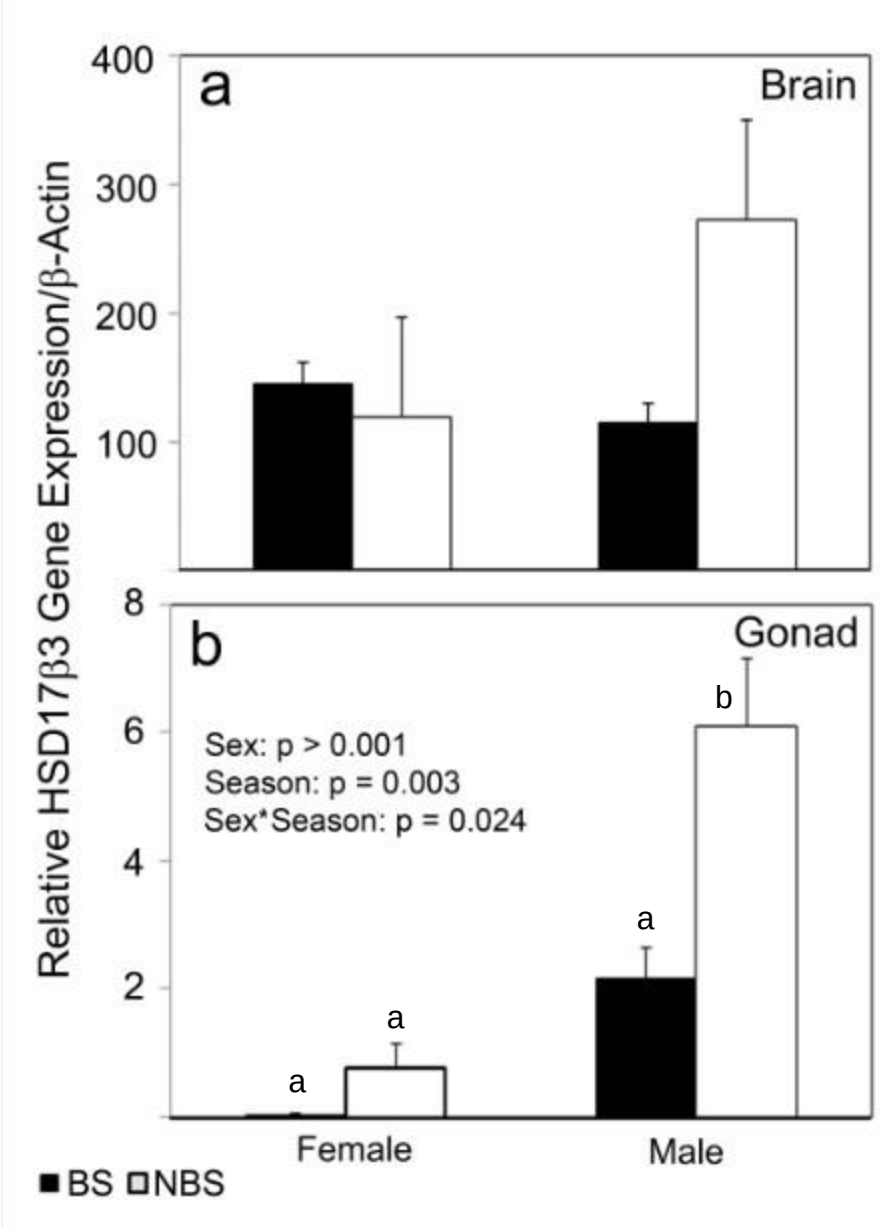

Figure 5. Relative HSD17β3 gene expression in the green anole lizard brain and gonads normalized to β-Actin. Breeding animals are depicted in black bars and non-breeding animals are depicted in white bars. (a) HSD17β3 expression in the brain was not different between sex or season. (b) HSD17β3 expression is increased in males compared to females, NBS compared to the BS, and there was also an interaction between sex and season. n = 6. Letters above bars denote statistical differences.

