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The Relationship Between Seasonal Breeding and Deiodinase Expression in the Green Anole Lizard

Hyejoo Kang
Minnesota State University, Mankato

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Hyejoo Kang

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This thesis has been examined and approved by the following members of the student's committee.

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Advisor

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Committee Member

_____
Committee Member

Abstract

Reproductive physiology and behavior is mainly regulated by the hypothalamus-pituitary-gonad (HPG) axis. Interestingly, abnormal levels of thyroid hormone (TH) results in the delayed onset of puberty and affects gonadal function of adults by altering HPG axis activity. Seasonally breeding animals undergo drastic hormonal and behavioral changes between breeding and non-breeding seasons. Green anole lizards (*Anolis carolinensis*), similar to other seasonally breeding animals, have increased sex steroid hormones, larger gonads, upregulated gonadal steroidogenic acute regulatory protein (StAR) mRNA and increased reproductive behavior during the breeding compared to non-breeding seasons. Relatively less is known regarding the regulation of gonadal TH in seasonal reproduction in reptiles. We examined whether the gonadal expression of enzymes involved in TH activation are altered in concert with seasonal regulation. Type 2 deiodinase (Dio2) mRNA, the TH activating enzyme, was upregulated in breeding male anole gonads compared to non-breeding males, while type 3 deiodinase (Dio3) mRNA, the TH deactivating enzyme, was upregulated in breeding female anole gonads. To study the association between the HPG axis and local activation of TH in regulating reproductive physiology, we manipulated the HPG axis during the non-breeding season by subcutaneously injecting luteinizing hormone (LH) and follicular stimulating hormone (FSH) in male and female green anoles. We examined mRNA expression of Dio2, Dio3 and StAR in gonads and measured plasma sex steroid hormone levels. LH and FSH injected males had significantly increased testes weight, StAR mRNA expression and testosterone levels, which indicates that gonadotropin injections were able to activate the HPG axis even during the non-breeding season. Surprisingly, Dio3 was upregulated in the

testes after LH and FSH injections, while Dio2 mRNA levels were unchanged compared to the vehicle injected group. This result suggests that there might be different roles of local TH activation in developing and maintaining fully grown gonads. Additionally, as determined through a mirror test, gonadotropin injections did not induce aggressive behavior in males despite their increased testosterone levels. Our findings support the role for thyroid hormone in regulating reproduction and contribute to a growing body of work examining the evolution of puberty and reproductive development.

Introduction

The hypothalamus – pituitary – gonad (HPG) axis is the main regulator of gamete production and steroidogenesis in many species, including humans. The hypothalamus releases gonadotropin releasing hormone (GnRH) to the anterior pituitary, which activates the secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH). Circulating LH enters gonadal tissue and triggers the production of steroidogenic acute regulatory protein (StAR), the rate-limiting step in steroidogenesis (Clark *et al.* 1994). Through increased StAR expression, sex steroid hormones, such as testosterone (T) and estradiol (E2), are produced from male and female gonads respectively.

Dysfunction of the HPG axis can result in reproductive disorders in both males and females and thyroid gland disorders seem to be associated with altering the HPG axis, impacting gonadal maturation as well as normal reproduction (Krassas *et al.* 2010). A recent study suggested that thyroid hormone (TH) disorders induced in adulthood affect spermatic function and testicular gene expression in male rats (Romano *et al.* 2017). Some clinical cases also reported that hypothyroid (abnormally low levels of TH) adult women tend to have menstrual irregularities and a lack of ovulation (Krassas *et al.* 1999). The production of thyroid hormone is regulated by thyrotropin-releasing hormone (TRH) secreted from the hypothalamic paraventricular nucleus (PVN) which stimulates the anterior pituitary gland (pars distalis) to synthesize thyroid-stimulating hormone (TSH) (Segerson *et al.* 1987). TSH is delivered to the thyroid gland where TH is produced and secreted (Haisenleder *et al.* 1992). TH is mainly secreted as thyroxine (T_4), the prohormone, whereas triiodothyronine (T_3) is the biologically active hormone

(Braverman *et al.* 1970). Activation and inactivation of TH is mediated by peroxidase enzymes called deiodinases that cleave specific iodines from TH (Kuiper *et al.* 2005). Type 2 deiodinases (Dio2) convert T_4 to the active form, T_3, while type 3 deiodinase (Dio3) convert T_4 to an inactive isoform, reverse triiodothyronine (rT_3) (Figure 1). Dio3 and Dio2 can also cleave one additional iodine from T_3 and rT3 respectively, which produces diiodothyronine (T_2) (Figure 1).

Local activation of T_3 by deiodinases in the brain and gonads is likely related to reproduction in many species, particularly in birds and mammals (Yoshimura 2013). However, the relationship between seasonal regulation of TH-related gene expression and gonadal development is still unclear and TH regulation in the gonads of other groups is less studied.

Thyroid hormone and reproduction

It is known that TH is essential for normal growth and sexual maturation in both males and females in many species including humans (Weber 2003). Especially in rodent models, altered TH levels during development exert a significant impact on gonadal morphology and function while gonadal function is mainly affected by abnormal TH levels in adults. Neonatal hypothyroidism in rats and mice results in increased testicular size and increased sperm production, while Sertoli cell maturation was delayed (Cooke *et al.* 1991; Hess *et al.* 1993; Joyce *et al.* 1993; De França *et al.* 1995). In contrast, neonatal hyperthyroidism induced by exogenous TH administration reduced testes size (Van Haaster *et al.* 1993). In female rats, prepubertal hypothyroidism results in reduced ovarian weight and disrupted folliculogenesis (Dijkstra *et al.* 1996). Hypothyroidism diagnosed later in the prepubertal period delayed the onset of puberty and sexual

maturation in girls (Pantsiouou *et al.* 1991). Furthermore, rats with adult-onset hypothyroidism had disrupted spermatic production and quality in males and irregular estrous cycle with decreased ovarian weight in females (Tohei *et al.* 1998; Romano *et al.* 2017). In addition, there is some evidence that indicates TH influences steroidogenesis in both sexes of rats and humans, such that hypothyroid adults have decreased levels of steroid hormones (Krassas *et al.* 2010; Romano *et al.* 2013) and increased T3 levels resulted in higher steroid hormone levels in mice (Manna *et al.* 1999; Liu *et al.* 2017).

TH action in tissue is regulated by deiodinases that modulate local TH availability. In rats, both types of deiodinases (Dio2 and Dio3) are expressed in the gonads throughout development and adulthood, although with different levels in activities (Bates *et al.* 1999). In ovaries and testes, Dio3 activity predominates during the developmental period and declines to adult levels while Dio2 activity is upregulated only during testicular development in males (Bates *et al.* 1999). Dio3 deficiency-induced hyperthyroidism results in cell proliferation arrest of neonatal mice testes and a 75% reduction in testes weight (Martinez *et al.* 2016). Changes in serum TH levels likely impacts deiodinase activity and gene expression in prepubertal and adult gonads, especially in males. In adult hypothyroid male mice, upregulated Dio2 activity in testes can compensate for low T_3 levels (Wagner *et al.* 2003). Hypothyroidism induced by thyroidectomy in adult rats leads to downregulated Dio3 mRNA expression in testes with compromised spermatogenesis (Romano *et al.* 2017). These results imply that T_3 signaling by deiodinases is associated with gonadal function during both development and adulthood. However, it is still unclear how deiodinase gene expression is regulated and how local activation of TH is related to gonadal function in adults.

TH and seasonal breeding

Seasonally breeding animals are excellent models to examine the regulation of reproduction, as these animals reproduce only under appropriate environmental conditions and halt reproduction when environmental conditions are no longer conducive to successful breeding. These behavioral changes have been associated with changes in the regulation of the HPG axis (Yoshimura 2013). The HPG axis is activated during the breeding season which results in a dramatic increase in gonadal weight, particularly in birds and reptiles (Dawson 1998; Wade 2011). In contrast, during the non-breeding season, gonads become inactive due to regression in size and weight. This change in gonadal size between the BS and NBS might be associated with TH activation or inactivation by deiodinase enzymes.

