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Role for androgens in determination of ovarian fate in the common snapping turtle, *Chelydra serpentina*

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ABSTRACT

Sex steroids are involved in sex determination in almost all vertebrates, including species with temperature-dependent sex determination (TSD). It is well established that aromatase and estrogens are involved in ovary determination in TSD species. In contrast, the role of non-aromatizable androgens in TSD is less clear. In this study, we used dihydrotestosterone (DHT) and an antagonist of the mammalian androgen receptor (flutamide) to examine the impact of androgens on sex determination in the snapping turtle. We incubated eggs at a male-producing temperature and treated embryos with drug delivery vehicle (5 L ethanol), DHT in vehicle, or flutamide in vehicle during the sex-determining period. We then measured expression of markers for ovarian and testicular development and genes involved in steroidogenesis. A subset of embryos and hatchlings were collected for histological analysis of gonad differentiation and sex determination. DHT and flutamide both induced ovarian development: 100% of vehicle-treated hatchlings had testes, while 60% of DHT-treated and 32% flutamide-treated hatchlings had ovaries. DHT and flutamide treatments also had feminizing effects on gene expression patterns and the structure of embryonic gonads. DHT treatment increased expression of *FoxL2*, androgen receptor, aromatase and several steroidogenic genes. Flutamide produced a similar, but weaker, pattern of gene expression. Genes involved in testis development (*Sox9* and *Amh*) were influenced by flutamide treatment. Our findings support the hypothesis that androgens and the androgen receptor are involved in ovary determination in the common snapping turtle.

1. Introduction

Many reptiles exhibit temperature-dependent sex determination (TSD), including numerous turtles, some lizards, and all crocodilians studied to date (Deeming, 2004; Ewert et al., 2004; Harlow, 2004; Lang and Andrews, 1994; Viets et al., 1993). Most of these species lack sex chromosomes. Instead, temperature of the embryo during the middle third of development determines sex. Evidence from all TSD reptiles studied to date indicates that sex steroid hormones are critical for sex determination. Administration of exogenous steroid hormones or hormone antagonists to developing embryos can interfere with the temperature signal and reverse the putative sex of embryos (Crews, 1994; Crews et al., 1996a; Lance, 2009; Matsumoto and Crews, 2012; Pieau and Dorizzi, 2004; Rhen and Schroeder, 2010). Sensitivity to hormone-induced sex reversal coincides with the temperature sensitive period for sex determination. Research implicates estrogens as crucial for ovarian development. Administration of 17ß-estradiol to embryos incubated at male-producing temperatures overrides the temperature signal and leads to ovarian development (Bull et al., 1988; Crews et al., 1991; Crews et al., 1996a; Díaz-Hernández et al., 2015; Díaz-Hernández et al., 2017; Dorizzi et al., 1991; Dorizzi et al., 1994; Freedberg et al., 2006; Gutzke and Bull, 1986; Kohno et al., 2015; Rhen and Lang, 1994; Wibbels et al., 1991; Wibbels et al., 1993). While estrogens are clearly involved in ovary formation, the impact of androgens in TSD reptiles is not as obvious because they can either act via the androgen receptor (AR) or be aromatized to estrogens and act via estrogen receptors (Rice et al., 2017).

Testosterone treatments produce a significant increase in the number of female hatchlings at temperatures that normally produce males or male-biased sex ratios (Crews and Bergeron, 1994; Crews et al., 1994; Rhen and Lang, 1994; Wibbels and Crews, 1992; Wibbels and Crews, 1995). Aromatase inhibitors block the feminizing effect of testosterone, presumably by preventing its conversion to 17ß-estradiol (Crews and Bergeron, 1994; Rhen and Lang, 1994; Wibbels and Crews, 1994). Given that aromatase expression and activity are very low at male temperatures (Smith et al., 1995; Rhen et al., 2007), it is not

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obvious how addition of substrate alone can induce ovary formation. One way to distinguish direct androgen effects on AR versus indirect effects via aromatization is to use compounds that act as "pure androgens". The natural testosterone metabolite, 5-dihydrotestosterone (DHT), is considered a "pure androgen" because it cannot be aromatized to estrogens (Murray et al., 1998; Swerdloff and Wang, 1998). In addition, DHT is a more potent AR activator than testosterone (Zhou et al., 1995).

Researchers have administered DHT to embryos to determine the effect of non-aromatizable androgens on TSD. In the red-eared slider turtle, *Trachemys scripta*, DHT had a masculinizing effect on embryos incubated at a temperature that normally produces a 1:1 sex ratio. However, DHT did not influence sex determination in red-eared sliders at a temperature that produces exclusively females (Wibbels and Crews, 1992; Wibbels and Crews, 1995). Similar results have been observed in the American alligator (*Alligator mississippiensis*; Lance and Bogart, 1994). In contrast, DHT had a feminizing effect on snapping turtle embryos, *Chelydra serpentina*, incubated at temperatures that produce a 1:1 sex ratio or a female-biased sex ratio (Rhen and Lang, 1994; Rhen and Schroeder, 2010). Furthermore, the anti-androgen flutamide had a feminizing effect on some snapping turtle clutches, but a masculinizing effect on others (Rhen and Schroeder, 2010). The conflicting effects of DHT and flutamide within and among TSD species indicates that androgen signaling should be studied more closely.

Interestingly, AR expression increases in gonads of snapping turtle embryos shifted from a male- to a female-producing temperature during the temperature sensitive period, suggesting a role for AR in ovary determination (Rhen et al., 2007). In fact, androgens act via AR to induce aromatase and Foxl2 expression in snapping turtle embryos incubated at a temperature that produces mixed sex ratios (Rhen and Schroeder, 2010). These findings may help explain the feminizing effect of testosterone at male-producing temperatures: testosterone may enhance its own conversion to estrogens by up-regulating aromatase expression. Although an androgen-dependent feed-forward mechanism might be involved in ovarian development, sensitivity to exogenous steroids can vary with incubation temperature (Crews, 1996). Thus, androgens may or may not have any effect on aromatase expression at male-producing temperatures where aromatase expression is very low.

The aim of this study was to determine the effects of DHT and flutamide on sex determination in snapping turtle embryos incubated at a male-producing temperature. In addition to gross morphological and histological analysis of gonadal phenotype, we assessed the effects of DHT and flutamide on key molecular markers of ovarian and testicular development. We measured expression of FoxL2, AR, and aromatase mRNA in embryonic gonads because these genes are expressed at significantly higher levels at female-producing than at male-producing temperatures in the snapping turtle (Rhen et al., 2007). Moreover, genes encoding FoxL2, AR and aromatase are critical for granulosa cell development and function in mammalian ovaries (Fisher et al., 1998; Loffler et al., 2003; Schmidt et al., 2004; Sen and Hammes, 2010). We hypothesized that DHT would induce FoxL2 and aromatase expression through a feed-forward loop involving the androgen receptor, leading to ovarian development at a male-producing temperature. Given that aromatase is a key steroidogenic enzyme, we also examined the effects of DHT and flutamide on expression of several other genes involved in steroid synthesis. Expression of these genes is essential for converting cholesterol into androgens that are substrates for aromatase (Payne and Hales, 2004). Finally we measured expression of Sox9 and Amh mRNA because these genes are normally expressed at a higher level in differentiating testes than ovaries in snapping turtle embryos (Rhen et al., 2007; Rhen et al., unpublished data). These genes are essential for Sertoli cell development and function in mammalian testes (Wilhelm et al., 2007). We hypothesized that DHT and flutamide would regulate expression of steroidogenic genes and repress genes involved in testis development.

