Changes in gonadal gene network by exogenous ligands in temperature-dependent sex determination

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Abstract

We examined the expression of candidate sex-determining genes in the red-eared slider turtle (*Trachemys scripta*) during the temperature-sensitive period (TSP). Aromatase and *Rspo1* were used as markers of ovarian differentiation and *Sox9* was used as a marker of testicular differentiation. Eggs were incubated at a male-producing temperature (26 °C or MPT) and a female-producing temperature (31 °C or FPT). First, eggs at the beginning of the TSP (stage 16) were topically treated with the steroid hormones 17β-estradiol (E_2), testosterone in combination with aromatase inhibitor (AI+T), the E_2 antagonist (ICI 182 780), and the androgen antagonist (flutamide). Secondly, gonads were removed at stage 16 and treated *in vitro* with E_2, AI+T, or hormone antagonists. At the FPT, AI+T *in ovo* suppressed aromatase and *Rspo1*, while activating *Sox9*. At the MPT, E_2 treatment rapidly increased aromatase and *Rspo1*, while suppressing *Sox9*. Treatment with the E_2 antagonist *in ovo* decreased aromatase at the FPT. Treatment with the androgen antagonist *in ovo* increased aromatase and *Rspo1* at early time points at MPT and decreased *Sox9* at MPT at later developmental stages. Treatment of isolated gonads cultured *in vitro* with AI+T at FPT decreased aromatase and *Rspo1* and E_2 increased the expression of these genes at MPT. *In vitro* treatment with E_2 antagonist suppressed aromatase and *Rspo1* expression at FPT. Overall, our results suggest that exogenous ligands dictate gonadal development by redirecting the expression of candidate sex-determining genes within the genetic cascades induced by temperature.

Key Words

- temperature-dependent sex determination
- gonad development
- steroid hormones
- aromatase
- *Sox9*
- *Rspo1*

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Introduction

Sex determination in vertebrates is a developmental process where the bipotential embryonic gonads differentiate into ovaries or testes depending on external or internal cues. Based on the susceptibility of the embryonic gonads to specific cues, the sex determination system can be divided into two categories: the genotypic sex determination (GSD) system and the environmental sex determination (ESD) system. Temperature-dependent sex determination (TSD) is a form of ESD, where the sex determination of the embryonic gonad is dictated by the incubation temperature of the eggs. In the red-eared slider turtle (*Trachemys scripta*), a well-studied TSD species, eggs incubated at 26 °C become males (male-producing temperature or MPT) while eggs incubated at 31 °C become females (female-producing temperature or FPT). The effect of the incubation temperature is only observed midway through development, also known as the temperature-sensitive period (TSP). Exogenous ligands

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(steroid hormones and steroid metabolism enzyme inhibitors) can override the effect of the incubation temperature if applied during this TSP window. Specifically, 17β-estradiol (E_2) treatment of the eggs induces ovarian development at the MPT, while testosterone along with aromatase inhibitor (AI) treatment induces testicular development at the FPT (Wibbels *et al*. 1991, Crews *et al*. 1996). Although exogenous ligands can override the effect of the incubation temperature, the role of endogenous sex steroid hormones in sex determination is still somewhat controversial in species with TSD. For example, administration of sex steroid hormone antagonists – E_2 antagonist applied at the FPT or androgen antagonist applied at the MPT – often fails to affect the gonad phenotype at hatching (Wibbels *et al*. 1991, Crews *et al*. 1996).