Photoperiodic effects on brain and testicular expression of deiodinase genes have been examined in many species (Yoshimura 2013). In Japanese quail (*Coturnix japonica*), the light-stimulated anterior pituitary (pars tuberalis) secretes TSH to the mediobasal hypothalamus (MBH), an area which appears to regulate photoperiodic control of seasonal reproduction (Yoshimura *et al.* 2003). In male Japanese quail, a long-day (LD) breeder, Dio2 mRNA is upregulated and Dio3 mRNA is downregulated in the MBH under LD conditions, with an opposite pattern of expression under short-day (SD) conditions (Ikegami *et al.* 2015). In contrast, in male European starlings (*Sturnus vulgaris*) there was no difference in hypothalamic Dio3 gene expression across the photoperiods, but Dio2 was highly expressed during a breeding photoperiod (Bentley *et al.* 2013). In mammals, LD-breeding Siberian hamsters had testicular growth with exogenous T_3 under the SD condition, whereas SD-breeding male sheep had higher Dio2

expression under LD conditions (Hanon *et al.* 2008; Henson *et al.* 2013). These results support the idea of a dual role for TH to both begin and terminate seasonal breeding. TH levels regulated by seasonal changes in deiodinase expression appear to affect seasonal breeding in males, although this has been less studied in female seasonal breeders.

Green anole lizards

Many reptiles breed seasonally and exhibit distinct gonadal changes during various phases of the reproductive cycle (Gautam *et al.* 2013). Green anole lizards (*Anolis carolinensis*) breed seasonally and are known for distinctive seasonal changes in morphology, hormone levels, behavior and gene expression such as StAR (Rosen & Wade 2001; Peek & Cohen 2018). During the breeding season, which occurs from April through August, the gonads become larger and produce steroid hormones, and the lizards display reproductive and territorial behaviors (Winkler & Wade 1998; Lovern *et al.* 2001; Jenssen *et al.* 2006). However, when they enter the non-breeding season, the gonads regress, steroid hormone levels decrease, and the lizards no longer display reproductive or territorial behaviors (Wade 2011). Unlike mammals, which sexually mature during puberty, reptiles do not appear to undergo a complete pubertal stage (Ball & Wade 2013). Instead, gonadal hormone synthesis and gametogenesis occurs just before the reproduction phase. Previous work in green anoles revealed that hyperthyroidism reduces the stimulatory response of dormant testis to long photoperiod conditions, which implies that excess levels of thyroid hormone might inhibit testicular recrudescence (Turner 1972), and suggests that thyroid hormone might play a role in seasonal changes of reproductive physiology and function. It is, however, not clear whether seasonal effects of TH and deiodinases contribute to seasonal changes in the gonads.

Experiment

We hypothesized that seasonal reproduction is regulated by local changes to thyroid hormone levels through seasonal changes in deiodinase expression. We examined Dio2 and Dio3 mRNA levels in the gonads to determine whether the mRNA expression of these enzymes is sexually and/or seasonally dimorphic. In addition, we investigated how HPG axis activation in non-breeding animals alters Dio2 and Dio3 expression levels. We hypothesized that deiodinase mRNA expression can be regulated by LH and FSH signaling at the gonads, which could lead to increased gametogenesis as well as steroidogenesis. Thus, we will examine the effect of exogenous LH and FSH on gonadal weight, deiodinase gene expression and StAR expression in female and male gonads.

Materials and Methods

Experiment 1: Seasonal deiodinase expression in the gonads

The gonads were collected from 6 female and 6 male lizards during the breeding and non-breeding seasons respectively. Dio2 and Dio3 mRNA expressions from gonads were quantified relative to β-actin and effects of season and sex were examined.

Experiment 2: LH or FSH manipulation of non-breeding lizards

Non-breeding female and male lizards were injected with a low dose of LH, FSH or vehicle (beginning January 2018) to examine which hormone is necessary for inducing seasonal gonadal changes. After injections (Figure 2), gonads were collected and the gonad mass was measured. Dio2, Dio3 and StAR mRNA expressions from gonads were quantified and compared across the injected groups. Steroid hormones levels were assayed (T in males and E2 in females) and male aggressive behavior was measured

using a mirror test before the tissue collection.

Experiment 3: LH and FSH manipulation of non-breeding male lizards

Male lizards were injected with a higher dose of LH+FSH or vehicle (mid-January 2018) to examine whether both LH and FSH can induce breeding in an otherwise non-breeding animal. After injections (Figure 2), gonads were collected and the gonad mass was measured. Gonadal Dio2, Dio3 and StAR mRNA expression was quantified and compared across the injected groups. Plasma T levels were determined and aggressive behavior was measured using a mirror test before the tissue collection.

Animals and tissue collection

For experiment 1, gonadal mRNA that was previously extracted for a different study was used (Peek & Cohen 2018). Briefly, female and male green anole lizards were ordered (Charles Sullivan) during the breeding (May 2015) and non-breeding seasons (October 2015). Lizards were dissected immediately upon arrival in the lab to collect the gonads and confirm breeding state. Breeding condition was confirmed by visual inspection of the reproductive system. Non-breeding females had small ovaries with no or small yoking follicles and small oviducts, whereas breeding females had enlarged ovaries with yoking follicles and large oviducts. Non-breeding males lizards had small testes, while breeding males had enlarged testes with a milky vas deferens.

For experiment 2 and 3, non-breeding green anole lizards were ordered (Candy's Quality Reptiles; end of November 2017) and housed in the lab for 43 days (experiment 2) and 60 days (experiment 3) prior to the first injection. All males were separately housed in glass terraria (39.5x22x26 cm) and 4-5 females were co-housed in common

terraria (77.5x32.5x32.5 cm) by injection group. Each cage had peat moss and sticks bars for substrate. All males were kept in visual isolation from each other and each cage was misted daily. Ambient temperatures ranged from 24°C during the day to 13°C at night (10:14 light/dark cycle). Full spectrum bulbs and heat lamps were provided directly above the cages to allow basking temperatures of 10°C above ambient. Calcium-dusted crickets were provided twice per week. Three male lizards were dissected to confirm non-breeding state (see above) before injection of gonadotropins (LH and FSH). On the day after the final injection (see below), all lizards were rapidly decapitated, and gonads and trunk blood were collected. Blood was collected using heparinized capillary tubes, then centrifuged to collect plasma, and stored at -20°C. Gonado-somatic index (GSI) was calculated [(gonadal weight) / (body weight) x 100%]. Gonads were frozen in cold methyl butane and stored at – 80°C.

LH and FSH injection

For experiment 2, each non-breeding female (n = 4-5) and male (n = 6) lizard was given a subcutaneous injection with saline (NaCl 0.99%, 1M NaOH; vehicle), LH (0.2 μg/g; Licht & Pearson 1969), or FSH (0.015 μg/g), using a 25 μl syringe (Hamilton Co.) (Figure 2). All lizards were given one injection every other day for 12 days (total of 6 injections).

For experiment 3, 6 non-breeding male lizards were given a subcutaneous injection of LH (2 μg/g) (Licht & Tsui 1975) and FSH (0.15 μg/g) (Licht & Papkoff 1971) on alternate days, using a 25μl syringe (Hamilton Co.) (Figure 2). In addition to the 6 males injected with saline from experiment 2, 3 additional males had subcutaneous injections with saline (NaCl 0.99%, 1M NaOH; vehicle) as a control group.

Aggressive behavior assay

On the 5th injection day, between 10 am and 12 pm (prior to receiving the injection), a mirror test was conducted for male lizards in all groups to determine aggressive behavior (Korzan *et al.* 2000). Each male was placed in the middle of a new terrarium with two wooden perches, and visually isolated from others. After a three-minute equilibration period, a mirror was placed on one side of the cage. Aggressive displays were recorded by video camera for 15 minutes after placing the mirror.

An observer blind to treatment recorded all aggressive behavior that occurred during the 15 minute test period. Aggressive behaviors of male anoles include dewlap extension, push-ups, lateral compression, color change, and/or post-orbital eyespot formation (Yang & Wilczynski 2003). We defined dewlap extension as a single extension of the red throat fan and push-up displays as vertical movement of the front portion of the body. Lateral compression was defined as the formation of a sagittal crest from the back of the neck towards the tail. Also, we tracked changes in the postorbital eyespot, which is an indicator of acute stress responses, and body color change (Yang *et al.* 2001). As each individual male displayed different types of aggressive behavior (dewlap extension, lateral compression, etc.), we also measured the latency to the first display of aggressive behavior.