2. Materials and methods

2.1. Egg Collection, Incubation, and hormone treatments

Animal experiments were carried out according to a protocol approved by the Institutional Animal Care and Use Committee at the University of North Dakota (Protocol #0905-1). Eggs were collected within 24 h of oviposition from eight snapping turtles nests throughout Minnesota in early June of 2010. Eggs from three snapping turtles nests were collected in early June of 2011 to replicate the experiment from 2010. Clutch sizes ranged from 29 to 76 eggs. We transported eggs to the animal quarters in the Biology Department at the University of North Dakota. We held eggs at ~20 °C for less than one week before clutches were assigned to experimental treatments. Eggs were washed in tepid water, candled, and infertile eggs removed. Eggs were placed in containers filled with moist vermiculite and then randomly positioned within foam box incubators and incubated at 26.5 °C, which produces exclusively males in this population (Rhen and Lang, 1994; Ewert et al., 2005). Prior to hormone treatments, a few eggs from each clutch and treatment group were randomly sampled to determine the developmental stage of the embryos (Yntema, 1968). Eggs were candled again for viability and eggs containing dead embryos were eliminated from the study.

Stage 17 is the middle of the temperature-sensitive period in this species and is the stage when embryos are most sensitive to temperature (Rhen et al., 2015). Hormone manipulations were performed at stage 17 of embryonic development to determine if DHT or flutamide had any influence on sex determination. Treatments included one vehicle-treated group (ethanol only) and two hormone-treated groups (either DHT or flutamide dissolved in ethanol). All solutions were topically applied to the vascularized upper surface of the eggshell as previously described (Crews et al., 1991). Eggs in the vehicle treatment group received a single dose of 5 µl of 95% ethanol, which does not have any detectable effect on sex determination in this species (Rhen and Lang, 1994). Eggs from the experimental groups received a single 50 µg dose of DHT (Cat no. A8380, Sigma-Aldrich, St. Louis, MO) dissolved in 5 µl 95% ethanol or a single 100 µg dose of flutamide (Cat no. F9397, Sigma-Aldrich) dissolved in 5 µl 95% ethanol. These doses were selected based on their effectiveness in previous studies conducted at temperatures that produce mixed sex ratios (Rhen and Lang, 1994; Rhen and Schroeder, 2010). After receiving treatments, all eggs were returned to incubators at a constant male-producing temperature of 26.5 °C.

2.2. Tissue collection and histology

In 2010, two eggs from each clutch and treatment group were sampled during embryonic stages 20–26. Eggs were opened and embryos quickly euthanized via decapitation. The adrenal-kidney-gonad (AKG) complex was removed, placed in RNA*later* (Ambion, Austin, TX) and stored at −20 °C. The gonads were micro-dissected from the mesonephros, taking care to remove any kidney tissue from the gonad. Only micro-dissected gonads were used for RNA isolation and subsequent gene expression experiments.

Eggs were sampled from 5 of 8 clutches in 2010 and 3 clutches in 2011 for histological examination of developing gonads. Eggs from three clutches in 2010 were not sampled for histology because there were not enough eggs to split between both the gene expression and histology studies. AKGs were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4 °C. Tissues were washed in PBS, dehydrated in ethanol, cleared in xylene, and embedded in paraffin according to standard protocols. AKGs were sectioned at 6 µm and mounted on HistoBond slides (VWR, Radnor, PA). Slides were deparaffinized in xylene, rehydrated in graded ethanol, and washed in PBS. Slides were stained with hematoxylin and eosin by standard protocols and images were taken using an Olympus BX-51 microscope equipped

with an Infinity 2 digital camera (Lumenera Corp., Ottawa, ON) using Rincon HD Software (Imaging Planet, Goleta, CA) to observe the morphology of developing gonads in the various treatment groups. We considered development of the cortex and follicles as an indication of ovary development. The observation of sex cords within the medulla was considered an indication of testis development. Prior work has shown that these morphological differences between developing ovaries and testes are visible by stage 20–21 (Rhen et al., 2015). We did not include the presence or absence of the Müllerian ducts as an indication of male or female development because treatments with DHT or flutamide can lead to retention of the Müllerian ducts in snapping turtles that develop testes (Schroeder and Rhen, unpublished data).

2.3. RNA isolation, DNAse treatment, and cDNA synthesis

Total RNA was extracted from each pair of gonads using RNAzol RT (Molecular Research Center, Cincinnati, OH). The RNAzol RT protocol was modified for the small amount of tissue. We used one-quarter the amount of liquid recommended by the manufacturer for tissue homogenization, RNA isolation, and recovery. We added 1 µl of precipitation carrier (Molecular Research Center, Cincinnati, OH) to the homogenate to assist with isolation of RNA because the expected yield was < 1 µg of total RNA. We added 15 µl of RNase-free water to dissolve the RNA pellet. Dissolved RNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Genomic DNA was removed by DNAse treatment after RNA extraction to ensure purity of the RNA.

Total RNA (150 ng) from each pair of gonads was reverse transcribed in a 20 µl reaction using the iScript cDNA Synthesis Kit, which contains a blend of oligo dT and random hexamers (BioRad, Hercules, CA). We diluted the cDNA to the equivalent of 0.5 ng input RNA/µl to include in real-time PCR reactions.

2.4. Primer selection and quantitative PCR

Primers for quantitative PCR for steroidogenic genes and Amh were developed from RNA-Seq data obtained from bipotential gonads for snapping turtles incubated at male-producing or female-producing temperatures (Rhen et al., unpublished data). The primers for each gene were designed using Primer Express 2.0 (Life Technologies, Grand Island, NY) with the following parameters: length 18–25 base pairs (bp), guanine-cytosine content near 50%, melt temperature ranging from 55 to 60 °C, and a short amplicon size (50–150 bp). Sequences and primers for Foxl2, aromatase, AR, and Sox9 have been previously reported (Rhen et al., 2007). All other primer sequences are listed in Table S1. All primers were purchased from Integrated DNA Technologies (Coralville, IA).