How environmental cues, such as temperature or exogenous ligands, engage the molecular mechanisms underlying gonadal development is not fully understood, although recent studies show that several genes (or gene networks) involved in the GSD system exhibit typical gonadal expression patterns in response to temperature in the red-eared slider. For example, aromatase, a gene that encodes the enzyme that catalyzes the conversion of androgens to estrogens, is upregulated at the FPT and downregulated at the MPT in the embryonic gonads (Ramsey *et al*. 2007). Aromatase expression and activity is not directly involved in sex determination in mammals; however, aromatase is sufficient to control gonadal sex during development in some nonmammalian vertebrates including birds and fish (Elbrecht & Smith 1992, Guiguen *et al*. 2010). *R-spondin1* (*RSPO1*), a gene involved in ovarian differentiation in mammals, is also highly expressed during the TSP in red-eared slider gonads at the FPT but is only expressed at low levels at the MPT (Smith *et al*. 2008). A transcription factor, sex-determining region on Y chromosome-box 9 (*Sox9*), a direct target of the transcription factor SRY during testicular differentiation in mammals, is upregulated at the MPT and downregulated at the FPT in embryonic gonads (Shoemaker *et al*. 2007*a*). Accumulating evidences suggest that *Rspo1* and *Sox9* are balancing factors to determine a unidirectional result of gonad phenotype in mammals (Huang *et al*. 1999, Barrionuevo *et al*. 2006, Parma *et al*. 2006, Chassot *et al*. 2008, Tomizuka *et al*. 2008). In this study, we examined the expression pattern of three candidate sex-determining genes in TSD, aromatase, *Rspo1*, and *Sox9*, in exogenous ligand-mediated sex determination in red-eared slider gonads. Slider eggs were topically treated with exogenous ligands at a corresponding temperature at stage 16, the beginning of the TSP. As previous studies of the red-eared slider show that treatment with aromatase inhibitor or testosterone alone during development produces limited or no male hatchlings (Wibbels *et al*. 1991, Crews & Bergeron 1994, Crews 1996), we used combined treatment with aromatase inhibitor and testosterone at the FPT to ensure mostly male hatchlings in the current study. The expression patterns were then examined during the following developmental time periods including hatching. Furthermore, we examined the effect of hormone antagonists on candidate sex-determining gene expression to understand the role of endogenous sex steroid hormones in gonadal determination. The effect of exogenous ligands and hormone antagonists on candidate sex-determining genes was also examined in isolated cultured gonads.

Materials and methods

Animals

Freshly laid red-eared slider turtle eggs were purchased from Clark Turtle Farms (Hammond, LA, USA) and maintained in accordance with humane animal practices under IACUC protocol # AUP-2011-00149. Eggs were collected daily within 24 h of laying so that all individual eggs in the shipment were roughly of equal developmental stages. Eggs were mixed, so clutches were equally represented in each experimental group. Eggs were stored at room temperature for 10 days, at which time all eggs were candled to assess viability. Viable eggs were randomly placed in trays with moistened vermiculite and incubated at either 26 °C (MPT) or 31 °C (FPT). Incubator temperatures were checked daily with thermometers, and temperature fluctuations were monitored with HOBO data loggers (Onset Computer Corp., Bourne, MA, USA). Embryos were allowed to develop until stage 16, the early TSP when all embryos are sensitive to temperature and exogenous ligands (Wibbels *et al*. 1991). Staging was according to external morphological characteristics according to the Greenbaum's staging series (Greenbaum 2002).

In ovo exogenous ligand treatment

At stage 16, eggs were treated as follows, after which the eggs were returned to their designated incubation temperature; at the FPT, eggs were treated with i) 10 μl EtOH as a vehicle control, ii) 100 μg testosterone (Sigma) combined with 75 μg non-steroid aromatase inhibitor, fadrozole (AI: Sigma), iii) 100 μg E_2 antagonist