Enzyme-linked immunosorbent assay (ELISA)

Plasma T and E2 levels were determined via a testosterone high sensitivity ELISA kit and 17β-estradiol high sensitivity ELISA kit (Enzo Life Sciences, Inc.). Samples from males were run using two T ELISA kits, with groups evenly distributed across plates. All female samples were run using one E2 ELISA kit. ELISAs were

performed according to manufacturer's instructions. Additional plasma standards were prepared at high (470 pg/ml), medium (117.5 pg/ml), and low (29.4 pg/ml) concentrations of T diluted in anole plasma (pooled from several males) for the T ELISAs. Similarly, plasma standards were prepared for the E2 ELISA at high (250 pg/ml), medium (125 pg/ml), and low (62.5 pg/ml) concentrations of E2 diluted in anole plasma (pooled from several females). Each plasma standard was run in duplicate and parallelism to the standard curve was demonstrated. Samples were also run in duplicate. Male average plasma volume was 55 µl ± 2.9 µl (range: 20 µl – 95 µl) and female average plasma volume was 36.5 µl ± 2.0 µl (range: 12 µl – 55 µl). Intra- and inter-assay coefficients of variance were determined using plasma standards, as appropriate.

Primer design

Specific primer sets for β-actin, Dio2, Dio3 and StAR were designed from the predicted sequences in the green anole genome (National Center for Biotechnology Information, NCBI) and purchased from Integrated DNA Technology (Table 1). Polymerase chain reaction (PCR) was performed to determine optimal primer concentrations (Table 1) and check primer specificity using Quick-load Taq 2X Master Mix (New England Biolabs, NEB) with the following reaction conditions: 95°C for 30 s followed by 40 cycles of 95°C (30 s), 60°C (1 min) and 68°C (1 min). The PCR products were run on a 1.5 % agarose gel to confirm the amplicon size and the presence of a single band. Amplicons were sequenced (GeneWiz) and the sequences were confirmed using BLAST NCBI.

RNA isolation and cDNA synthesis

Gonads from the lizards were weighed, homogenized in QIAzol (Qiagen) and

incubated with chloroform. RNA was isolated using the RNeasy Lipid Tissue Mini Kit (Qiagen), as per manufacturer's instructions. An additional on-column DNase I (Qiagen) treatment at room temperature for 15 minutes was used to remove genomic DNA. Isolated RNA was concentrated using ethanol precipitation and reconstituted with 20 µl of RNAse free water at 65°C. RNA purity and concentration was determined by NanoDrop. RNA was run on a 1.5% agarose gel with 1% bleach to visually confirm RNA quality (Aranda *et al.* 2012). All samples were of high quality with a 260/280 ratio close to 2.0. Isolated RNA was stored at -80°C and 1 µg of RNA was reverse transcribed to cDNA using the ProtoScript II First Strand cDNA Synthesis Kit (NEB). cDNA was stored at -20°C until use.

qPCR

Quantitative real-time polymerase chain reaction (qPCR) was conducted using the relative standard curve method. Standard curves were run for each gene using a 5-fold serial dilution of cDNA (100 to 0.8 ng/µl) to determine sample concentration and confirm primer efficiencies (Table 1).

Each qPCR reaction contained 40 ng/µl total cDNA, PowerUp SYBR Green PCR master mix (ThermoScientific) and the optimal concentration of forward and reverse primers (Table 1). Each sample was run in duplicate and one gene (including the standard curve, water controls and samples) was assayed on each 96-well plate.

The expression of target genes was analyzed using the StepOnePlus (Applied Biosystems) real-time PCR machine. The initial denaturation step was at 95°C (10 min) followed by 40 cycles of 95°C (15 sec) and a combined annealing and extension step of 60°C (1 min). Then, a melt curve was conducted with steps at 95°C (15 sec), 60°C (1

min), and 95°C (15 sec) to confirm the presence of a single amplicon.

Data analysis

All data are presented as mean ± SEM. The qPCR efficiencies of individual genes (β-Actin, Dio2, Dio3 and StAR) were calculated using the equation, $E = 10^{-1/slope}$ (Pfaffl 2001). Ideal efficiencies are approximately 2.0, as DNA should be doubled at each cycle (Pfaffl 2001). The relative standard curve method was used to quantify the mRNA expression of each gene. mRNA expression was calculated by a logarithmic equation derived from the standard curve of each gene and normalized to β-actin.

Statistical analysis was conducted using SPSS software with $\alpha = 0.05$. We first conducted t-tests to compare the two male vehicle groups from experiment 2 and 3, and we found that these two groups did not differ in testicular weight ($t_7 = 1.14$, $p = 0.293$) or GSI ($t_7 = 2.12$, $p = 0.072$). Therefore, we combined all vehicle individuals for subsequent analysis. Levene's test was performed to confirm equal variance across groups. Two-way ANOVAs were used to examine the effects of sex, season effects on gene expression and a Tukey-b post hoc test was used to examine significant differences across groups. T-tests were conducted to compare hormone levels and behaviors of breeding with non-breeding seasons. One-way ANOVAs were used to determine the treatment effects on gene expression, hormone levels, GSI, and behaviors. T-tests were conducted to compare the LH+FSH injection group with the vehicle injection group. If the data had unequal variance (as detected by Levene's test), we used Mann-Whitney U tests to compare groups. For all injection groups, we used Pearson's correlations to examine the relationship between hormone levels, StAR mRNA expression and gonad size, with only

significant relationships reported. Grubb's test was performed to detect outliers, which were subsequently removed from the data set.

Results

Experiment 1: Sexually and seasonally dimorphic expression in gonadal deiodinase expression

Dio2 mRNA expression was significantly upregulated in breeding compared to non-breeding gonads ($F_{(1, 18)} = 13.41$, $p = 0.002$; Figure 3A). There was also a significant sex difference such that males had higher gonadal expression of Dio2 mRNA than females ($F_{(1, 18)} = 5.88$, $p = 0.026$). We also detected an interaction between sex and season in gonadal Dio2 mRNA expression ($F_{(1, 18)} = 6.37$, $p = 0.021$). Tukey-b post hoc tests confirmed that breeding male gonads had highly upregulated Dio2 mRNA expression compared to other groups.

We detected significantly upregulated Dio3 mRNA expression in breeding compared to non-breeding gonads ($F_{(1, 16)} = 24.55$, $p < 0.001$; Figure 3B). Also, there was a sex difference such that female gonads had higher Dio3 mRNA expression compared to males ($F_{(1, 16)} = 10.55$, $p = 0.005$). Lastly, there was an interaction between season and sex ($F_{(1, 18)} = 9.238$, $p = 0.008$), with breeding female gonads having the highest Dio3 mRNA across groups.

To confirm the validity of the T and E2 ELISAs, we examined T (in males) and E2 (in females) levels in breeding and non-breeding lizards. As has been shown previously (Lovern *et al.* 2001), breeding males had higher T levels compared to non-breeding males ($t_{10} = 3.670$, $p = 0.004$; Figure 4A). We were unable to detect a difference

in E2 levels between breeding and non-breeding females ($t_9 = 0.283$, $p = 0.783$; Figure 4C).

We examined aggressive behavior in breeding and non-breeding males to confirm the validity of the mirror test. As has been shown previously (Jenssen *et al.* 2006), breeding males displayed higher levels of aggressive behavior compared to non-breeding males ($t_8 = 3.059$, $p = 0.016$; Figure 4B).

Experiment 2: LH or FSH injections did not induce breeding in non-breeding lizards

For males, LH or FSH injections did not induce testicular growth ($F_{(2,17)} = 0.03$, $p = 0.973$; Figure 5A). We detected an effect of treatment on StAR mRNA expression ($F_{(2,17)} = 4.50$, $p = 0.027$; Figure 5B) in the gonads, such that LH injections induced increased StAR mRNA expression in testes compared to FSH injections, although the effect of LH was not significantly different from the vehicle injection group. Additionally, there were no effects of treatment on T levels ($F_{(2,16)} = 1.77$, $p = 0.203$; Figure 5C) and the latency to the first aggressive behavior ($F_{(2,18)} = 0.47$, $p = 0.631$; Figure 5D).

In females, there were no effects of LH or FSH treatments compared to the vehicle control on GSI ($F_{(2,11)} = 2.11$, $p = 0.167$; Figure 6A), StAR mRNA expression ($F_{(2,10)} = 0.83$, $p = 0.464$; Figure 6B), and E2 levels ($F_{(2,11)} = 0.71$, $p = 0.513$; Figure 6C).