Quantitative PCR was used to measure mRNA expression of select genes in gonads of control and hormone-treated embryos. We evaluated expression of genes involved in ovarian development [aromatase (Cyp19a1), forkhead box L2 (Foxl2), and androgen receptor (AR)], testicular development [anti-Müllerian hormone (Amh) and SRY-box 9 (Sox9)], and several key steroidogenic genes [steroidogenic acute regulatory protein (StAR), cholesterol side chain cleavage enzyme (Cyp11a1), 3ß-hydroxysteroid dehydrogenase (3ß-Hsd), cytochrome P450 family 17 subfamily A member 1 (Cyp17a1), hydroxysteroid 17-beta dehydrogenase (17ß-Hsd), and steroid-5-α reductase (Srd5a1)]. We also measured 18S rRNA as a control for variation in efficiency of RNA extraction and cDNA synthesis (i.e., a "reference gene"). In brief, each PCR contained 5 µl of 2× SsoFast EvaGreen supermix (BioRad, Hercules, CA), 200 nM of forward and reverse primer, 2 µl of cDNA (equivalent of 1 ng total RNA input), and water to bring the total reaction to 10 µl. Reactions were run on the CFX 384 Real-Time PCR Detection System (BioRad, Hercules, CA). The thermal profile was 95 °C for 30 sec to activate the DNA polymerase followed by 40 cycles of two-step PCR (95 °C for 5 sec and 61 °C for 10 sec).

Rigorous standard curves across eight orders of magnitude were used to quantify gene expression in absolute terms as described in Rhen et al. (2007). Efficiencies of each real-time PCR reaction were estimated from the slope of our standard curves and ranged from 90 to 110 % with all regression lines having an $R^2 > 0.990$. Controls lacking reverse transcriptase or RNA template were prepared to test for contamination from genomic DNA and exogenous DNA (i.e., PCR products). A melting temperature analysis was added at the end of each real-time PCR reaction to verify that a single product was amplified for each gene.

2.5. Statistical analysis

We used JMP 13.1.0 software for all statistical analyses (SAS Institute, Cary, NC). We analyzed hatchling sex ratios using a nominal logistic model. Hormone treatment, year, and clutch nested within year were independent variables and the number of males and number of females were the dependent variable for analysis of sex determination. We did not test for a clutch by hormone treatment interaction because there were only 2–5 hatchlings per clutch in each treatment group.

We analyzed gene expression in embryonic gonads using hormone treatment and developmental stage as main effects in a 2-way ANOVA with a randomized complete block design. Clutch identity was a blocking factor because there were only 1.1 embryos (on average) from each clutch in each treatment/stage combination. All gene expression values were log10 transformed to meet the assumptions of ANOVA. Residuals of each ANOVA were assessed for outliers using Cook's distance and outliers were removed if necessary. We used Ct values for 18S rRNA as a covariate to control for potential variation in the quality of input RNA as well as variation in the efficiency of the reverse transcription reaction. When treatment effects were significant ($p < 0.05$), we used the Dunnett's test to compare DHT and flutamide treatment groups to the vehicle control group (i.e., a correction for multiple comparisons). When developmental stage was significant, we used Tukey's HSD for post hoc comparisons among stages. Sample sizes for experimental groups are shown in each figure. We checked assumptions for each ANOVA by examining residuals.

3. Results

3.1. DHT and flutamide induce ovarian development

Hormone treatment ($LR^2 = 40.1$, df = 2, $p < 0.0001$) influenced sex ratio of hatchling turtles. All ethanol-treated turtles developed testes (29 males: 0 females), as expected for turtles incubated at 26.5 °C. DHT treatment induced ovarian development in 59.6% (12 males: 17 females) of turtles incubated at 26.5 °C. Flutamide induced ovarian development in 32.1% (19 males: 9 females) of turtles incubated at 26.5 °C. Clutch also affected hatchling sex ratios ($LR^2 = 31.7$, df = 6, $p < 0.0001$). Sample sizes were too small for a rigorous test of the clutch by hormone interaction, but there appeared to be differences in responsiveness among clutches (Table 1). Hatchling sex ratios did not differ between years ($LR^2 = 5 \times 10^{-8}$, df = 1, p = 0.99).

The effects of DHT and flutamide on embryonic gonads paralleled their effects on hatchling sex ratio. All embryos treated with ethanol vehicle had testes with well-developed seminiferous tubules containing germ cells (Fig. 1A). Some embryos treated with DHT and flutamide developed testes with seminiferous tubules that were very similar in appearance to ethanol controls (Fig. 1C and 1E, respectively). An ovary from a control female incubated at 31 °C is shown for comparison; note large meiotic oocytes in the cortical layer (Fig. 1B). DHT and flutamide treatments induced ovarian development in some embryos (Fig. 1D and 1F, respectively), as evidenced by a thickened cortex containing numerous follicles with meiotic oocytes. In contrast to testes from ethanol, DHT, and flutamide treated embryos (Fig. 1A, C, and E, respectively), ovaries from DHT and flutamide treated embryos did not have organized tubules within the medullary region (Fig. 1D and F, respectively).

Table 1
Sex ratios for hatchling snapping turtles that were treated with ethanol, DHT or flutamide during the sex-determining period. Sex ratios are shown as percent male for each clutch. The number in parentheses is the total number of hatchlings from that treatment and clutch.

Clutch (Year Sampled)	Vehicle	DHT	Flutamide
2 (2010)	100.0 (3)	0.0 (3)	33.3 (3)
6 (2010)	100.0 (3)	33.3 (3)	66.7 (3)
7 (2010)	100.0 (3)	100.0 (3)	50.0 (2)
9 (2010)	100.0 (3)	100.0 (3)	100.0 (3)
21 (2011)	100.0 (3)	100.0 (3)	100.0 (3)
23 (2011)	100. 0 (4)	50.0 (4)	100.0 (4)
26 (2011)	100.0 (5)	0.0 (5)	40.0 (5)
30 (2010)	100.0 (5)	0.0 (5)	60.0 (5)

3.2. Expression of genes involved in ovarian development

We measured expression of Foxl2, aromatase, and AR in differentiating gonads from vehicle-treated embryos and embryos treated with DHT or flutamide. Expression of Foxl2 mRNA was affected by hormone treatment ($F_{2,167} = 13.4$, $p < 0.0001$), but not by developmental stage ($F_{6,167} = 1.1$, $p = 0.34$) or the treatment by stage interaction ($F_{12,167} = 0.65$, $p = 0.80$). Expression of Foxl2 mRNA was significantly higher in gonads from DHT-treated embryos compared to vehicle-treated embryos ($p < 0.0001$), but there was no difference between flutamide-treated and vehicle-treated embryos ($p = 0.67$) (Fig. 2A). There was significant variation in FoxL2 expression among clutches of eggs produced by different females ($F_{8,167} = 5.0$, $p < 0.0001$). 18S rRNA was a significant covariate ($F_{1,167} = 12.3$, $p = 0.0006$).