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ICI 182 780 (Sigma), or iv) 500 μg ICI 182 780. At the MPT, eggs were treated with i) 10 μl EtOH as a vehicle control, ii) 10 μg E_2 (Sigma), iii) 1 mg androgen antagonist, flutamide (Sigma), or iv) 2.5 mg flutamide. All chemicals were dissolved in 10 μl 100% EtOH and topically applied to the surface of the eggs. The dosages used were based on previous studies and on the solubility of the specific agents (Crews & Bergeron 1994, Wibbels & Crews 1994, Wu *et al.* 2009, Barske & Capel 2010). These eggs were incubated until the embryos reached stages 17, 19, 21, 23, 25, or hatching, at which time gonads were collected to be processed individually ($n=6$–8 gonads, i.e. three to four individuals/stage/treatment). Gonads from stage 16 embryos incubating at the FPT and MPT were collected before the treatments to determine a basal gene expression level. Gonads were placed individually into tubes containing 800 μl Trizol (Life Technologies), vortexed, and stored at −80 °C until RNA extraction. Sex was macroscopically diagnosed in hatchling turtles before processing for gene expression (Crews & Bergeron 1994). All treatments and sex diagnosis were coded and so blind to the investigators.

In vitro exogenous ligand treatment in tissue culture

Red-eared slider embryonic gonads were isolated and cultured as described previously (Shoemaker-Daly *et al.* 2010). Briefly, at stage 16 (Day 0), embryonic gonads were dissected under sterile conditions. The isolated gonads were immediately transferred to a floating 0.4 μm Millicell membrane (Millipore, Billerica, MA, USA) placed on the surface of a 24-well plate (BD Falcon, Franklin Lakes, NJ, USA) filled with cell culture media composed of Leibovitz's L-15 medium (+L-glutamine, phenol red-free; Life Technologies) with 10% charcoal-stripped fetal bovine serum (FBS; Sigma) and 1% antibiotic/antimycotic (Life Technologies). Isolated gonads from eggs incubated at the FPT were treated with i) 10 μM AI combined with 1 μM testosterone or ii) 10 μM ICI 182 780 and allowed to develop at the FPT. Gonads from eggs incubated at the MPT were treated with i) 100 nM E_2 or ii) 10 μM flutamide and allowed to develop at the MPT. All chemicals were dissolved in EtOH and adjusted to a final concentration of 0.03%. Both incubation temperatures included two negative control conditions: i) vehicle control of 0.03% EtOH and ii) negative control gonads without any treatment to reinforce the validity of EtOH as a proper negative control for subsequent gene expression analysis. The dosages used were chosen based on previous *in vitro* studies performed using other cell types (Yano *et al.* 1995, Bhattacharyya *et al.* 2006, Jolly *et al.* 2006, La Sala *et al.* 2010, Yilmaz *et al.* 2011). Culture plates were incubated in a self-contained growth chamber under sterile condition. On the day of the tissue culture at stage 16 (Day 0), gonads were also extracted and immediately placed in individual tubes containing 800 μl Trizol solution, vortexed, and stored at −80 °C until RNA extraction (baseline control). The cell culture media were changed every 2 days, and the same concentration of exogenous ligands or controls was added to the fresh media in each well. Gonads were collected in 800 μl Trizol on day 1, 4, 8, 12, 16, or 20, vortexed, and placed at −80 °C until RNA extraction.

Total RNA extraction, cDNA synthesis, and quantitative real-time PCR

Total RNA was extracted with Trizol by following the manufacturer's protocol (Life Technologies). Subsequently, total RNA was treated with DNA-Free Turbo DNase I (Life Technologies) and reverse transcribed into cDNA using the iScript Kit (Bio-Rad). Relative gene expression was quantified using SYBR green (Life Technologies) on the ABI PRISM 7900HT real-time PCR cycler (Life Technologies). Samples were run in triplicate in quantitative real-time PCR (qPCR), and the median values were used for analysis. Relative gene expressions were measured using previously published aromatase, *Rspo1*, and *Sox9* primers specific to sliders and normalized to the gene expression of protein phosphatase 1 (*PP1*), a housekeeping gene expressed constitutively relative to the genes of interest in slider gonads (Ramsey *et al.* 2007, Shoemaker *et al.* 2007*a*, Smith *et al.* 2008). The specificity of qPCR was validated by melting curve analysis. The obtained qPCR data were analyzed using the ΔCT method and the gene expression fold changes between control and treatment groups were calculated by setting the lowest values of the group as onefold. For functional landscape analysis, qPCR data were represented as a percentage maximum in each gene and plotted by Matlab (MathWorks, Natick, MA, USA).

