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Tissue-specific gene regulation corresponds with seasonal plasticity in female testosterone

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ABSTRACT

Testosterone (T) is a sex steroid hormone that often varies seasonally and mediates trade-offs between territorial aggression and parental care. Prior work has provided key insights into the 'top-down' hypothalamic control of this seasonal plasticity in T, yet mechanisms acting outside of the brain may also influence circulating T levels. We hypothesized that peripheral mechanisms may be especially critical for females, because peripheral regulation may mitigate the costs of systemically elevated T. Here, we begin to test this hypothesis using a seasonal comparative approach, measuring gene expression in peripheral tissues in tree swallows (*Tachycineta bicolor*), a songbird with intense female-female competition and T-mediated aggression. We focused on the gonad and liver for their role in T production and metabolism, respectively, and we contrasted females captured during territory establishment versus incubation. During territory establishment, when T levels are highest, we found elevated gene expression of the hepatic steroid metabolizing enzyme CYP2C19 along with several ovarian steroidogenic enzymes, including the androgenic 5α-reductase. Despite these seasonal changes in gene expression along the steroidogenic pathway, we did not observe seasonal changes in sensitivity to upstream signals, measured as ovarian mRNA abundance of luteinizing hormone receptor. Together, these data suggest that differential regulation of steroidogenic gene expression in the ovary is a potentially major contributor to seasonal changes in T levels in females. Furthermore, these data provide a unique and organismal glimpse into tissue-specific gene regulation and its potential role in hormonal plasticity in females.

1. Introduction

Individuals are often confronted by energetic, temporal, or mechanistic limitations that generate trade-offs between mating effort and parental investment (Stiver and Alonzo, 2009; Trivers, 1972). Hormones, such as the sex steroid testosterone (T), are generally considered key mediators of these trade-offs (Ketterson et al., 1992; Wingfield et al., 1990). Decades of research in male vertebrates suggest that elevated levels of T in circulation are associated with enhanced mating effort and territory defense (Eens et al., 2007; Onyango et al., 2013; Sasvari et al., 2009), but negatively related to parental care (McGlothlin et al., 2007; Smorkatcheva et al., 2010), and these patterns are further supported when T is experimentally elevated (reviewed in Lynn, 2008, 2016). Accordingly, T levels often decrease in circulation as the breeding season progresses from acquiring territories and mates to caring for offspring (Goymann et al., 2007; Hirschenhauser and Oliveira, 2006; Wingfield et al., 1990).

While we know far less about these patterns in females, it is becoming clear that, at least in some systems, females may face similar T-mediated trade-offs (de Jong et al., 2016; Ketterson et al., 2005; Rosvall, 2013a, 2013b; but see Goymann and Wingfield, 2014). Female-female competition is common (Clutton-Brock, 2009; Rosvall, 2011; Stockley and Bro-Jørgensen, 2011), particularly early in the breeding season (Rosvall, 2008; Sandell and Smith, 1997; Slagsvold and Lifjeld, 1994). Territorial aggression in females typically increases with experimental elevations of T (Chiver and Schlinger, 2017; Rosvall, 2013a; Sandell, 2007; Zysling et al., 2006; but see Elekonich and Wingfield, 2000), and female aggression has been positively correlated with natural variation in circulating T (Cain and Ketterson, 2012; Langmore et al., 2002; but see Jawor et al., 2006a). However, females can incur costs associated with experimentally elevated T, even within physiological ranges, such as reduced maternal care (O'Neal et al., 2008; Rosvall, 2013a), delayed breeding (Clotfelter et al., 2004; de Jong et al., 2016; Goerlich et al., 2010), and decreased reproductive output (Gerlach and Ketterson, 2013). Therefore, it may be beneficial for females to change circulating T levels alongside seasonal changes in the

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demands of social competition, reproduction, and maternal care (Ketterson et al., 2005), and yet these proximate mechanisms in females are unresolved.

From an evolutionary perspective, selection acting on females should favour mechanisms that allow for greater phenotypic independence (Ketterson et al., 2009), where one or another trait can become less dependent on the direct actions of circulating T due to tissue-specific variation in T production or sensitivity. In principle, this would enable more precise control of T or specific T-mediated traits without necessitating systemically elevated T (Rosvall, 2013b), and it may explain why a relationship between systemic T levels and life-history traits is not always apparent in females (Goymann and Wingfield, 2014). The ability to locally regulate steroid production and sensitivity in neural tissues has previously been demonstrated in both males and females (Soma, 2006; Soma et al., 2008; Voigt and Goymann, 2007), and similar processes may also occur in peripheral tissues (Schmidt et al., 2008; Soma, 2006). Indeed, females have all the cellular and molecular machinery to produce and respond to T in peripheral tissues (MacManes et al., 2017; Staub and De Beer, 1997). Moreover, at least in mammals, androgen-responsive gene networks in females show a high degree of modularity in peripheral tissues (van Nas et al., 2009), suggesting that females may more precisely regulate functionally connected T-mediated gene networks in the periphery.