Hormone treatment ($F_{2,167} = 11.4$, $p < 0.0001$) and developmental stage ($F_{6,167} = 3.0$, $p = 0.008$) influenced expression of aromatase mRNA, but the treatment by stage interaction did not ($F_{12,167} = 0.78$, $p = 0.67$). Aromatase mRNA expression was significantly higher in gonads from DHT-treated embryos compared to vehicle-treated embryos ($p < 0.0001$) (Fig. 2B). Expression of aromatase mRNA did not differ significantly between flutamide-treated and vehicle-treated embryos ($p = 0.10$) (Fig. 2B). Tukey's HSD test revealed that aromatase expression was significantly higher in stage 26 embryos than in stage 20 embryos, but there were no differences among any other stages. Clutch identity affected aromatase expression ($F_{8,167} = 5.0$, $p < 0.0001$) and 18S rRNA was a significant covariate ($F_{1,167} = 14.0$, $p = 0.0003$).

Expression of AR mRNA was affected by hormone treatment ($F_{2,167} = 8.0$, $p = 0.0005$), but not by developmental stage ($F_{6,167} = 1.1$, $p = 0.39$) or the treatment by stage interaction ($F_{12,167} = 1.26$, $p = 0.25$). Expression of AR mRNA was significantly higher in gonads from DHT-treated embryos compared to vehicle-treated embryos ($p = 0.0002$), but the difference between flutamide-treated and vehicle-treated embryos was not statistically significant ($p = 0.10$) (Fig. 2C). There was significant variation in AR expression among clutches ($F_{8,167} = 3.0$, $p = 0.004$). 18S rRNA was a significant covariate ($F_{1,167} = 54.9$, $p < 0.0001$).

3.3. Expression of genes involved in steroidogenesis

Expression of StAR mRNA was influenced by hormone treatment ($F_{2,167} = 21.4$, $p < 0.0001$) and developmental stage ($F_{6,167} = 3.4$, $p = 0.003$). DHT treatment increased StAR expression ($p < 0.0001$), but flutamide did not ($p = 0.30$) (Fig. 3A). Tukey's HSD test revealed that StAR mRNA expression was significantly higher in stage 22 and 23 embryos than in stage 24 embryos, but there were no differences among any other developmental stages. The interaction between hormone treatment and stage did not affect StAR mRNA expression ($F_{12,167} = 1.1$, $p = 0.40$). However, clutch identity affected StAR expression ($F_{8,167} = 7.3$, $p < 0.0001$) and 18S rRNA was a significant covariate ($F_{1,167} = 10.1$, $p = 0.002$).

Hormone treatment affected Cyp11a1 mRNA expression ($F_{2,167} = 8.1$, $p = 0.0004$). DHT increased Cyp11a1 expression in comparison to vehicle controls ($p = 0.0002$), while flutamide had no detectable effect on Cyp11a1 mRNA expression ($p = 0.09$) (Fig. 3B). Developmental stage ($F_{6,167} = 1.4$, $p = 0.21$) and the interaction between treatment and stage ($F_{12,167} = 0.8$, $p = 0.65$) had no impact on Cyp11a1 expression. The blocking factor and the covariate had significant effects on Cyp11a1 expression: clutch identity ($F_{8,167} = 4.7$, $p < 0.0001$), levels of 18S rRNA ($F_{1,167} = 32.6$, $p < 0.0001$).

Expression of 3ß-Hsd mRNA was significantly influenced by hormone treatment ($F_{2,167} = 3.8$, $p = 0.02$), but not by developmental stage ($F_{6,167} = 2.0$, $p = 0.07$) or the interaction between treatment and stage ($F_{12,167} = 0.69$, $p = 0.76$). Levels of 3ß-Hsd were significantly higher in gonads from embryos treated with DHT than in vehicle controls ($p = 0.02$) (Fig. 3C). Flutamide had no detectable effect on expression of 3ß-Hsd mRNA ($p = 0.07$) (Fig. 3C). There was significant variation in 3ß-Hsd expression among clutches ($F_{8,167} = 7.0$, $p < 0.0001$). 18S rRNA as a significant covariate ($F_{1,167} = 54.5$, $p < 0.0001$).

Hormone treatment ($F_{2,167} = 1.2$, $p = 0.3$) and the interaction between hormone treatment and developmental stage ($F_{12,167} = 0.63$, $p = 0.81$) had no impact on Cyp17a1 mRNA expression. However, there were significant developmental changes in Cyp17a1 expression ($F_{6,167} = 6.7$, $p < 0.0001$). Tukey's HSD test revealed that expression of Cyp17a1 increased gradually with developmental stage. The blocking factor and the covariate had significant effects on Cyp17a1 expression: clutch identity ($F_{8,167} = 5.9$, $p < 0.0001$), 18S rRNA ($F_{1,167} = 9.9$, $p = 0.002$).

Expression of 17ß-Hsd mRNA was not affected by hormone treatment ($F_{2,167} = 1.3$, $p = 0.28$), developmental stage ($F_{6,167} = 1.1$, $p = 0.36$), or the interaction between hormone treatment and stage ($F_{12,167} = 1.4$, $p = 0.18$). Clutch influenced 17ß-Hsd expression ($F_{8,167} = 3.1$, $p = 0.003$) and 18S rRNA was a significant covariate ($F_{1,167} = 45.9$, $p < 0.0001$).

Expression of Srd5a1 mRNA was influenced by hormone treatment ($F_{2,167} = 3.6$, $p = 0.03$), but not by developmental stage ($F_{6,167} = 1.75$, $p = 0.11$) or the interaction between hormone treatment and stage ($F_{12,167} = 0.93$, $p = 0.52$). Expression of Srd5a1 was higher in gonads from embryos treated with DHT than in vehicle controls ($p = 0.02$) (Fig. 3D). Flutamide had no impact on Srd5a1 expression ($p = 0.55$) (Fig. 3D). Clutch influenced Srd5a1 expression ($F_{8,167} = 3.0$, $p = 0.004$) and levels of 18S rRNA were a significant covariate ($F_{1,167} = 49.9$, $p < 0.0001$).

3.4. Expression of genes involved in testicular development

We also measured expression of Sox9 and Amh in gonads from vehicle-treated embryos and embryos treated with DHT or flutamide. Hormone treatment ($F_{2,167} = 4.9$, $p = 0.009$) influenced expression of Sox9 mRNA, but developmental stage ($F_{6,167} = 1.0$, $p = 0.42$) and the treatment by stage interaction did not ($F_{12,167} = 1.4$, $p = 0.19$). Expression of Sox9 mRNA was higher in flutamide-treated embryos than vehicle control embryos ($p = 0.01$), but there was no difference between DHT-treated and vehicle control embryos ($p = 0.95$) (Fig. 4A). Clutch identity affected Sox9 expression ($F_{8,167} = 9.8$, $p < 0.0001$). 18S rRNA was a significant covariate in the model ($F_{1,167} = 20.7$, $p < 0.0001$).