Experiment 2: Upregulated Dio2 mRNA in testes in response to LH or FSH injections

We detected an effect of treatment on Dio2 mRNA expression in the testes ($F_{(2,17)} = 6.67$, $p = 0.007$; Figure 7A), with higher levels of Dio2 mRNA in testes after either LH or FSH injection compared to vehicle controls. There was no effect of treatment on Dio2 mRNA levels in ovaries ($F_{(2,10)} = 1.01$, $p = 0.398$; Figure 7B).

There were no effects of treatment on Dio3 mRNA expression in either the testes ($F_{(2,17)} = 2.40$, $p = 0.121$; Figure 7C) or ovaries ($F_{(2,10)} = 3.04$, $p = 0.093$; Figure 7D).

There were no significant correlations between GSI, T levels, testicular StAR gene expression and aggressive behavior in males (all $r^2 \leq 0.06$, $p \geq 0.301$; data not shown). In females, there were no significant correlations between GSI, E2 levels and ovarian StAR gene expression (all $r^2 \leq 0.06$, $p \geq 0.408$; data not shown).

Experiment 3: LH+FSH injections induced some breeding-like parameters in non-breeding males

Males injected with LH+FSH had a larger GSI compared to vehicle controls ($t_{13} = 2.46$, $p = 0.029$; Figure 8A). There was also a significant increase in StAR mRNA expression in testes after LH+FSH injections compared to vehicles ($U = 0$, $n_1 = 9$, $n_2 = 6$, $p < 0.001$; Figure 8B). Systemic T levels were also significantly increased in the LH+FSH group compared to controls ($t_{12} = 3.34$, $p = 0.006$; Figure 8C). However, there was no effect of treatment on the latency to the first aggressive display ($t_{13} = 0.32$, $p = 0.758$; Figure 8D). Plasma T concentration was highly correlated with gonadal StAR mRNA expression ($r^2 = 0.44$, $p = 0.010$; Figure 9). All other comparisons were not significant (all $r^2 \leq 0.15$, $p \geq 0.164$; data not shown).

Experiment 3: Testicular Dio3 mRNA expression is upregulated in response to LH+FSH injections

In the testes, we detected a significant increase in Dio3 mRNA levels in the LH+FSH injected group compared to the vehicle controls ($t_{13} = 3.68$, $p = 0.003$; Figure 10B). In contrast, there was no significant difference detected in testicular Dio2 mRNA

expression between groups ($t_{13} = 0.95$, $p = 0.359$; Figure 10A).

Discussion

The present study was designed to test the hypothesis that deiodinase (Dio2 and Dio3) mRNA expression patterns vary with sex and season, and whether expression levels of these enzymes interact with seasonal changes in gonadotropin levels (LH and FSH) to impact seasonal breeding morphology, steroid hormone levels and behavior. We found that Dio2 and Dio3 mRNA expression patterns are seasonally and sexually dimorphic, which suggests that differential activation of thyroid hormone locally might play a role in seasonal breeding. Also, increased testes size, steroidogenic enzyme mRNA expression, and Dio3 mRNA expression were induced after LH+FSH injections in non-breeding males. Our results suggest that local thyroid hormone may interact with gonadotropins to control seasonal changes in gonadal function.

Breeding testes may have higher TH activation by upregulating Dio2

Although neural Dio2 mRNA is known to be critical for reproduction in birds and mammals (Yoshimura *et al.* 2003; Yasuo *et al.* 2006, 2007; Ikegami *et al.* 2015), the role of Dio2 in the gonads has not been well studied. To our knowledge, this study is the first to examine Dio2 mRNA expression in reptilian gonads. We found that Dio2 mRNA expression in the testes was approximately three times higher in breeding compared to non-breeding male lizards, while females maintained low Dio2 mRNA expression levels in the ovaries across seasons (Figure 3). High Dio2 mRNA expression in breeding testes suggests that there might be higher gonadal activation of TH during the breeding season. Similarly, Dio2 mRNA was highly expressed, with high Dio2 activity in the germ cells of

adult rat testes (Wajner *et al.* 2007), with higher Dio2 protein expression during the breeding season in golden hamster (*Mesocricetus auratus*) testes (Verma & Haldar 2016). Furthermore, Dio2 knockout zebrafish (*Danio rerio*) had impaired fertilization and reduced T production (Houbrechts *et al.* 2019), suggesting that Dio2 (and TH signaling) might be important for adult testes function. These results imply that increased levels of gonadal T3 through Dio2 expression might play role in maintenance of normal testicular functions, steroidogenesis and gametogenesis.

Breeding ovaries may restrict TH activation by upregulating Dio3

We found that Dio3 mRNA expression was about four times higher in breeding compared to non-breeding ovaries, with very low expression in the testes across seasons (Figure 3). High expression of Dio3 mRNA in breeding ovaries suggests that restricting gonadal TH activation may be important for ovarian function in green anoles. This was an unexpected result, as hypothyroidism is known to be associated with irregular estrus cycles and reduced E2 production in both rats and zebrafish (Tohei *et al.* 1998; Houbrechts *et al.* 2019). Furthermore, T3 is known to increase E2 production and follicular development from mouse granulosa cells in the presence of FSH (Liu *et al.* 2017). However, work in seasonally breeding ewes (*Ovis aries*) reported that thyroidectomy at the end of the breeding season prevented the transition to anestrus (the period of sexual quiescence) and the suppression of the HPG axis, which suggests the necessity of TH in suppressing the HPG axis and inducing the transition to anestrus (Moenter *et al.* 1991). These results support the idea that breeding lizard ovaries might restrict thyroid hormone activation by increasing Dio3 expression in order to maintain reproduction and prevent the transition to non-breeding.

TH levels have been shown to fluctuate seasonally in a variety of seasonally breeding animals. For example, ewes have increased T4 and T3 levels during the non-breeding compared to breeding season (Peeters *et al.* 1990), while female frog (*Rana perezi*) plasma T4 and T3 levels are increased during the breeding compared to non-breeding season (Gancedo *et al.* 1995). Furthermore, exogenous T3 injections have been demonstrated to upregulate Dio3 mRNA expression in the gonads of another frog species (*Silurana tropicalis*), which may be a mechanism to maintain normal T3 levels in the gonads (Campbell & Langlois 2018). Although seasonal levels of TH have not been directly measured in green anoles, studies have shown that thyroid activity is increased in green anole and western fence (*Sceloporus occidentalis*) lizards in warmer compared to colder environmental temperatures (Lynn *et al.* 1965; Chiu *et al.* 2007), which suggests seasonal variation in TH levels in lizards. Thus, increased Dio3 expression in breeding green anole ovaries might function in a compensatory manner to counteract rising endogenous TH levels during the breeding season. Taken together, these data suggest that, during the breeding season, T3 production might be restricted to allow for appropriate ovarian reproductive function, although more work is needed to examine this possibility.

Low levels of LH or FSH induce increased Dio2 mRNA

We detected increased Dio2 mRNA expression in the LH or the FSH injected lizards, with no changes in GSI, StAR mRNA expression and plasma T levels compared to vehicle controls (Figures 5 and 7). Likely, the LH and FSH doses were too low to induce testicular recrudescence from a non-breeding to breeding state, but were enough to induce changes in Dio2 levels. Martinez et al. (2016) has suggested that Dio2 is not as

critical as Dio3 in testicular development, with Dio2 knockout mice rarely displaying deficits in fertility or testicular structure. Similarly, pubertal Japanese quail (*Coturnix japonica*) with regressed gonads maintain high levels of Dio2 mRNA until the gonad begins transitioning to breeding (Ikegami *et al.* 2015). Taken together, these data suggest that Dio2 might be important for maintaining gonadal state (i.e., breeding or non-breeding). Additional studies will be needed to test this idea.

LH+FSH injections induced a breeding-like testis

We observed that LH+FSH injections induced approximately 3 times higher T production compared to vehicle controls, which was coupled with increased testicular growth and StAR mRNA expression in testes in LH+FSH treated lizards (Figures 8 and 9). Circulating LH is known to induce StAR expression in the gonads, which is the rate limiting step of steroidogenesis (Clark *et al.* 1994; Kallen *et al.* 1998). Thus, increased LH levels during the breeding season lead to an increased StAR response, which is followed by an increase in steroid hormone production in a variety of seasonally breeding animals such as horses (*Equus caballus*), tree swallows (*Tachycineta bicolor*), Japanese quail, European sea bass (*Dicentrarchus labrax*), and green anole lizards (Rocha *et al.* 2009; Kozi *et al.* 2012; Ikegami *et al.* 2015; Peek & Cohen 2018; Bentz *et al.* 2019). Similarly, our data revealed that gonadotropins induced steroidogenesis in non-breeding green anole males.