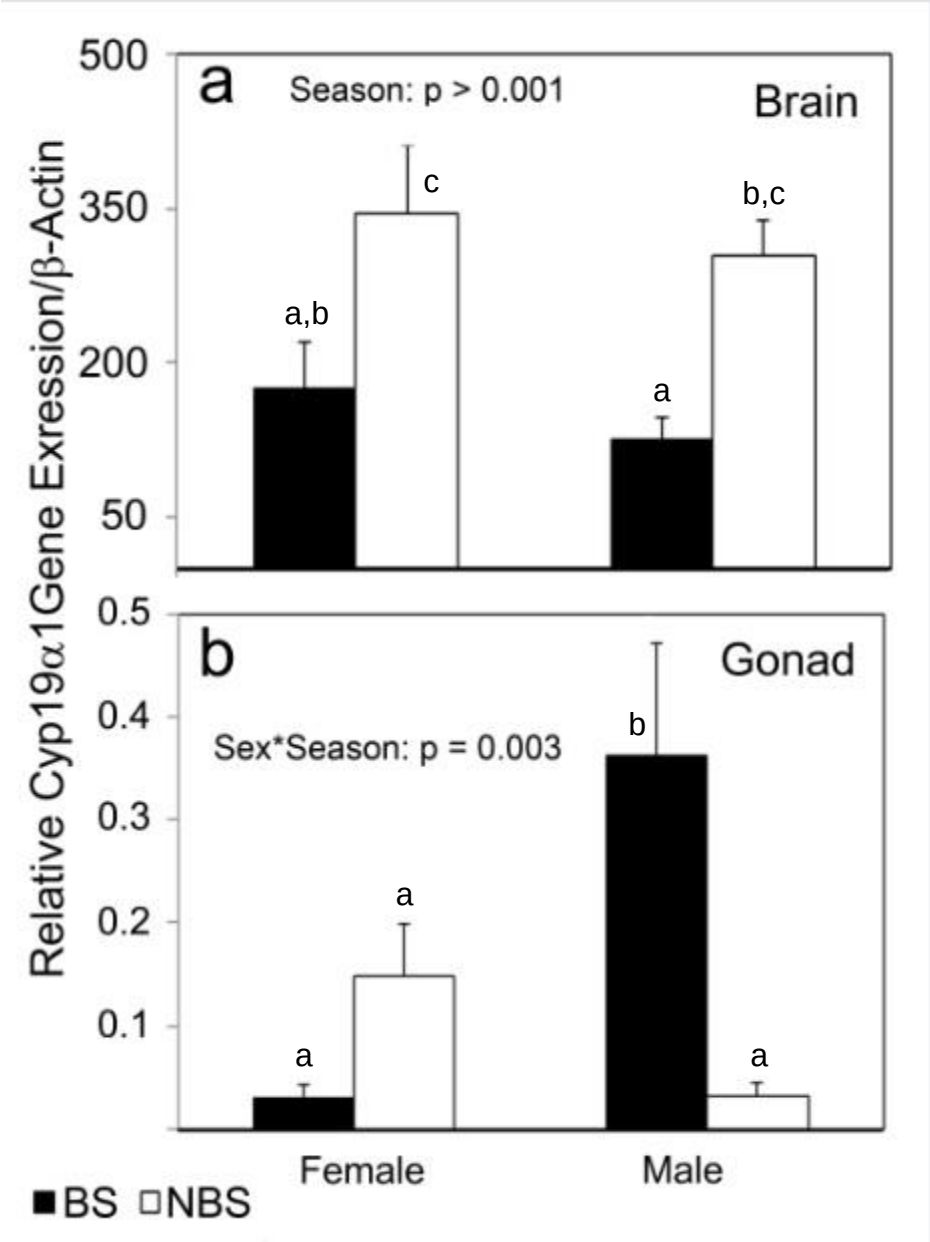

Figure 6. Relative Cyp19α1 gene expression in the green anole lizard brain and gonads normalized to β-Actin. Breeding animals are depicted in black bars and non-breeding animals are depicted in white bars. (a) Cyp19α1 expression in the brain was not different between sexes, but there was increased expression in the NBS compared to the BS. (b) Cyp19α1 expression in the gonad was not different between sex and season, but there was an interaction. n = 6. Letters above bars denote statistical differences.

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