Expression of Amh mRNA in developing gonads was affected by hormone treatment ($F_{2,167} = 8.5$, $p = 0.0003$). Flutamide increased Amh expression ($p = 0.04$), but DHT had no detectable effect when compared to vehicle controls ($p = 0.16$) (Fig. 4B). Developmental stage ($F_{6,167} = 1.5$, $p = 0.18$) and the treatment by stage interaction ($F_{12,167} = 1.0$, $p = 0.44$) did not affect Amh expression. There was significant variation in Amh mRNA expression among clutches

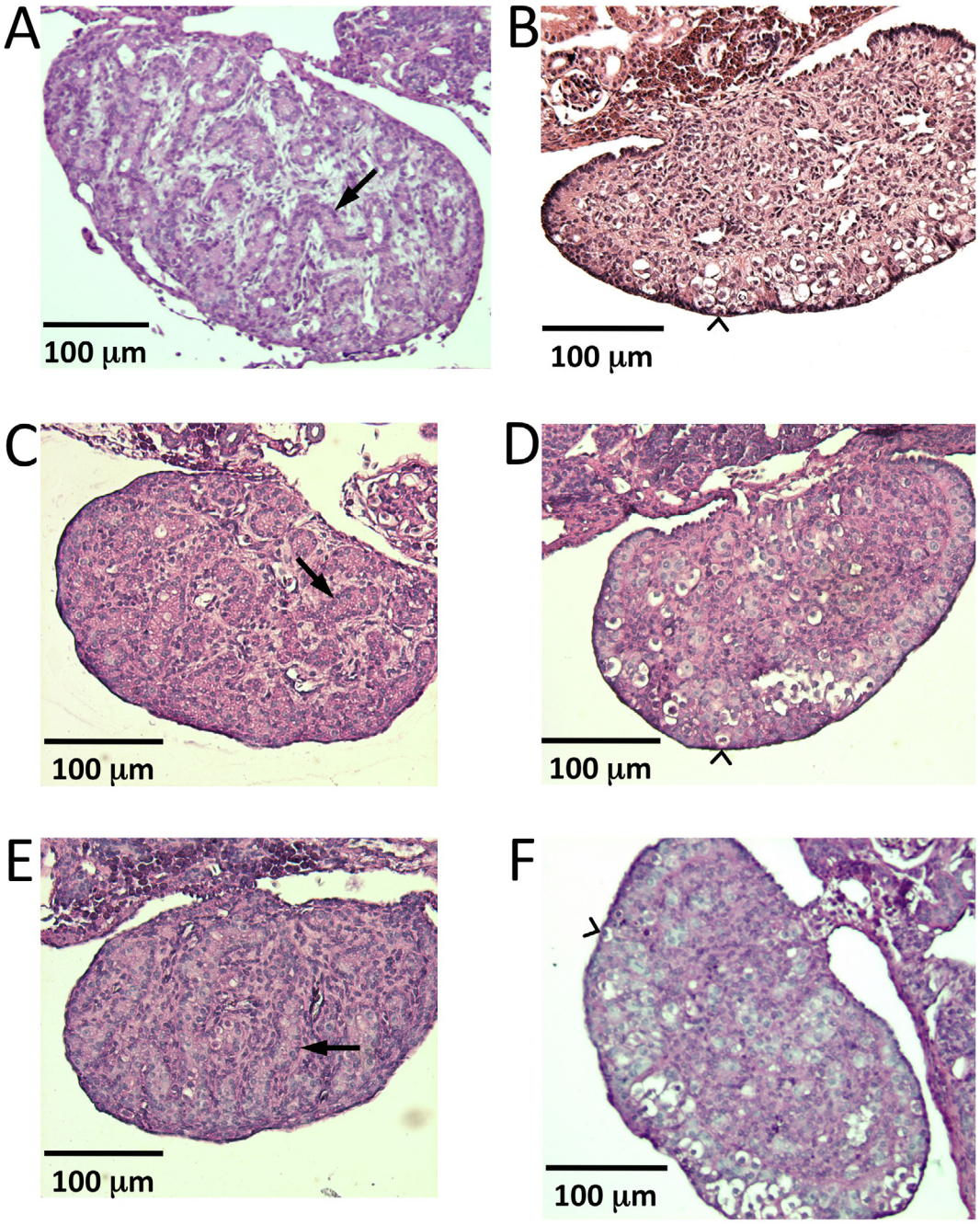

Fig. 1. Representative photomicrographs of gonads from snapping turtle embryos at stage 24. Gonads in panels A, C, D, E, and F were from embryos incubated at 26.5 °C, which normally produces 100% males. The gonad in panel B was from an embryo incubated at 31 °C, which normally produces 100% females. Differentiating testes from embryos treated with A) ethanol control, C) DHT, or E) flutamide at stage 17 of development. Arrows indicate seminiferous tubules in testes. Differentiating ovaries from A) an untreated control female at 31 °C and 26.5 °C embryos treated with D) DHT, or F) flutamide at stage 17 of development. Chevrons indicate meiotic oocytes in ovaries.

($F_{8,167} = 3.6$, $p = 0.0008$). 18S rRNA was a significant covariate ($F_{1,167} = 19.6$, $p < 0.0001$).

4. Discussion

The involvement of sex steroids in sex determination has been extensively studied in fish, amphibians, and reptiles (Devlin and Nagahama 2002; Nakamura 2010; Ramsey and Crews, 2009). Sex steroids are implicated in sex determination because of their ability to cause sex reversal. In general, estrogen treatments induce ovarian development in individuals that would otherwise develop testes (e.g., in genetic males or embryos incubated at male-producing temperatures in TSD species) (Devlin and Nagahama 2002; Nakamura, 2010; Ramsey and Crews, 2009). In contrast, non-aromatizable androgens typically induce testicular development in individuals that would normally develop ovaries (e.g., in genetic females or embryos incubated at female-producing temperatures in TSD species). However, these patterns are not universal. DHT, for instance, induces ovarian development in snapping turtles incubated at temperatures that produce mixed sex ratios (Rhen and Lang, 1994; Rhen and Schroeder, 2010).