Although LH+FSH injections induced a breeding-like testes with increased circulating T levels, male aggressive behavior was not enhanced (Figure 8). Our treatment paradigm was relatively short (two weeks), which may not have been long enough to induce neural changes necessary to alter aggressive behavior (e.g. dewlap

extensions, push ups, and lateral compression) (Kabelik *et al.* 2008). Additionally, there is evidence in a number of species, including green anoles, that aggression is not necessarily dependent on circulating T levels, but may be dependent on other parameters, such as social cues (Yang & Wilczynski 2003; Korzan & Summers 2004).

LH+FSH injections induced increased Dio3 mRNA in testes

We found that LH+FSH injections induced significant upregulation of Dio3 mRNA expression in non-breeding male testes compared to vehicles (Figure 10), suggesting that non-breeding males with acute HPG axis activation may be restricting TH activation in the testes by increasing Dio3 mRNA expression. This contrasts with our data from unmanipulated breeding males, with upregulated Dio2 mRNA expression. Unlike mammals, reptiles do not appear to undergo puberty and, instead, gonadal maturation occurs seasonally during the breeding season, with regression to a non-mature state in the non-breeding season in response to changes in photoperiod and HPG axis activity (Ball & Wade 2013). Thus, the transition to breeding from non-breeding gonads with upregulated HPG axis activity can be compared to the maturation of mammalian gonads (i.e., puberty). Thus, lizards treated with LH+FSH for short periods likely have developing testes instead of fully matured testes. High Dio3 mRNA levels in developing lizard testes are similar to high Dio3 activities detected in the developing testes of neonatal and weaned rats and mice (Bates *et al.* 1999; Hernandez *et al.* 2006). Similarly, Dio3 knockout mice have impaired sperm production as well as reduced testicular weight, which implies that restriction of T3 signaling by Dio3 during development is essential for normal testis maturation and function (Martinez *et al.* 2016). Furthermore, Dio3 mRNA is highly expressed in Japanese quail testes during the early stage of testicular

recrudescence with increased activity of HPG axis, indicating the presence of low testicular T3 at the beginning of recrudescence (Ikegami *et al.* 2015). Together with previous studies, our data suggest that there may be a potential inhibitory effect of TH on testicular recrudescence, and maturing testes increase Dio3 expression in order to reduce TH signaling. Additional work is needed to test this idea.

Conclusion

Relatively little is known about thyroid activation or inactivation in the gonads and how this might play a role in seasonal changes of reproductive functions. Dio2 mRNA was upregulated in breeding lizard testes, which suggests that breeding testes may have increased TH activation, while Dio3 mRNA was upregulated in breeding ovaries, potentially restricting TH signaling. However, acute HPG axis activation by LH+FSH injections induced increased Dio3 mRNA levels, which was accompanied by early testicular recrudescence as determined by increased testicular growth, StAR mRNA and plasma T. This suggests different roles of locally activated TH in the gonads, where TH might inhibit testicular recrudescence, but is important for the maintenance of breeding testes. Future work is needed to elucidate how HPG axis activation alters deiodinase gene expression in both males and females and what role locally produced TH has in seasonal breeding.

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Figures and Tables

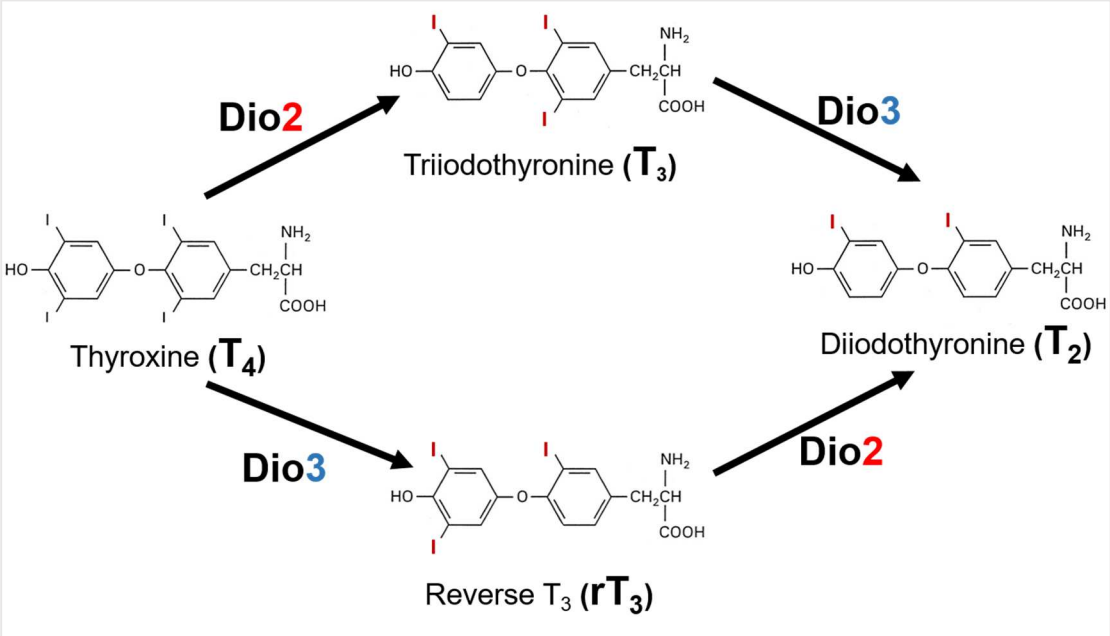

Figure 1. TH activation and inactivation is controlled by deiodinase enzymes. With specific iodine removal, Dio2 activates TH by converting T_4 to T_3 and Dio3 inactivates TH by converting T_4 to rT_3. Dio3 and Dio2 convert T_3 and rT_3, respectively, to T_2.

Experiment	Group	Injection Dose	N
1	BS	No injection	♂: 6, ♀: 6
	NBS	No injection	♂: 6, ♀: 6
2	Vehicle	-	♂: 6, ♀: 4
	LH	0.2µg/g	♂: 6, ♀: 5
	FSH	0.015µg/g	♂: 6, ♀: 5
3	Vehicle	-	♂: 3
	LH + FSH	2µg/g LH + 0.15µg/g FSH	♂: 6

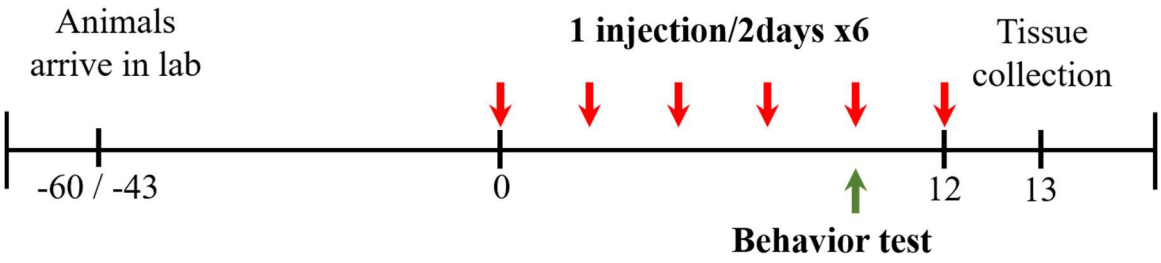

Figure 2. Experimental design. Lizards in experiment 1 were unmanipulated. For experiments 2 and 3, lizards were injected once every two days (total of 6 injections) with vehicle, LH, FSH, or LH+FSH.

Table 1. Target genes with primer sequences, amplicon size, primer concentration and qPCR efficiencies.