Seasonal plasticity in T production is regulated primarily by the hypothalamo-pituitary-gonadal (HPG) axis, which is activated when external stimuli, such as changing daylengths, trigger the hypothalamus to release gonadotropin-releasing hormone (GnRH), which stimulates pituitary release of luteinizing hormone (LH) that binds to LH receptor (LH-R) in the gonad. This prompts a series of enzymatic steps to produce and secrete T (Fig. 1a). The seasonal regulation of this tropic hormonal cascade is often studied using GnRH challenges, which indicate that gonadal responsiveness to upstream signals varies seasonally and may be sex-specific. While males typically retain the ability to produce T to some degree throughout the breeding season (Apfelbeck and Goymann, 2011; Jawor et al., 2006b), females eliminate or dramatically reduce T production during parental phases (DeVries and Jawor, 2013; George and Rosvall, 2018; Jawor et al., 2007), despite still producing other upstream hormones like LH (Jawor et al., 2007). These data suggest females modulate T responsiveness downstream of the hypothalamus or pituitary, perhaps in the gonad. However, most studies focus on seasonal variation in the 'top-down' effects of the hypothalamus on T production (e.g., Calisi et al., 2016; Dixit et al., 2017), although expression of several gonadal steroidogenic enzymes (e.g., StAR, P450scc, and CYP17) has also been linked to variation in T secretion (Huffman et al., 2012; Luo et al., 2005; Rosvall et al., 2016; Zmuda et al., 2001). While the mechanisms regulating these enzymes are well-studied (LaVoie and King, 2009; Miller, 1988; Payne and Hales, 2004; Stocco et al., 2005), their seasonal variability, particularly in females, remains uncertain.

T clearance may also play a role in regulating seasonal plasticity in T levels, but has received far less attention. Steroid metabolism largely occurs in the liver, where T and other steroids are conjugated to make them water soluble prior to clearance from the body. This process is catalysed by UDP-glucuronosyltransferase (UGT; i.e., glucuronidation), cytochrome P450 (CYP; i.e., hydroxylation), and sulfotransferase (SULT; i.e., sulfonation) enzymes (Mueller et al., 2015; Wilson and Leblanc, 2000; Fig. 1b). Many of these enzymes not only have a high affinity for androgens (e.g., UGT2B17, Dehennin and Matsumoto, 1993; Sten et al., 2009; Turgeon et al., 2001; CYP2C19, Yamazaki and Shimada, 1997), but they have been linked to variation in circulating T levels (e.g., UGT2B17, Jakobsson et al., 2006; CYP2C19, Löfgren et al., 2009; SULT1B1, Peterson et al., 2014) and behavior (e.g., CYP2C19, Ishii et al., 2007). Thus, peripheral tissues outside of the HPG axis, such as the liver, have the potential to regulate the amount of T available in circulation, yet far less is known about how liver metabolism may change over the breeding season.

In this study, we tested the hypothesis that seasonal changes in female T levels may be facilitated by tissue-specific changes in T production and metabolism in the periphery. Our study subjects, female tree swallows (*Tachycineta bicolor*), fiercely compete for limited nesting cavities early in the breeding season (Rosvall, 2008), prior to producing a single brood that females alone incubate. Critically, they demonstrate T-mediated trade-offs between aggression and maternal care (Rosvall, 2013a). Thus, even though there is ambiguity about the general applicability of T-mediated trade-offs in females (Goymann and Wingfield, 2014), there is very little ambiguity about it in the species used in the current study. In particular, aggression in female tree swallows is enhanced by exogenous T, and T levels elevated within physiologically relevant ranges have detrimental effects on incubation and hatching success (Rosvall, 2013a), indicating there should be fitness benefits to having elevated T early in the breeding season (i.e., greater likelihood of securing a territory) paired with fitness costs

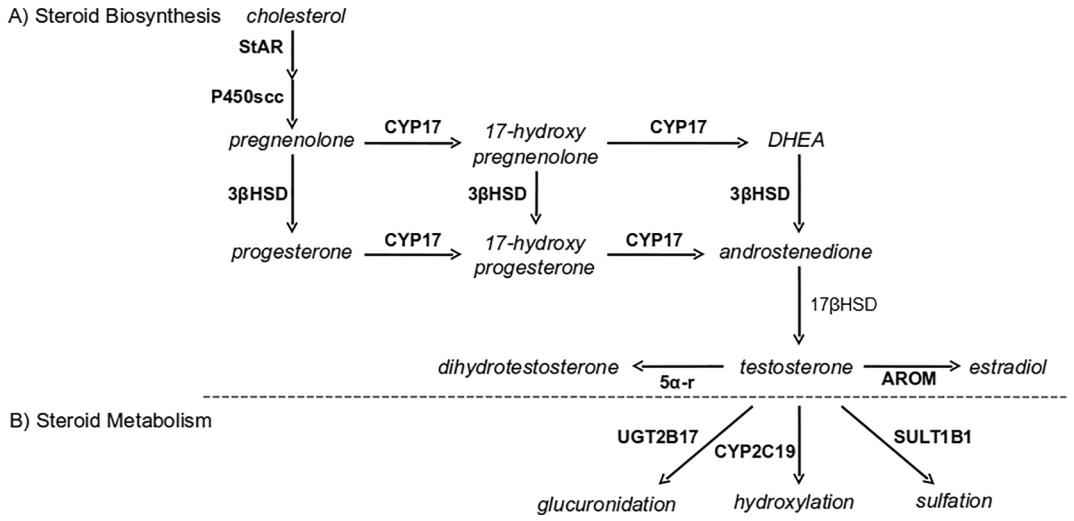

Fig. 1. Simplified diagram of sex steroid (A) biosynthesis and (B) metabolism. Genes of interest are highlighted in bold. Abbreviations: StAR, steroidogenic acute regulatory protein; P450scc, cytochrome P450 side-chain cleavage; CYP17, cytochrome P450 17α-hydroxylase/17,20lysase; 3βHSD, 3β-hydroxysteroid dehydrogenase/isomerase; 17βHSD, 17β-hydroxysteroid dehydrogenase; DHEA, dehydroepiandrosterone; AROM, cytochrome P450 aromatase; 5α-r, 5α-reductase; UGT2B17, UDP-glucuronosyltransferase 2B17; CYP2C19, cytochrome P450 2C19; SULT1B1, sulfotransferase 1B1. The metabolic processes shown here are relevant for T as well as other steroid hormones.