Here we explore the mechanism underlying this paradoxical effect by testing whether DHT or flutamide alter gene expression in embryonic gonads and sex-reverse snapping turtles at a temperature that produces 100% male hatchlings. We found DHT treatment induced

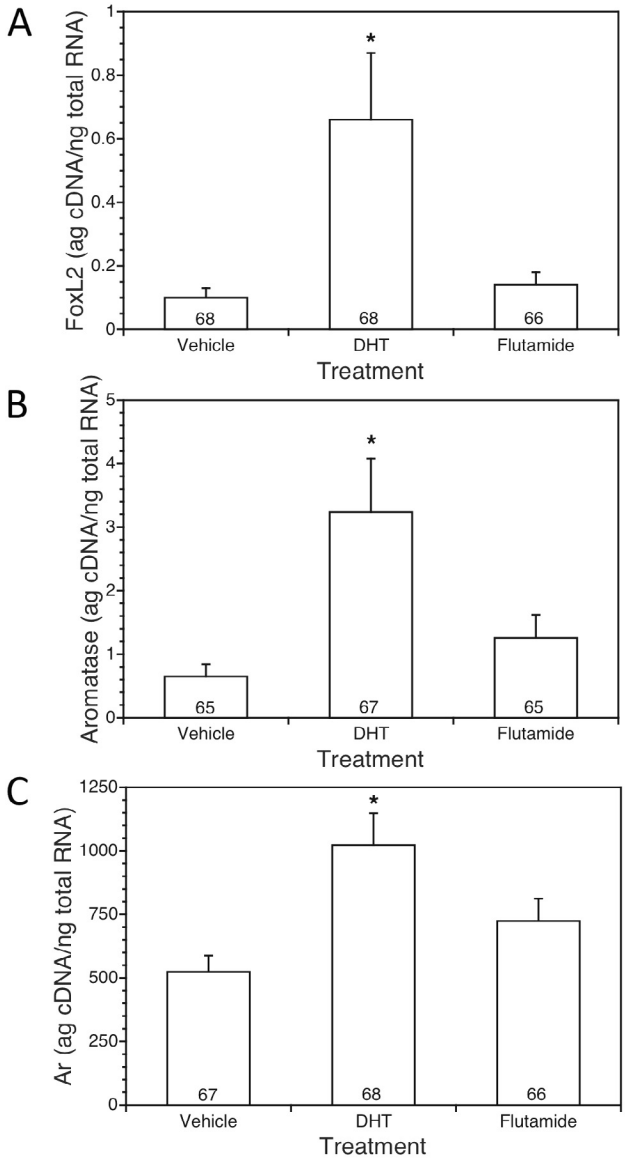

Fig. 2. Expression of A) *FoxL2,* B) aromatase, and C) *Ar* mRNA in gonads from snapping turtle embryos treated with vehicle, DHT, or flutamide. Expression values are means + 1 SEM. Sample sizes for each group are presented in each column. Asterisks indicate a significant difference ($p < 0.05$) between the treatment group and the vehicle control.

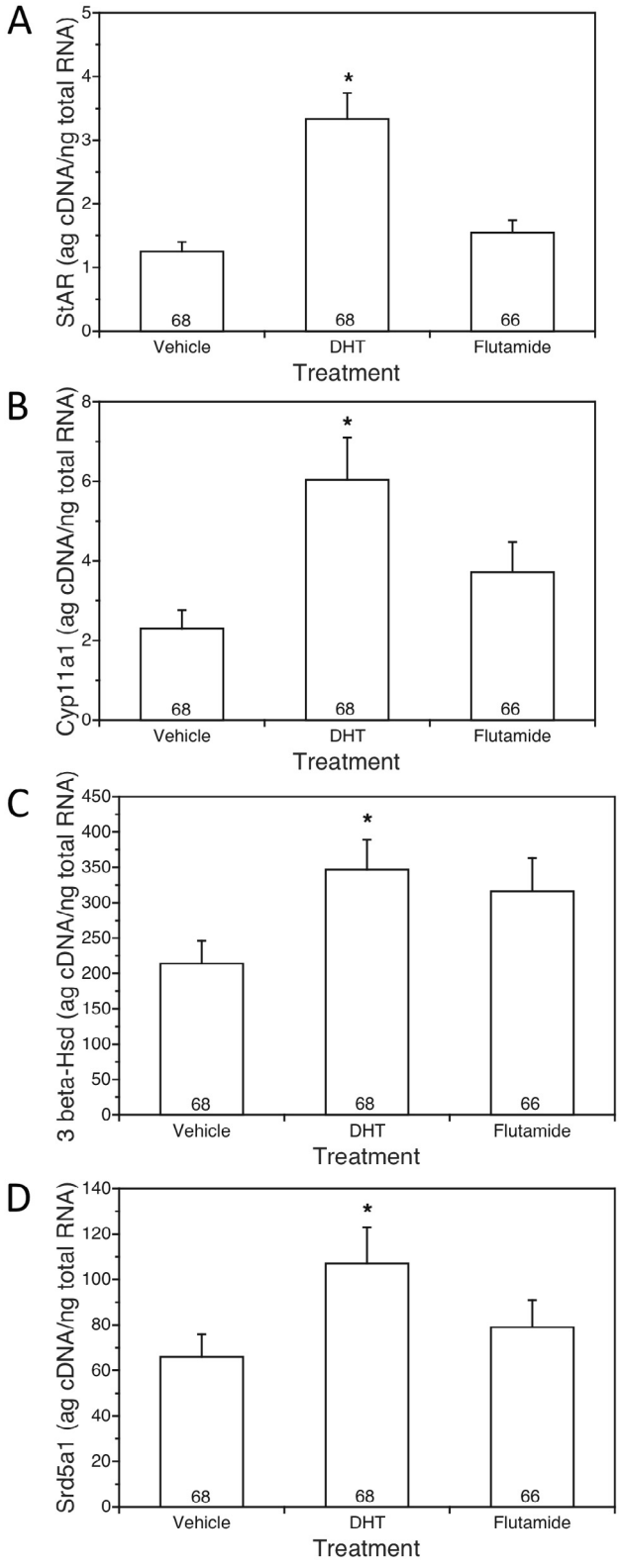

Fig. 3. Expression of A) *StAR*, B) *Cyp11a1*, C) *3ß-Hsd*, and D) *Srd5a1* mRNA in gonads from snapping turtle embryos treated with vehicle, DHT, or flutamide. Expression values are means + 1 SEM. Sample sizes for each group are presented in each column. Asterisks indicate a significant difference ($p < 0.05$) between the treatment group and vehicle control.

ovarian development in ~60% of hatchlings, while flutamide induced ovarian development in ~32% of hatchlings. Gonads from DHT and flutamide treated embryos also exhibit clear evidence of feminization in that an ovarian cortex developed and contained follicles with meiotic oocytes. This is the first study to demonstrate that DHT or flutamide can override the effects of a 100% male-producing temperature in snapping turtle embryos. These results are consistent with prior studies at temperatures that produce mixed sex ratios (Rhen and Lang, 1994; Rhen and Schroeder, 2010).