Gene	NCBI ID #	Primer Sequence	Amplicon Size (bp)	Concentration (µg/µl)	Efficiency
Dio2	100553484	F: AGCCTTACAAAAGAGCAAGC R: CACACCAGCCTTCGTGTCTT	166	0.2	2.03
Dio3	103279636	F: TGGGTTCAGAGGAGAGTTGC R: CAGAATGGACCTCCCAGGTG	247	0.2	1.96
StAR	100554580	F: CACTCGCTGGAGATCCCTACC R: CCACCTGCGTCTGGG	112	0.05	2.14
β-Actin	100337533	F: GACGAGGCGCAGAGTAAAG R: TCAGGGCAACTCTCAACTC	131	0.1	2.04

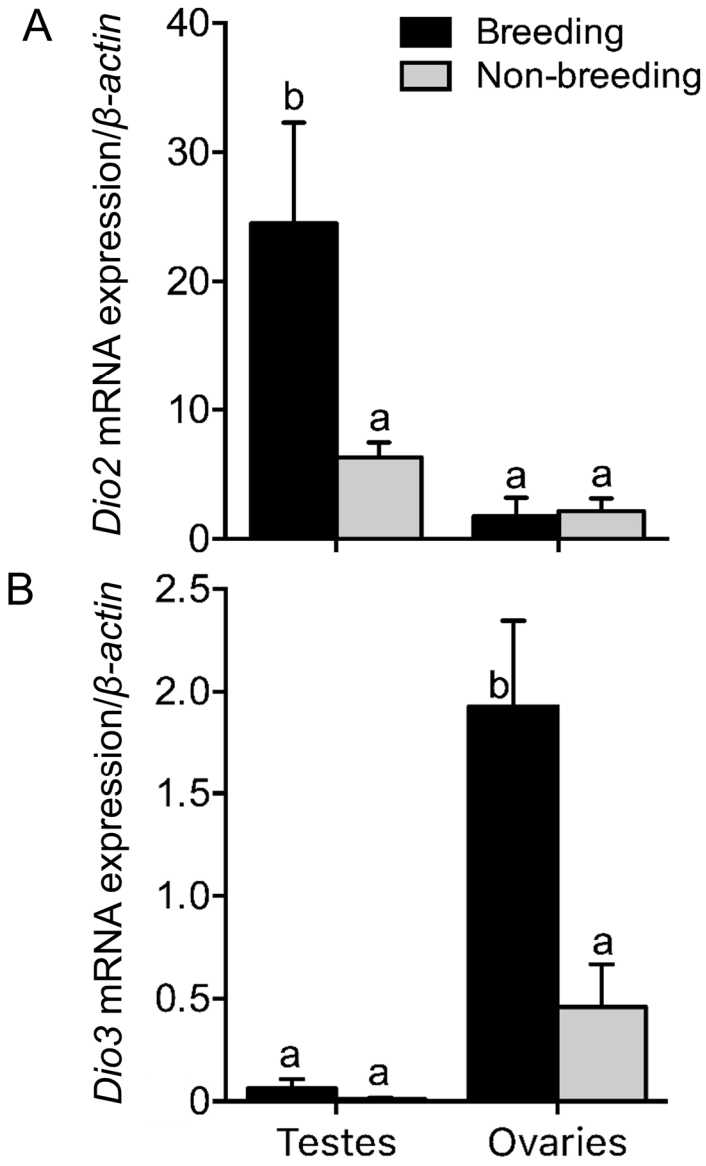

Figure 3. Deiodinase mRNA expression (arbitrary units) in breeding and non-breeding gonads. (A) Dio2 expression in the breeding male gonads was significantly upregulated compared to other groups. (B) Dio3 expression in the gonads was increased in breeding female gonads compared to other groups. n = 5-6 per group. Letters above bars denote statistical differences between groups. Two-way ANOVAs, $p \leq 0.05$

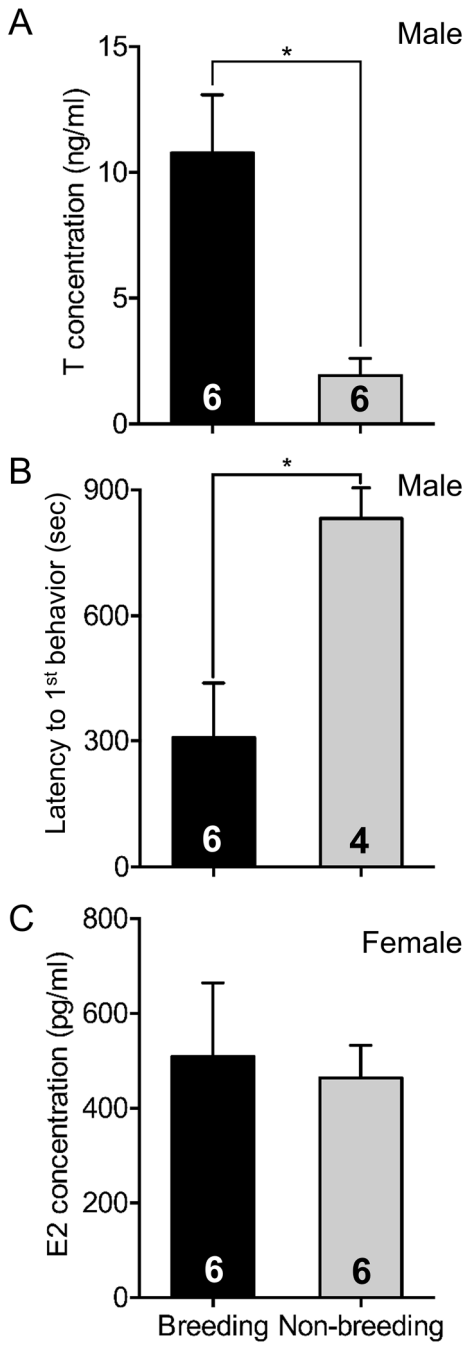

Figure 4. Steroid hormone ELISAs and aggressive behavior tests were validated in this species. (A) T levels were higher in breeding compared to non-breeding males (B) Breeding males had a shorter latency to the first aggressive behavior display compared to non-breeding males. (C) E2 levels were not different between breeding and non-breeding females. Numbers in the bars denote the sample size. T-test, *$p \leq 0.05$

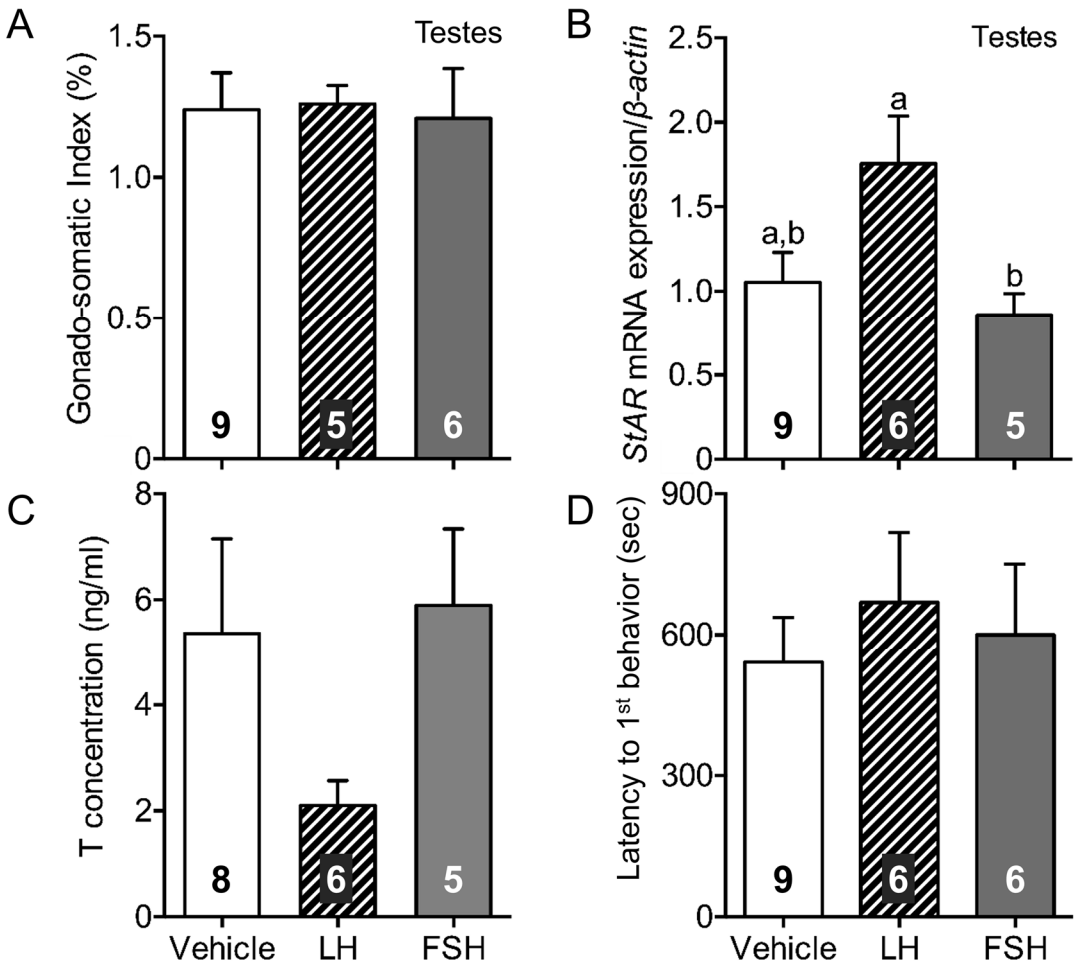

Figure 5. Testicular response to LH or FSH injections. (A) LH or FSH injections did not induce changes in the gonado-somatic index (GSI) of testes. (B) StAR mRNA levels (arbitrary units) in testes were higher in the LH injected group compared to the FSH injected group, but these did not differ from the vehicle injected group. (C,D) LH or FSH injections did not induce changes in T levels or aggressive behavior. Numbers in the bars denote the sample size. Letters above bars denote statistical differences between groups. One-way ANOVAs, $p \leq 0.05$