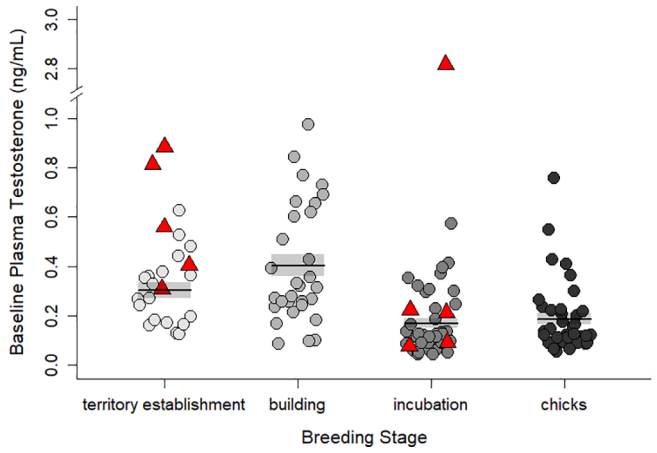

Fig. 2. Baseline plasma testosterone (ng/mL) and breeding stage in female tree swallows from the current study (represented by triangles) and data reproduced from George and Rosvall (2018; circles, means, and SEM), which measured T in females from the same population in the same year. The black line is the mean and shading denotes SEM.

during parental phases. Consistent with this potential trade-off, female T levels decline as the breeding season progresses, with marked T production during territory establishment and nest building, followed by a precipitous decline during incubation (Fig. 2; circles, means, and SEM reproduced from George and Rosvall, 2018). In the current study, we quantified two major components of peripheral gene regulation that may contribute to these seasonal changes in T. First, in the ovary, we measured expression of several genes know to influence sex steroid production (Fig. 1a), including LH-R as a proxy for gonadal sensitivity to upstream signals; StAR, which is responsible for positioning cholesterol for steroidogenesis; P450scc, which converts cholesterol into pregnenolone; and 3βHSD and CYP17, which convert pregnenolone into more active hormones. We also measured ovarian AROM and 5α-r, which convert T into 17β-estradiol and the potent androgen 5α-dihydrotestosterone (5αDHT), respectively. Second, focused on the liver, we quantified expression of genes that code for steroid metabolizing enzymes (Fig. 1b): UGT2B17, CYP2C19, and SULT1B1. We predicted that ovarian steroidogenesis would decline and that liver metabolism would increase to facilitate declining T in later breeding stages.

2. Methods

2.1. General field methods

We monitored breeding tree swallows located in Monroe and Brown County, Indiana, USA (39°9N, 86°31 W) between April and July 2016. We collected females between 900 and 1330 hr in their nestboxes during territory establishment or incubation. Females captured during territory establishment (April 4–14) were actively engaging in aggressive interactions at nest sites to defend territory ownership (KAR, pers. obs.), as is characteristic of this breeding stage (Rosvall, 2008). None of these females had a complete nest, nor did any other females in this study population, suggesting that they were not yet fertile. For reference, the earliest egg laying in the population began on April 25, with the peak of laying during the first week of May. Females captured during incubation (May 16–31) all had a brood patch and warm eggs, and had completed laying 3–11 days earlier (avg. 7.2 days ± 1.6) out of the typical 14-day incubation period. Females were sexed based on the presence of a brood patch, wing chord length, behavior, and/or plumage coloration (Stutchbury and Robertson, 1987). Sex was also confirmed post-mortem, described below. This study was approved by the Bloomington Institutional Animal Care and Use Committee under protocol #15–004.

2.2. Tissue collection

Ten females (n = 5 territory establishment and n = 5 incubation stage) were euthanized with an overdose of isoflurane, followed by decapitation, and tissues were immediately collected. Gonad and liver were frozen on powdered dry ice and transferred to −80 °C in the lab. Trunk blood was stored on ice and centrifuged before plasma was separated and stored at −20 °C. Brain, pectoral muscle, and spleen were also collected and snap-frozen for another study. We qualitatively assessed reproductive status by visual inspection of the ovaries. All females captured during territory establishment had recrudesced ovaries with small white follicles. All incubating females had small white follicles as well, and two of them also had small yellow follicles. One of these incubating females had several yellow follicles, was the earliest collected (May 16), and had completed laying eggs the most recently (3 days earlier); the other female had one small yellow follicle and had completed a clutch 11 days earlier.