Statistical analysis

Because of the asymmetric distribution, the nonparametric test was used to analyze all data. Gonad sex ratio and qPCR data were analyzed using χ^2 (Fisher's exact) and Wilcoxon rank-sum test respectively by JMP 8.0 (SAS Institute, Cary, NC, USA). All P values acquired by qPCR data are shown in Supplementary Table 1, see section on supplementary data given at the end of this article. Functional landscape

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analysis was performed using MultiDimBio package in R (Scarpino *et al*. 2013). A P value <0.05 was considered to be statistically significant.

Results

Exogenous ligands, not steroid hormone antagonists, can override the incubation temperature on gonad sex ratio at hatching

In the red-eared slider, a single treatment of exogenous ligands at the beginning of TSP, stage 16, is sufficient to override the ambient temperature and produce a specific gonad phenotype (Wibbels *et al*. 1991, Crews *et al*. 1996).

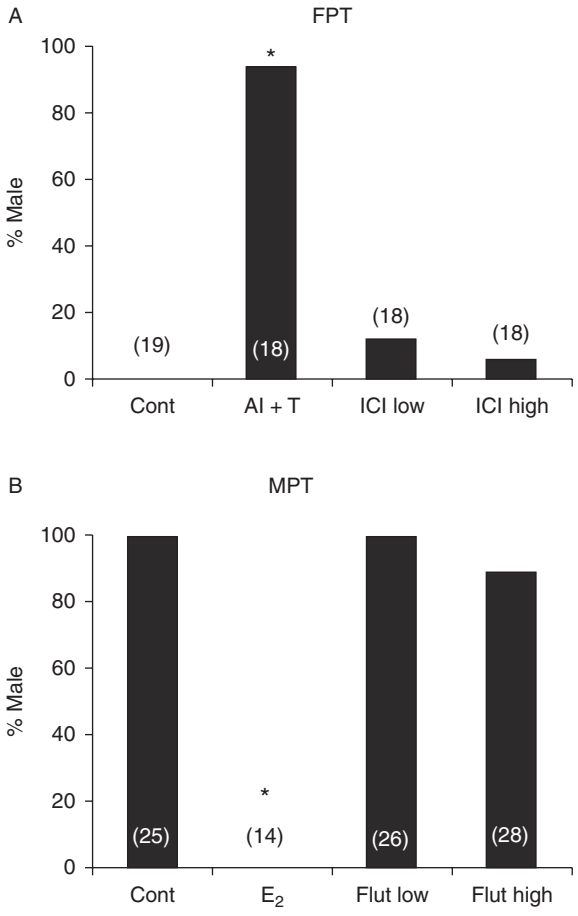

Figure 1
Percentage of male hatchlings in exogenous ligand- and steroid hormone antagonist-treated embryos. Eggs were topically treated with exogenous ligands or hormone antagonists at stage 16, and the gonad phenotype at hatching was examined. (A) FPT and (B) MPT. Values in parentheses indicate the number of individuals. Asterisks indicate statistically significant differences from control groups analyzed by χ^2 (Fisher's exact) test. Cont, vehicle control; ICI, E_2 antagonist ICI 182 780; Flut, androgen antagonist flutamide.