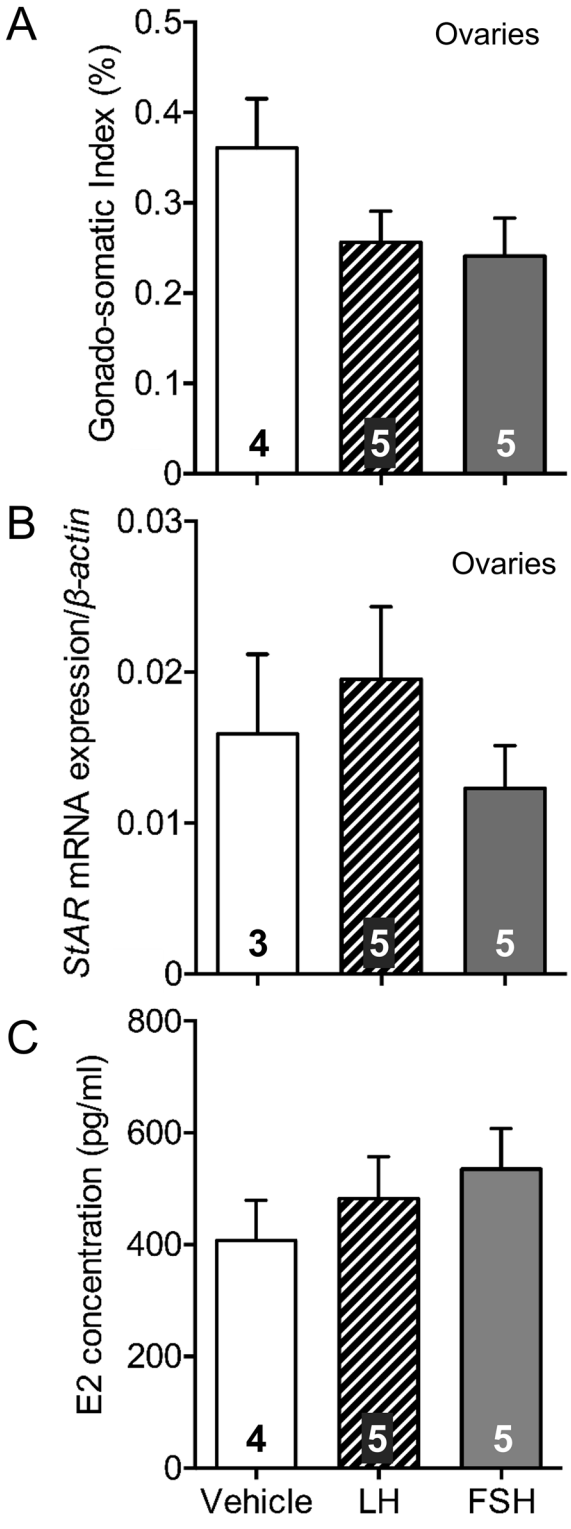

Figure 6. Ovarian response to LH or FSH injections. LH or FSH injections in females did not induce changes in (A) GSI, (B) StAR mRNA expression (arbitrary units) or (C) E2 levels. Numbers in the bars denote the sample size. One-way ANOVAs, $p \geq 0.167$

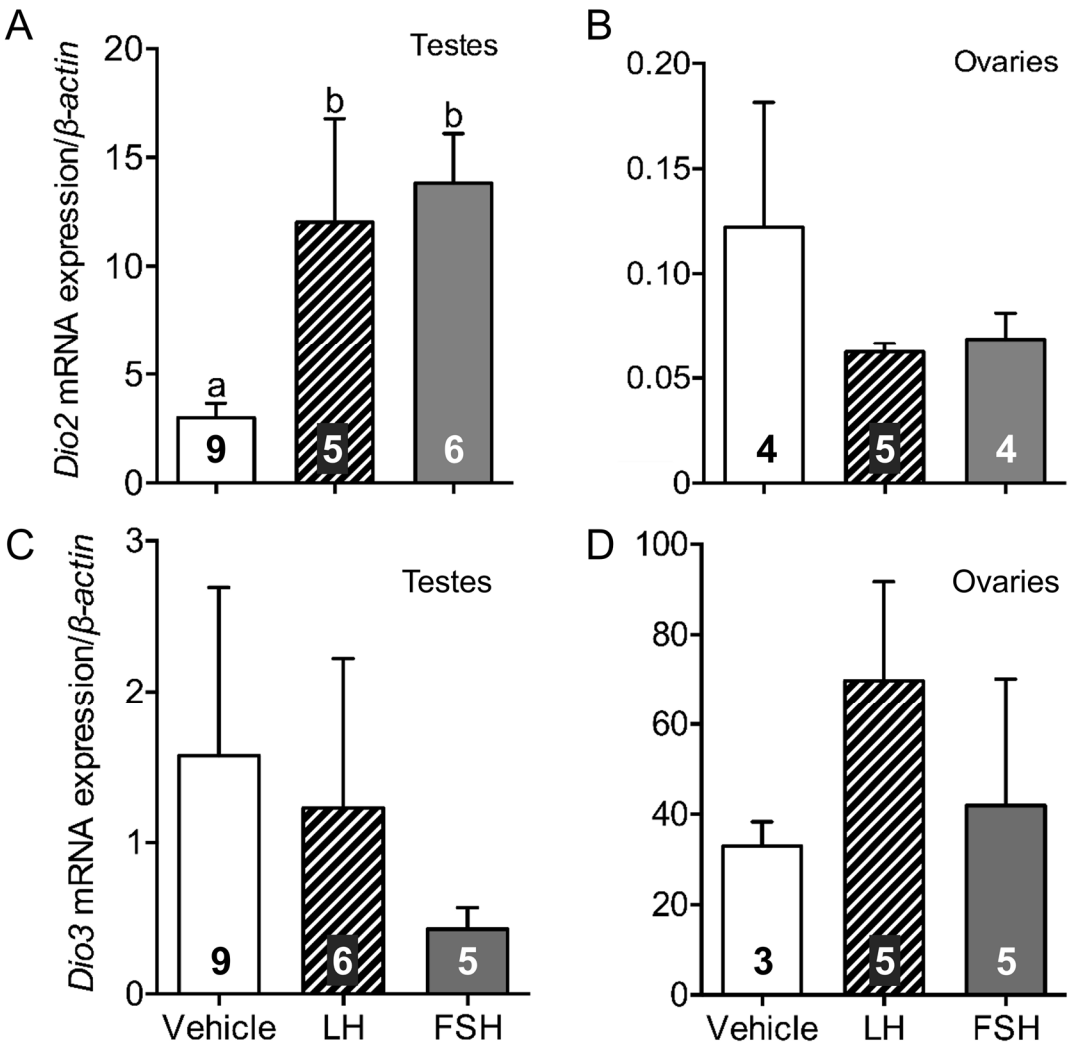

Figure 7. Deiodinase mRNA expression (arbitrary units) in testes and ovaries in response to LH or FSH injections. (A, B) Dio2 mRNA expression was upregulated in the testes of animals injected with LH or FSH compared to vehicle controls, with no difference in females. (C, D) Relative Dio3 mRNA expression did not differ across injection groups in both testes and ovaries. Numbers in the bars denote the sample size. Letters above bars denote statistical differences across groups. One-way ANOVAs, $p \leq 0.05$