2.3. Plasma testosterone analysis

We quantified T concentrations using 40 µl of plasma using enzyme immunoassays (Enzo #ADI-900-176, Farmingdale, NY, USA), which were recently validated for female tree swallows (George and Rosvall, 2018). All samples were extracted 3× with ether and plated in duplicate. Anti-testosterone had a cross-reactivity of 16.4% with androstenedione, 7.6% with 19-hydroxytestosterone, 2.70% with DHT, and < 1% with all other steroids. Samples were run on two plates. Average intra-assay variation was 4.8%, and inter-assay variation was 5.4%. We did not calculate or correct for extraction efficiency of individual samples, but we estimated efficiencies to be 94.6% ± 1.3 SEM based on spike-recovery experiments conducted in parallel.

2.4. Quantitative PCR

We extracted RNA from gonad and liver tissue using the phenol-chloroform-based Trizol method, following the manufacturer's instructions (Invitrogen, Carlsbad, CA). We quantified total RNA with spectrophotometry and treated 1 µg RNA with DNAse (Promega, Madison, WI) and RNasin Ribonuclease Inhibitor (Promega, Madison, WI) for reverse-transcription with oligo dT primers and Superscript III (Invitrogen, Carlsbad, CA).

The resulting cDNA was used in quantitative real-time PCR (qPCR) to measure mRNA abundance of LH-R, StAR, P450scc, 3βHSD, CYP17, AROM, and 5α-r in the gonad, and CYP2C19, SULT1B1, and UGT2B17 in the liver. We did not include 17βHSD in this study because 17βHSD has several isoforms that both synthesize and metabolize T, and there is some suggestion in the literature that avian isoforms may function differently than the more well characterized mammalian isoforms (London, 2013; London and Clayton, 2010; Mindnich et al., 2004). Our analyses therefore include most other major genes involved in sex steroid synthesis, conversion, and metabolism. We used the reference gene peptidylprolyl isomerase A (PPIA) for normalization.

Most of these primers have been previously published in other songbirds (Abolins-Abols et al., 2018; Bergeon-Burns et al., 2014; Rosvall et al., 2012, 2016), but we designed new primers for 5α-r, CYP2C19, SULT1B1, and UGT2B17 for this study (see Table S1 for all primer sequences and accession numbers). All primers were validated using a serial dilution (replication efficiencies: 103.15% ± 1.69; Table S1) and melt curves were checked for a single product. All qPCR reactions (25 µl) were run in duplicate, alongside no template controls (NTCs), in a Stratagene MX3000p thermocycler (Agilent Technologies, Santa Clara, CA) using PerfeCta SYBR Green SuperMix with low ROX (Quanta Biosciences, Gaithersburg, MD). Each well contained 2.5 µl cDNA diluted 1:50 (except P450scc which was 1:10), or 2.5 µl water for NTCs, and primers diluted to 0.3 uMin for a total volume of 25 µl. cDNA dilutions were selected to optimize efficiency across all samples within

Table 1
Linear models testing differences in mRNA abundance (relative fold change calculated with the $2^{-\Delta\Delta Ct}$ method) between breeding stages (territory establishment and incubation) while controlling for time of capture. Estimates (β) use territory establishment as the reference and significant values ($p < 0.05$) are in bold. Adjusted p values (p_{adj}) with a Benjamini-Hochberg correction are also presented.

Tissue	Dependent Variable	Independent Variable	β (SE)	$F_{2,7}$	p	p_{adj}
Gonad	LH-R	Stage	−0.02 (0.15)	0.02	0.90	0.90
		Time	−0.001 (0.001)	0.38	0.56	0.58
	StAR	Stage	−1.12 (0.38)	8.45	**0.02**	0.07
		Time	0.002 (0.002)	1.41	0.27	0.58
	P450scc	Stage	1.93 (3.74)	0.20	0.67	0.85
		Time	−0.03 (0.02)	1.80	0.22	0.58
	3βHSD	Stage	−5.06 (1.87)	7.15	**0.03**	0.07
		Time	0.01 (0.01)	0.34	0.58	0.58
	CYP17	Stage	−3.28 (0.75)	18.14	**0.004**	**0.03**
		Time	0.01 (0.004)	4.50	0.07	0.49
	AROM	Stage	−0.11 (0.33)	0.13	0.73	0.85
		Time	−0.001 (0.002)	0.62	0.46	0.58
	5α-r	Stage	−0.29 (0.12)	6.10	**0.04**	0.07
		Time	−0.001 (0.001)	0.60	0.46	0.58
Liver	CYP2C19	Stage	−0.31 (0.13)	6.73	**0.04**	0.12
		Time	−0.003 (0.001)	12.91	**0.01**	**0.03**
	SULT1B1	Stage	−0.68 (0.40)	2.87	0.13	0.20
		Time	−0.0002 (0.002)	0.01	0.94	0.94
	UGT2B17	Stage	−0.01 (0.41)	0.01	0.93	0.93
		Time	−0.003 (0.002)	1.79	0.22	0.33

a tissue. Thermocycling conditions were: 10 min at 95 °C, followed by 40 cycles of 95 °C for 30 s, 60 °C for 1 min, and 70 °C for 30 s. A final melting phase of 95 °C for 1 min, 55 °C for 30 s, and 95 °C for 30 s was run to confirm single-product specificity of each sample.