Feminizing effects were also observed at the molecular level. DHT increased expression of key ovarian markers, Foxl2 and aromatase, as well as AR in embryonic gonads at a male-producing temperature. Although AR and aromatase expression in flutamide-treated embryos was between vehicle-treated and DHT-treated embryos, it did not differ significantly from the vehicle group after correction for multiple comparisons. Weak flutamide induction of aromatase mRNA in the current study is similar to that reported in a previous study (Rhen and Schroeder, 2010). Whilst flutamide is generally considered an AR antagonist, it has partial agonist activity in some systems (Nyugen et al., 2007; Haendler and Cleve, 2012). Steroid receptor modulators often have mixed agonist/antagonist properties on different genes and/

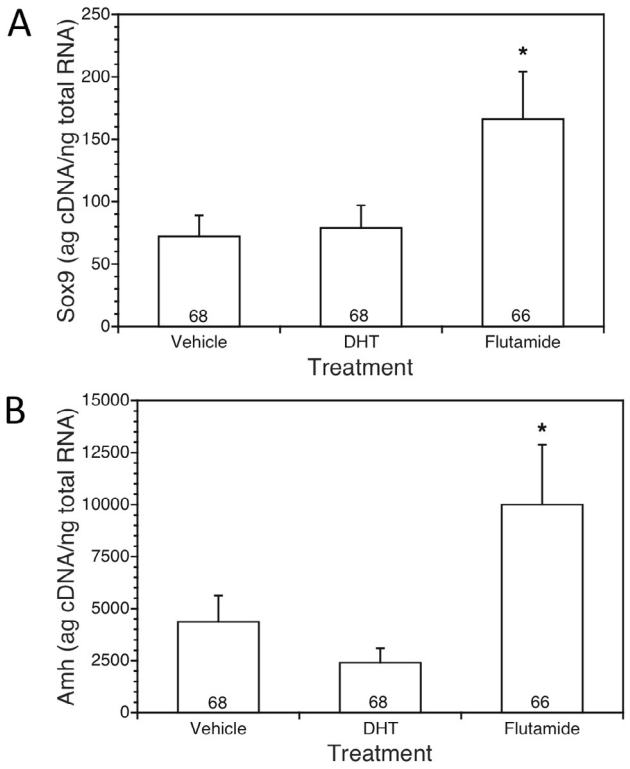

Fig. 4. Expression of A) *Sox9* and B) *Amh* mRNA in gonads from snapping turtle embryos treated with vehicle, DHT, or flutamide. Expression values are means + 1 SEM. Sample sizes for each group are presented in each column. Asterisks indicate a significant difference ($p < 0.05$) between the treatment group and vehicle control.

or tissues (Nilsson and Gustafsson, 2002; Solomon et al., 2019), which could explain the conflicting effects of flutamide on gene expression in the current study. Indeed, previous work shows that flutamide has different effects on DHT induction of different genes in the snapping turtle: flutamide did not block DHT induced aromatase expression, but completely blocked DHT induced FoxL2 expression (Rhen and Schroeder, 2010). In other words, flutamide acted as a partial AR agonist for aromatase, but as a complete AR antagonist for FoxL2.

Future studies examining the role of androgens and AR in ovary determination should test whether the mixed agonist/antagonist effects of flutamide are dose-dependent or are an inherent property of this compound. If flutamide displays mixed effects regardless of dose, it would be critical to find another compound that acts as a pure AR antagonist in the snapping turtle. Several other nonsteroidal anti-androgens, including bicalutamide, nilutamide, enzalutamide, and ARN-509, are highly effective in mammals (Solomon et al., 2019) and are good candidates for study in the snapping turtle. Identification of a pure antagonist for the snapping turtle AR would enable loss-of-function studies at female-producing temperatures to complement the gain-of-function studies with DHT described here.

In contrast to flutamide, DHT effects on gene expression patterns are consistently feminizing (with the sole exception of Sox9, which was not affected by DHT). In a cell culture model, snapping turtle FoxL2 potentiates the effect of DHT on aromatase expression (Guo and Rhen, 2017). Together, these observations support the hypothesis that androgens and AR are part of a feed-forward loop that includes Foxl2 and aromatase (i.e., androgens induce FoxL2; both androgens and FoxL2 induce aromatase) (Fig. 5). In turn, aromatase converts androgens into estrogens, which are key regulators of ovary determination in TSD reptiles (Dorizzi et al., 1994; Lance, 1997; Lance, 2009; Pieau et al., 1995; Pieau and Dorizzi, 2004; Rhen and Lang, 1994). Based on the finding that DHT also increased AR expression in the current study, we suggest that androgens and AR may form a positive feedback loop that reinforces the feed-forward loop described above (Fig. 5).

This type of feed forward regulatory motif confers both reversibility and stability to transitions in cell fate (Doncic and Skotheim, 2013). An analogous situation occurs in TSD where there is a period of reversibility when changes in temperature alter the probability of cells/gonads taking on alternative fates, but eventual loss of reversibility when cell/gonad fate is determined. We propose a model in which an overall shift in expression of steroidogenic genes causes the loss of reversibility (i.e., establishment of cell/gonad fate), but is not involved in initial sensing of the environmental signal. Indeed, the dynamics of steroidogenic gene expression in temperature shift studies indicate that this class of genes does not directly sense temperature. Expression of steroidogenic genes (StAR, Cyp17a1, aromatase) does not increase until the third or fourth day of exposure to a female-producing temperature (Rhen et al, 2007; Rhen et al., unpublished results), which coincides with irreversible determination of ovarian fate (Rhen et al., 2015).

We therefore measured expression of other genes that encode steroidogenic enzymes to test whether androgens might have a widespread effect on steroidogenesis in embryonic gonads at a male-producing temperature. Treatment with DHT increased expression of all genes studied except Cyp17a1 and 17ß-Hsd. StAR mediates the rate-limiting step in steroidogenesis by delivering cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane where Cyp11a1 resides. Cyp11a1 produces pregnenolone, which is then metabolized by 3ß-Hsd to produce progesterone. Although Cyp17a1 and 17ß-Hsd were not regulated by DHT, both mRNAs are expressed in embryonic gonads. These genes are involved in conversion of progestins to androgens. Finally, treatment with DHT increased both aromatase and Srd5a1 expression, which respectively produce estrogens and DHT. Expression of Cyp11a1, 3ß-Hsd, aromatase, and AR in flutamide-treated embryos was in between vehicle- and DHT-treated embryos, but the flutamide effect was not significant after correction for multiple comparisons. Based on overall expression patterns, gonads induced to develop into ovaries by DHT (or flutamide) appear more steroidogenically active than gonads developing into testes at the same temperature.

In contrast, DHT administration had a masculinizing effect on red-eared slider turtle embryos at a temperature that produces mixed sex ratios (Crews et al., 1996B). This suggests the role of androgens in ovarian determination is not conserved among turtle species. Data in other groups also suggests the role of androgens in sex determination may be species-specific. For example, administration of DHT to channel and blue catfish increases the production of females (Davis et al., 1992), while DHT has a masculinizing effect in many other fish species (Chiasson and Benfey, 2007; Devlin and Nagahama, 2002). Davis et al. (1992) reported that the feminizing effect of DHT depended on the dose and timing of administration.