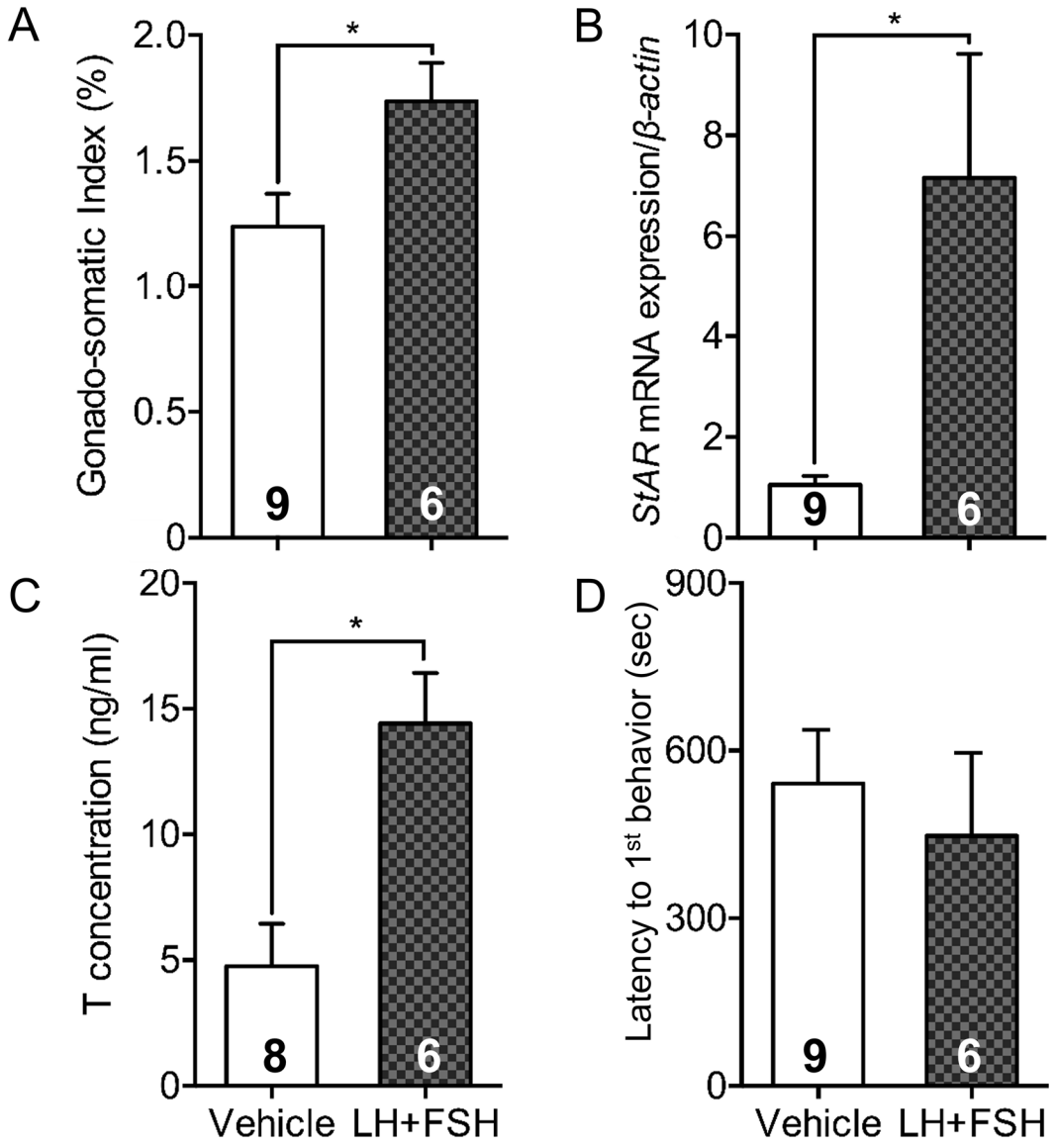

Figure 8. Testicular response to LH+FSH injections. (A) LH+FSH injections increased the GSI compared to vehicle controls. LH+FSH injections increased (B) StAR mRNA expression (arbitrary units) and (C) plasma T levels compared to vehicle controls. (D) The latency to the first aggressive behavior did not differ between groups. Numbers in the bars denote the sample size. T-test, *$p \leq 0.05$

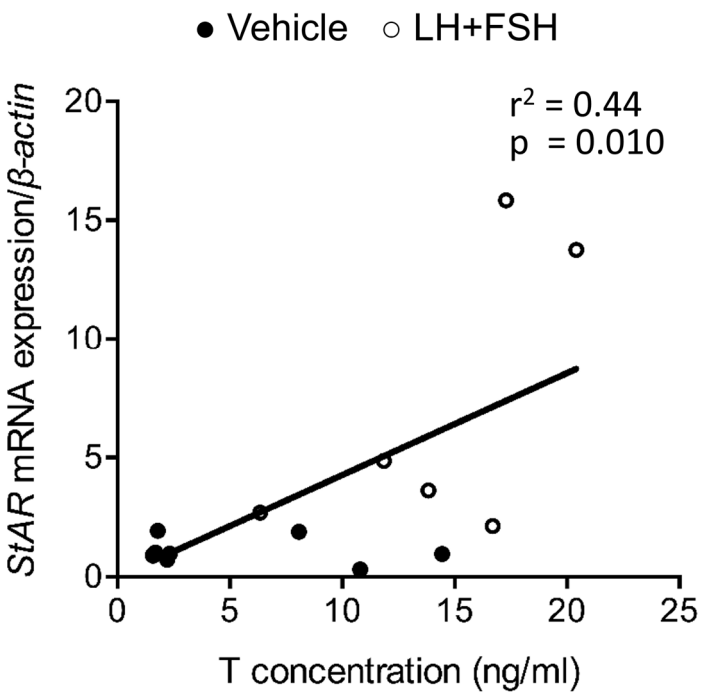

Figure 9. Correlation between testicular responses after LH+FSH injections. Plasma T concentration was highly correlated with StAR mRNA expression (arbitrary units). $p \leq 0.05$

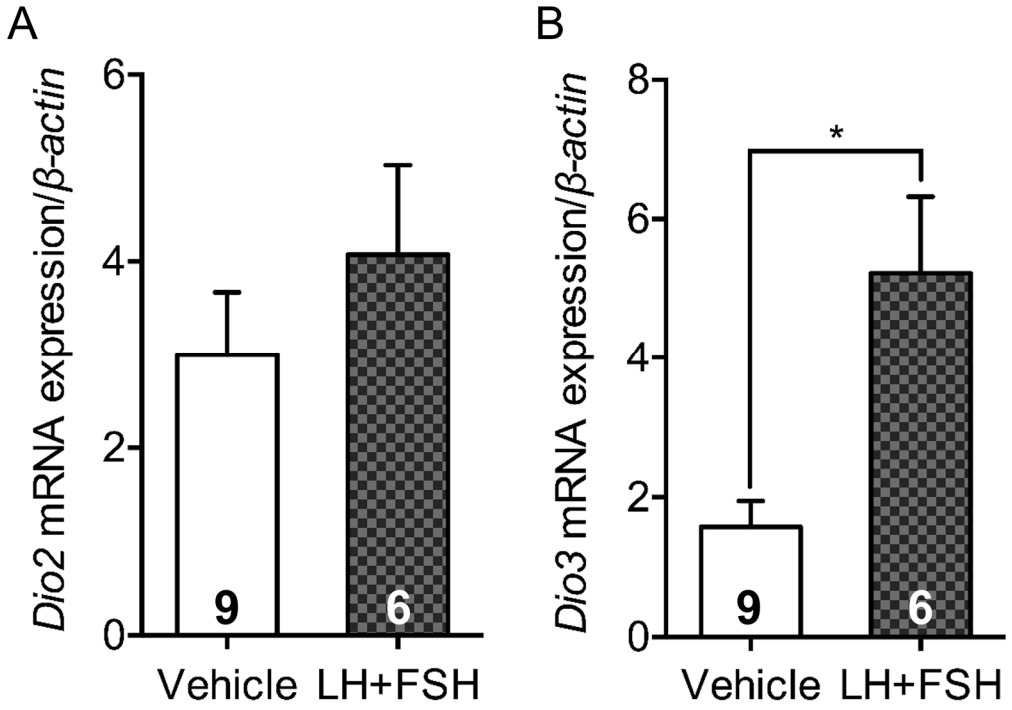

Figure 10. Testicular deiodinase mRNA expression (arbitrary units) after LH+FSH injections. (A) Dio2 mRNA expression was not different between vehicle and LH+FSH groups. (B) Dio3 mRNA expression was significantly increased in LH+FSH injected lizards compared to vehicle controls. Numbers in the bars denote the sample size. T-test, *$p \leq 0.05$

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