We used MxPro software (v.4.10, Agilent) to calculate mRNA abundance using the comparative Ct method ($2^{-\Delta\Delta Ct}$), which reports mRNA abundance for each gene of interest as the fold change in expression compared to a calibrator sample and normalized to an internal reference gene (Livak and Schmittgen, 2001). The calibrator for each tissue was cDNA derived from a single incubating female ovary, and we used PPIA as an internal reference, because this gene is known to be a reliable reference gene in birds (Zinzow-Kramer et al., 2014), which we confirmed in the current study. While $2^{-\Delta\Delta Ct}$ is ideal for quantifying relative mRNA abundance within genes for non-model organisms, it does not allow for comparisons among genes.

2.5. Statistical analyses

Statistical analyses were performed using R (v. 3.4.3, R Core Team, 2017). We first examined seasonal differences in baseline T between territory establishment and incubation breeding stages using a linear model. To quantify seasonal changes in mRNA expression of all genes of interest in the gonad and liver we performed linear models for each gene in each tissue. In all models, relative mRNA abundance (i.e., fold change quantified as $2^{-\Delta\Delta Ct}$) was the dependent variable and breeding stage was the independent variable. We included the time of capture as an independent variable, because peripheral tissues can differ in circadian patterns of transcription to meet changing daily physiological demands (Storch et al., 2002). We were unable to use multivariate analyses due to sample size restrictions (i.e., because we had 5 individuals per group and more than 5 genes of interest, which creates robustness issues because n < the number of dependent variables; Tabachnick and Fidell, 2007). Therefore, we analysed each gene in a separate linear model, and we report standard and adjusted p-values using Benjamini-Hochberg corrections (Benjamini and Hochberg, 1995). Multiple comparison corrections have the advantage of minimizing type 1 error, but they do so at the expense of type 2 error (Rothman, 1990), and studies with small samples sizes, such as ours, are more prone to type 2 errors (Johnson, 1999). Further, from a biological standpoint, several lines of evidence suggest that genes along the steroidogenic pathway are regulated by different transcription factors (LaVoie and King, 2009; Payne and Hales, 2004; Peter and Dubuis, 2000), and they vary in their response to pharmacological manipulations with various hormones and secondary messengers (Mamluk et al., 1999; Payne and Youngblood, 1995; Stocco et al., 2005), suggesting that they should be treated independently. As a consequence, we report both p-values and adjusted p-values and interpret results cautiously based on the body of evidence. We report all results as mean ± one standard error (SEM), unless otherwise noted, and we tested for homogeneity of variances (Levene's test; all $p > 0.15$) and normality of linear model residuals (Shapiro-Wilks test).

In most cases residuals were normally distributed (Shapiro-Wilks: all $p > 0.14$), the exceptions were the analysis of SULT1B1 mRNA expression ($p = 0.01$) and plasma T concentration ($p = 0.001$). In both instances there was one significant outlier (Grubbs test: SULT1B1, $p < 0.001$; plasma T, $p < 0.001$). The T outlier was an incubating female that had most recently begun incubation and still had several yellow follicles, and T levels that were 3x higher than the next highest T level in the study. The SULT1B1 outlier was a female captured during territory establishment, whose SULT1B1 values were 2x higher than the next highest value. Therefore, we re-ran these models by performing a robust linear regression using the function lmRob from the R package robust (Wang et al., 2017). Robust regressions can detect outliers and give them less weight to control for their potentially inflated influence (Rousseeuw and Leroy, 1987; Wilcox, 2011).

3. Results

3.1. Seasonal changes in plasma testosterone

Females collected for gene expression analyses showed higher T levels during territory establishment (0.59 ng/ml ± 0.11) compared to incubation (0.15 ng/ml ± 0.04 without outlier; 0.68 ng/ml ± 0.53 with outlier). Robust regression, which allowed for inclusion of the outlier, showed that female T levels significantly declined from territory establishment to incubation ($t_{1,8} = 3.22$, $p = 0.01$; triangles in Fig. 2). Ordinary least squares regression did not support this result ($F_{1,8} = 0.03$, $p = 0.87$); however, residuals were not normally distributed unless the outlier was removed, and then the results were similar ($F_{1,7} = 11.42$, $p = 0.01$).