In accordance with this finding, the treatments of exogenous ligands were able to override the temperature effect. AI+T at the FPT mostly produced testis phenotypes except one exhibited ovary-like structure with an oviduct (Fig. 1A). Blocking endogenous E_2 with a low dosage of ICI 182 780 at the FPT produced all ovarian phenotypes except for two eggs that exhibited testes with oviducts; with high doses, all eggs displayed ovarian phenotypes except one that had testes without an oviduct (Fig. 1A). E_2 at the MPT produced 100% ovarian phenotypes at hatching (Fig. 1B). Blocking endogenous testosterone with a low dose of flutamide at the MPT did not have any effect on gonad phenotype, whereas a high dose of flutamide produced three eggs that exhibited ovarian phenotypes with oviduct formation (Fig. 1B). None of the individuals in control groups failed to show the predicted gonad sex according to the ambient temperature. Further, no intersex gonads (e.g. ovotestes) were observed in the treatment groups.

Treatment with testosterone and aromatase inhibitor at FPT reversed the temperature-specific expression patterns of candidate sex-determining genes *in ovo*

The expression of candidate sex-determining genes in the control group exhibited a typical temperature-specific expression pattern at FPT: i) an ovarian marker, aromatase exhibited a gradual increase at the beginning of stage 17, which peaked at stage 21 and gradually decreased untill hatching; ii) another ovarian marker, *Rspo1*, began to increase after stage 23 and peaked at hatching; and iii) a testicular marker, *Sox9*, exhibited a low expression throughout development at the FPT (Fig. 2A). These observations are in agreement with previous findings (Ramsey *et al*. 2007, Shoemaker *et al*. 2007*a*,*b*, Smith *et al*. 2008), although the current study examines an extended developmental period including hatching. When eggs were topically treated with AI+T at stage 16, the expression of aromatase and *Rspo1* decreased as early as stage 17, and they remained at low levels throughout development (Fig. 2A). The expression of *Sox9* in the AI+T-treated group exhibited a gradual increase throughout development as observed typically at the MPT (Fig. 2A). Next, we performed functional landscape analysis to examine a functional change in the relationship among genes. In this analysis, a transcriptional change in three genes within an individual was considered as one unit, i.e. a functional landscape, and compared between treatments at specific developmental time points (Scarpino *et al*. 2013). Such analysis will provide us with biologically different aspects of gene functionality

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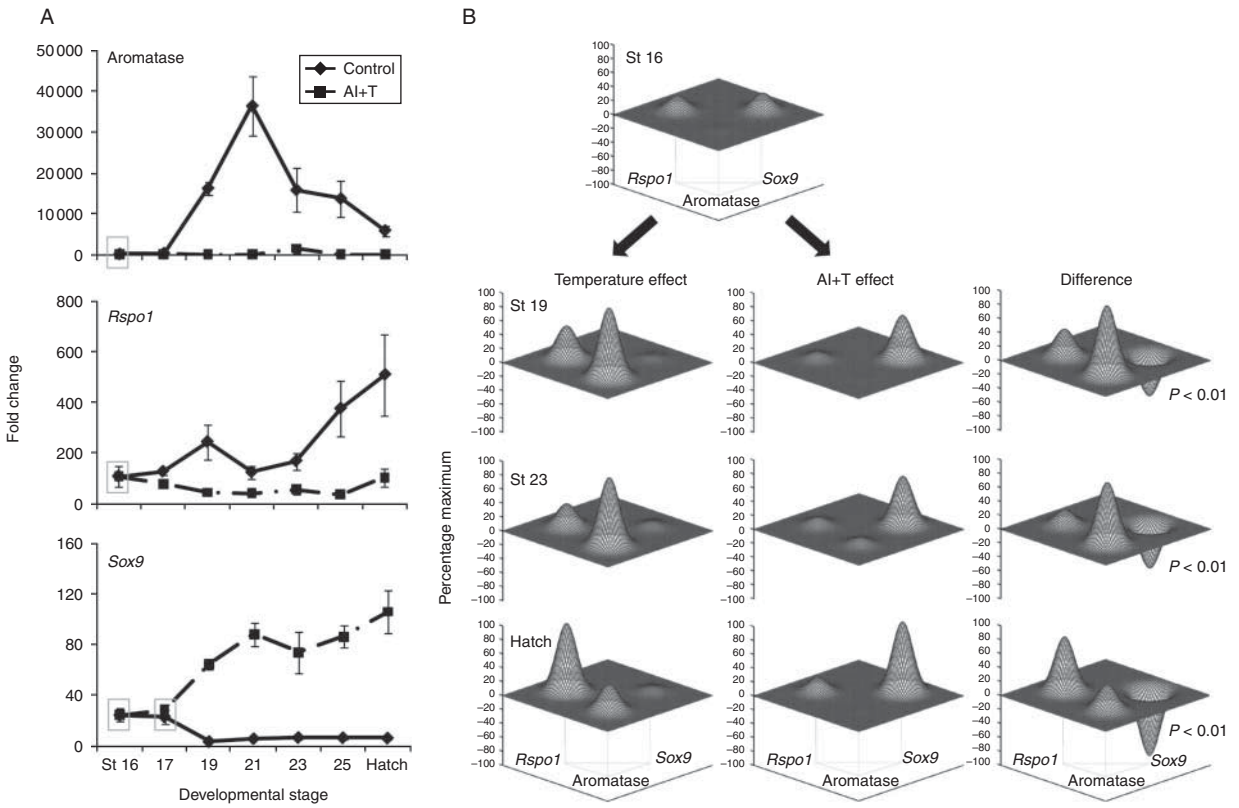