Although androgens and AR have yet to be implicated in ovarian determination in other TSD reptiles, there is increasing evidence that androgens play an evolutionarily conserved role in ovarian differentiation and function in vertebrates. AR is expressed at higher levels in developing ovaries than testes in chickens and reptiles (Katoh et al., 2006; Ramsey and Crews, 2007; Ramsey and Crews, 2009; Rhen et al., 2007). An appropriate level of androgen signaling is critical for normal ovarian development in mammals (Sellix and Sen, 2017). Fetal exposure to high levels of androgens is associated with polycystic ovarian syndrome (PCOS) in mammals (Norman et al., 2007). PCOS is characterized by an increase in the number of developing follicles compared to normal ovaries (Webber et al., 2003; Steckler et al., 2005). On the other hand, low androgen levels or lack of AR function leads to premature ovarian failure (POF) in mammals (Shiina et al., 2006). POF is characterized by amenorrhoea and early loss of ovarian function (Goswami and Conway, 2005). Previous studies in snapping turtles revealed a phenotype similar to PCOS: ovaries from individuals treated with DHT during embryogenesis had increased numbers of developing

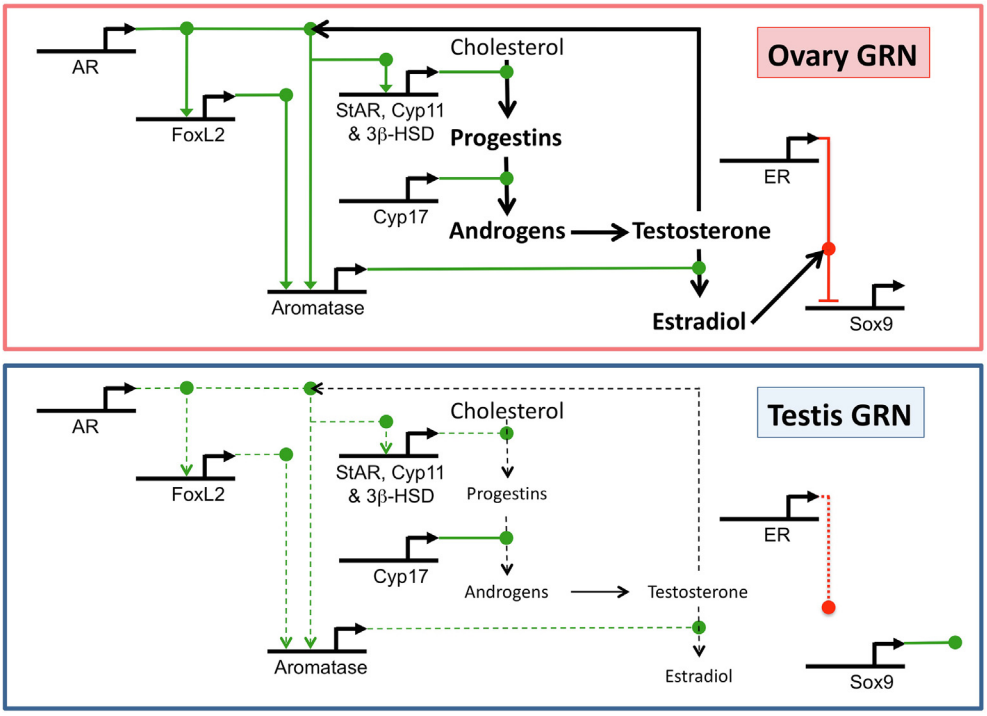

Fig. 5. Model of gene regulatory network for sex determination in snapping turtle embryos. Genes are shown as black lines. Gene products are shown as filled circles. Green lines ending in arrowheads indicate positive regulation of the target gene. Red lines ending in bars indicate repression of the target gene. Solid versus dashed lines indicate difference in expression/activity of gene or gene products between developing ovaries and testes.

follicles (Rhen et al., unpublished results). Furthermore, Amh and many of the genes encoding steroidogenic enzymes have been implicated in the development of PCOS (Durlinger et al., 2002; Luense et al., 2011; Prapas et al., 2009; Pellatt et al., 2010). Elevated expression of these genes in the developing ovary from DHT-treated snapping turtles may lead to the characteristic ovarian phenotype of PCOS. Further analyses of these genes in control and DHT-induced ovaries are warranted.

We also examined Sox9 and Amh because both genes are expressed in Sertoli cells in the developing testes of birds, reptiles, and mammals (Carré-Eusèbe et al., 1996; di Clemente et al., 1992; Oreal et al., 1998; Smith et al., 1999; Vigier et al., 1989; Western et al., 1999). DHT had no effect on Sox9 or Amh mRNA levels compared to the vehicle controls. However, flutamide treatment increased expression of Sox9 and Amh. These findings appear contradictory because DHT and flutamide induced ovarian development at the male-producing temperature used in this study. One potential explanation is that the medullary region of the gonad regresses over an extended period in developing ovaries (i.e., it continues after hatching) (Rhen et al., 2015). In agreement with this idea, Sox9 expression gradually decreases in gonads committed to an ovarian fate (Rhen et al., 2007). While this hypothesis could explain the ineffectiveness of DHT, it does not explain the flutamide-induced increase in Sox9 and Amh expression.

An alternative explanation is that there is genetic variation in androgen signaling in the snapping turtle. Though we did not design the current study to test for clutch by hormone treatment interactions, there appear to be differences in responsiveness to DHT and flutamide among clutches (Table 1). These treatments produced females in some clutches while other clutches did not respond (i.e., males were produced). Moreover, flutamide increased production of females in some clutches but decreased production of females in other clutches at a temperature that produces mixed sex ratios (Rhen and Schroeder, 2010). Given that flutamide has mixed agonist/antagonist effects on gene expression (i.e., aromatase vs. FoxL2) and clutch-specific effects on sex determination in the snapping turtle, identification of genetic variants in AR might provide mechanistic insight into androgen signaling via AR (Chamberlain et al., 1994; Choong et al., 1996; Dejager et al., 2002; Giovannucci et al., 1997; Mifsud et al., 2000).

Variation in the effect of flutamide within snapping turtles parallels species differences in the effect of DHT on sex determination. That is, flutamide induces ovarian development in some snapping turtle clutches, but testicular development in other clutches at temperatures that produce mixed sex ratios. Likewise, DHT induces ovarian development in snapping turtles but testicular development in red-eared slider turtles at temperatures that produce mixed sex ratios. Future studies of molecular evolution of AR and androgen signaling within and among TSD species will help us understand the role of androgens in ovary determination and function. The data presented here are consistent with the idea that genetic variation in AR sensitivity or activity may be associated with hyper-androgenism in the ovary (Ibáñez et al., 2003; Legro et al., 1998; Westberg et al., 2001).

In summary, our findings support the hypothesis that androgens and AR play a role in determination of ovarian fate in the common snapping turtle by increasing expression of a suite of steroidogenic genes, including aromatase. Although androgens are important in ovarian development, too much or too little androgen production in females leads to disease phenotypes, suggesting that the production of androgens and estrogens is highly regulated during development of normal ovaries. Genetic variation in androgen signaling within and among species is also important to understand. Future studies of DHT and flutamide effects on transcriptional activity of the snapping turtle AR using an androgen response element reporter could shed light on the mixed antagonist/agonist properties of flutamide. Likewise, identification of a "pure antagonist" for the snapping turtle AR would help define the role of androgens more precisely.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.05.003.

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