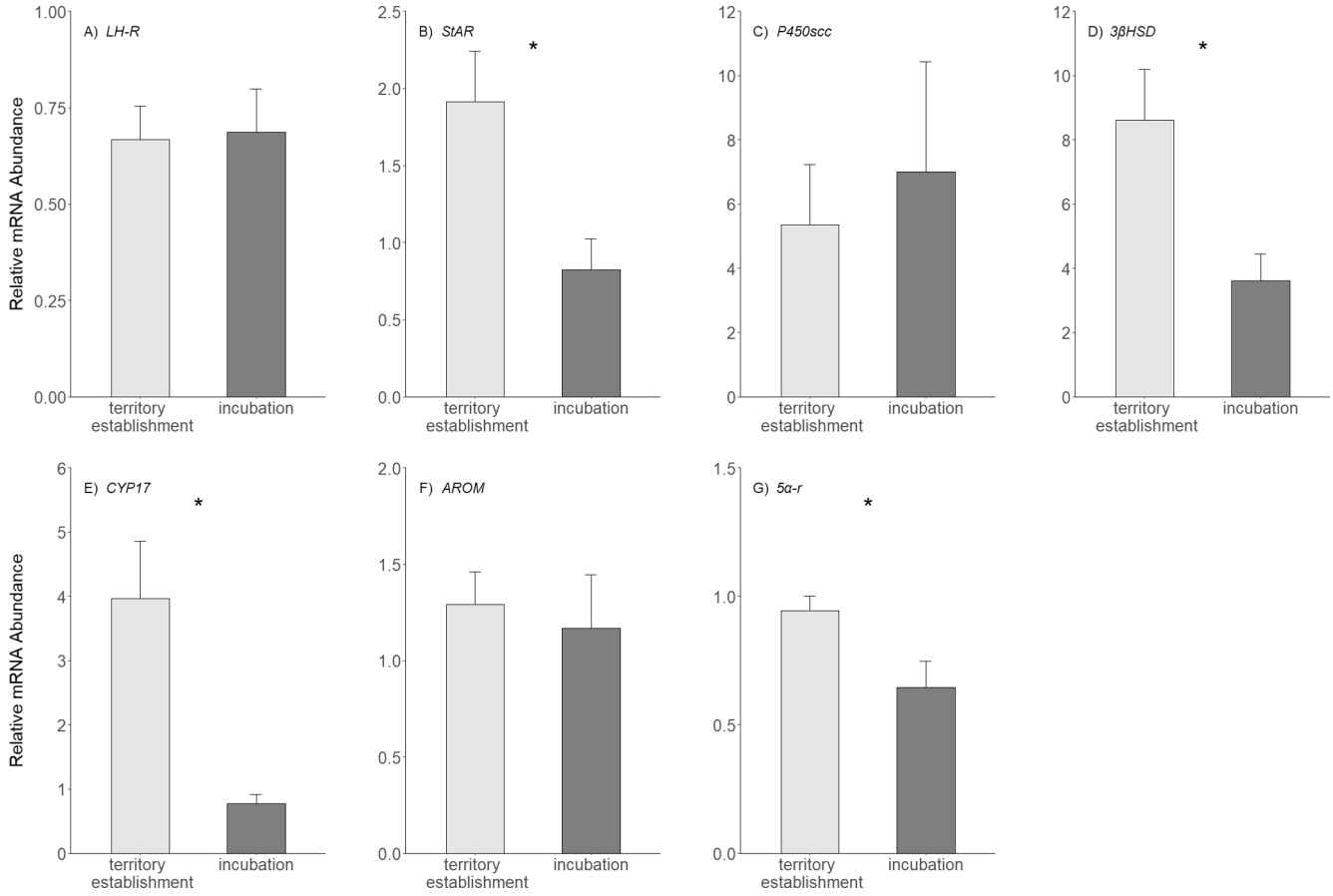

Fig. 3. Abundance of mRNA of steroidogenic genes in the gonad during territory establishment (light gray; n = 5) and incubation (dark gray; n = 5). Relative mRNA abundance is a unitless quantity ($2^{-\Delta\Delta Ct}$), which depicts mRNA abundance in each sample normalized to a reference gene (PPIA) and relative to a calibrator sample separately for each gene. Values differing by 1 unit differ by 1 fold change. Error bars represent SE and * denotes a significant difference between breeding stages ($p < 0.05$) when tests are not corrected for multiple comparisons.

3.2. Seasonal changes in gonad gene expression

In the gonad, StAR, 3βHSD, CYP17, and 5α-r had significantly lower expression during incubation compared to territory establishment, whereas LH-R, P450scc, and AROM did not significantly differ between breeding stages (Table 1; Fig. 3). Time of day did not relate to gene expression in the gonad (Table 1). After Benjamini-Hochberg corrections, only CYP17 was still significantly differently expressed ($p = 0.03$); StAR, 3βHSD, and 5α-r showed strong trends ($p = 0.07$; Table 1).

3.3. Seasonal changes in liver gene expression

Expression of steroid-metabolizing enzymes in the liver also changed over the breeding season: CYP2C19 was significantly lower during incubation, but SULT1B1 and UGT2B17 did not significantly differ between stages (Table 1; Fig. 4). When a robust regression was applied to the analysis of SULT1B1 to account for the outlier, the outcome did not change (stage: $t_{1,7} = 1.82$, $p = 0.11$; time: $t_{1,7} = -0.78$, $p = 0.46$), and SULT1B1 did not significantly differ in gene expression between stages. Time of capture was only related to CYP2C19, such that females caught later in the morning had lower gene expression; however, the effect size was low, almost two orders of magnitude lower than the effect of breeding stage (Table 1). After Benjamini-Hochberg corrections, the only liver result that remained significant was time of capture for CYP2C19 (Table 1).

4. Discussion

We found several significant seasonal changes in peripheral gene expression that may contribute to seasonal plasticity in T levels in females. These observational data are consistent with the hypothesis that seasonal regulation of T production occurs at the level of the gonad, in addition to upstream regulation that is known to occur in the brain (i.e. Calisi et al., 2016; Tsutsui et al., 2010). While our findings should be interpreted cautiously given small sample sizes, females that were establishing territories had lower gene expression of StAR, CYP17, and 3βHSD than incubating females, which may explain why ovaries becomes unresponsive to exogenous stimulation of the HPG axis during parental phases (George and Rosvall, 2018; Jawor et al., 2007), in contrast to males that typically retain the ability to produce T (Apfelbeck and Goymann, 2011; Jawor et al., 2006b). 5α-r, the enzyme that converts T to 5αDHT, was also highly expressed during territory establishment, but not during incubation, further suggesting that androgen production may be important during early breeding stages. Our study is also among the first to collectively consider how these mechanisms regulating gonadal steroidogenesis change alongside seasonal changes in the liver, a peripheral tissue that may actively influence the degree to which T can bring about a behavioral effect. In the liver, we found seasonal declines in mRNA abundance for one key metabolic enzyme (CYP2C19). This pattern suggests that hepatic metabolism of T is highest when T levels are also higher, although this result dissipated after correcting for multiple comparisons. Below, we discuss these findings in light of how peripheral regulation of steroid synthesis and

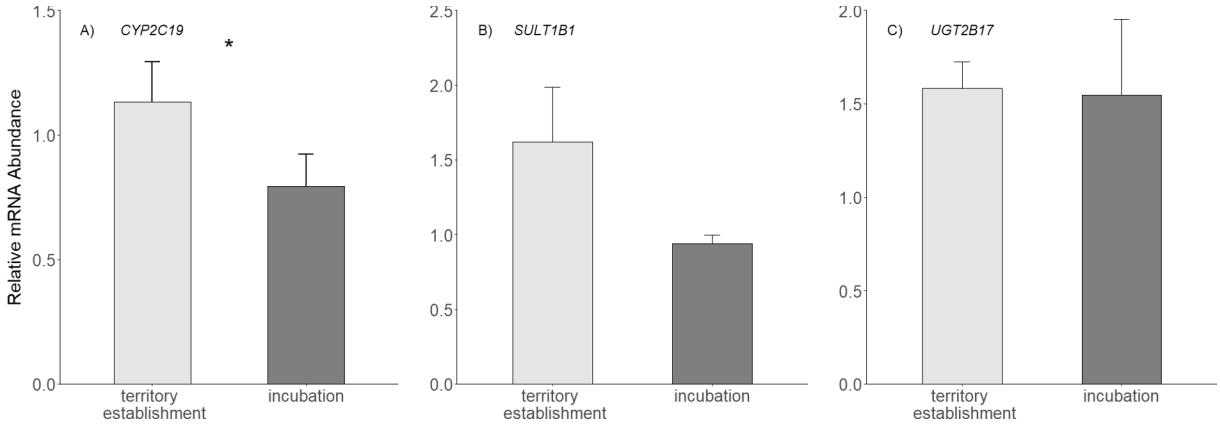

Fig. 4. Abundance of mRNA of steroid-metabolizing genes in the liver during territory establishment (light gray; n = 5) and incubation (dark gray; n = 5). Relative mRNA abundance is a unitless quantity ($2^{-\Delta\Delta Ct}$), which depicts mRNA abundance in each sample normalized to a reference gene (PPIA) and relative to a calibrator sample separately for each gene. Values differing by 1 unit differ by 1 fold change. Error bars represent SE and * denotes a significant difference between breeding stages ($p < 0.05$) when tests are not corrected for multiple comparisons.

metabolism may contribute to seasonal changes in female physiology and behavior.

4.1. Seasonal changes in gonadal steroidogenesis

During territory establishment, ovaries expressed more mRNA for StAR, CYP17, 3βHSD, and 5α-r, compared to incubating females. This difference in expression ranged from approx. 1.5× greater expression of 5α-r, up to 4× greater expression of CYP17 during territory establishment (Fig. 3). To the degree that mRNA predicts abundance of these enzymes, lower expression of these steroidogenic genes during incubation may contribute to the significantly lower T levels found in incubating females. More specifically, our data suggest that seasonal regulation of T production could be mediated, in part, by the decreased flux of cholesterol into the mitochondria via StAR, as well as decreased conversion of pregnenalone ("the mother of all steroids") into active steroids via 3βHSD and CYP17. While our sample size inhibited our ability to make robust, direct comparisons between gene expression and circulating T within breeding stages, both StAR and CYP17 expression have been linked with T secretion in studies conducted in males (e.g., Huffman et al., 2012; Luo et al., 2005; Rosvall et al., 2016; Zmuda et al., 2001), and all of these genes have been linked with sex steroid production in the biomedical literature (LaVoie and King, 2009; Payne and Youngblood, 1995). Additionally, theory suggests that these enzymes are likely to be regulators of flux through a pathway due to their position (Rausher, 2013; Wright and Rausher, 2010): StAR prepares the first step of steroidogenesis, and 3βHSD and CYP17 catalyze branching steps. While elevations in StAR and 3βHSD could also relate to increased production of progesterone, this is unlikely because progesterone secretion typically peaks just prior to ovulation when follicles are fully developed (Rehder et al., 1986; Sharp, 1980; Sockman and Schwabl, 1999), and the females we collected during territory establishment all had small, white undeveloped follicles. Furthermore, progesterone also generally decreases territorial (Goymann et al., 2008; Kohlert and Meisel, 2001) and reproductive (Leboucher et al., 2000) behaviors in females, which is inconsistent with observations that aggressive interactions are common during this time (George and Rosvall, 2018). Direct measures of progesterone and other hormones are necessary to link our gene expression data with the full suite of sex steroids produced by the ovary, but our findings are nevertheless an important step towards understanding seasonal hormone regulation in females.

One of the most exciting patterns of gene expression we observed was that 5α-r was highly expressed in ovaries during territory establishment, but less so during incubation. This observation, along with enhanced expression of other upstream enzymes, suggests that females may be actively producing the potent androgen 5αDHT during territory establishment. Notably, none of the females collected during territory establishment were yolking eggs, and so hormone production at this stage cannot simply relate to egg production. Instead, we argue that androgens may serve a behavioral function at this stage, similar to how male androgen levels exceed what is needed for reproduction because they facilitate territorial aggression early in the breeding season (Goymann et al., 2007; Wingfield et al., 1990). Further, 5αDHT, once considered an androgenic 'dead end', can be converted into estrogenic metabolites, indicating 5αDHT could influence behavior via either estrogen or androgen receptor activity (Handa et al., 2009; Morali et al., 1994). Direct measurements of these steroid hormones are needed to validate these ideas, but our findings nevertheless suggest a potential role for 5αDHT, or its metabolites, early in the breeding season before egg laying has begun.

These seasonal changes in gonadal gene expression collectively demonstrate that at least some seasonal regulation of T production may occur lower along the HPG axis (i.e., at the level of the gonad) in addition to other upstream regulation at the hypothalamus and pituitary. This idea has been indirectly supported for years by observations that exogenous GnRH does not increase T levels even when it elevates LH in circulation (Jawor et al., 2007). While males typically remain capable of elevating T to some degree throughout the breeding season (Apfelbeck and Goymann, 2011; Jawor et al., 2006b), females essentially eliminate their ability to elevate T in response to exogenous GnRH during parental phases (DeVries and Jawor, 2013; George and Rosvall, 2018; Jawor et al., 2007), despite still producing LH (Jawor et al., 2007). In support of the idea that females regulate T production lower on the HPG axis, we show that ovarian sensitivity to LH (i.e., LH-R mRNA abundance) does not change seasonally, despite a decrease in steroidogenesis in the ovary. While this finding does not negate the critical role of LH in regulating T production, prior work in other female songbirds suggests that variation in plasma LH levels or LH-R mRNA abundance does not account for continuous, quantitative variation in T production (Rosvall et al., 2013). Instead, like the LH surge required to start ovulation, there may be a threshold of LH release or LH sensitivity needed to produce T, such that a dose-dependent relationship between the two is not expected (Adkins-Regan, 2005).

This apparent ability to regulate T production at the end of the tropic hormone cascade could act as a biological 'fail-safe' to prevent elevated systemic T at a time when its pleiotropic effects would be too costly (i.e. during parental phases). Peripheral regulation lower along the HPG axis may also allow for more precision, as it would avoid triggering the suite of traits affected by upstream hormones. For

example, GnRH stimulates release of both LH and follicle-stimulating hormone, whereas enzymes later in the cascade yield changes in a more limited set of hormones. One alternative interpretation is that variation in T is the cause, not consequence, of these seasonal patterns in gene expression, based on autoregulatory processes (Melmed et al., 2011). Although we cannot address this issue in this study, other experimental work demonstrates that T treatment tends to decrease expression of steroidogenic genes (LaVoie and King, 2009; Payne and Youngblood, 1995), which is not consistent with the patterns we observed here. Thus, while top-down regulation is clearly important for HPG axis function (Calisi et al., 2016; Tsutsui et al., 2010), and there are certainly other peripheral organs that may influence the level of androgens in circulation (e.g., muscles, Fuxjager et al., 2013, 2017 adrenals, Soma, 2006; Soma and Wingfield, 2001), our data suggest that differential regulation of steroidogenic gene expression in the ovary may also contribute to seasonal changes in T levels in females.

4.2. Seasonal changes in hepatic steroid metabolism

Alongside these changes in gonadal steroid synthesis, we also observed seasonal changes in steroid metabolic processing in the liver. Most notably, we found that mRNA abundance of CYP2C19, which performs hydroxylation of T and other steroids prior to excretion (Choi et al., 2005; Yamazaki and Shimada, 1997), was roughly 1.5x higher during territory establishment than incubation. We had hypothesized that females would *increase* T metabolism later in the breeding season, as a means to increase clearance of T when it is most costly. Our results instead suggest that CYP2C19 activity may track, rather than drive, T levels, though caution is warranted due to small sample sizes. In support of this interpretation, however, exogenous T can increase hepatic expression of this gene (Löfgren et al., 2009), apparently due to greater availability of substrate to be metabolized. Thus, clearance of T (or other steroids) via hydroxylation by CYP2C19 may be related to seasonal changes in T, but hepatic steroid breakdown is not necessarily a mechanism used to disproportionately reduce circulating T levels later in the breeding season. Additionally, two alternative pathways of steroid metabolism did not show robust evidence of seasonal changes: UGT2B17, a catalyst of glucuronidation, and SULT1B1, a sulfotransferase, did not differ between early- and late- breeding stage females. Nevertheless, these findings do not exclude these metabolic processes from playing a role in seasonal T variation. Future work should include greater statistical power that may well highlight significant changes, as well as quantification of other isoforms or other genes involved in hepatic steroid metabolism. In particular, while the sulfotransferase we measured (SULT1B1) is one of the most abundant isoforms (Riches et al., 2009) and shows a sex-specific response to experimentally elevated T (Peterson et al., 2014), other isoforms have higher affinities for steroids (Mueller et al., 2015), but they are not well characterized in birds. Ultimately, more research is needed on the functional effects of T synthesis and metabolism in the periphery, in both males and females. Our data provide a first step toward testing the hypothesis that these peripheral mechanisms of T processing may operate in females, perhaps as an adaptive mechanism of seasonal hormonal plasticity.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2018.10.001.

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