Figure 2

The effect of testosterone and aromatase inhibitor on the expression of aromatase, *Rspo1*, and *Sox9* at the FPT. (A) Changes in gene expression in exogenous ligand-treated slider gonads during development *in ovo*. Eggs at the FPT were topically treated with AI+T at stage 16. Gonads were collected at stage (St) 17, 19, 21, 23, 25, and hatching. The relative gene expression levels of aromatase, *Rspo1*, and *Sox9* were measured by qPCR and analyzed using the ΔCT method. All values were normalized to a housekeeping gene, *PP1*, and are represented as a fold change. Each time point included $n=6$–8 gonads/treatment/time point. Data represented as the mean ± S.E.M. Grey boxes indicate no statistical difference between the two treatments within a developmental stage by Wilcoxon rank sum test. Points without the boxes were significantly different. All *P* values from statistical analysis are shown in Supplementary Table 1. (B) Landscape analysis for a functional change in the relationship among aromatase, *Rspo1*, and *Sox9*. Values from qPCR were represented as a percentage maximum in each gene and plotted by developmental time points. Difference represents a subtraction of a treatment group (AI+T effect) from a control group (temperature effect) in each stage.

(expression vs interaction), enabling us to follow the 'network' differences in gene expression that covary from one individual to another. The treatment with AI+T completely altered the relationship of three gene expressions at the FPT as early as stage 19 (Fig. 2B). Early stage (stage 19) was particularly marked with a presence or absence of prominent aromatase peak followed by a gradual increase in *Rspo1* and in *Sox9* in control FPT and in AI+T treatment group respectively (Fig. 2B).

Treatment with E_2 at the MPT reversed the temperature-specific expression patterns of candidate sex-determining genes *in ovo*

Next, we examined the effect of E_2 at the MPT on the expression patterns of candidate sex-determining genes, aromatase, *Rspo1*, and *Sox9*, in red-eared slider gonads. In the control group, aromatase, *Rspo1*, and *Sox9* genes exhibited typical expression patterns found in MPT: suppressed expression of aromatase and *Rspo1* and a corresponding increase in the expression of the testicular marker *Sox9* (Fig. 3A). The topical addition of E_2 at stage 16 reversed the gene expression pattern typically observed at the MPT. The expression of the ovarian marker aromatase increased in response to E_2 at stage 23, which was a later developmental time point and a weaker intensity than the one normally observed at the FPT (Fig. 3A). The expression of *Rspo1* was increased as early as stage 17 in rapid response to E_2 with an exception of stage 21 (Fig. 3A). *Sox9* expression was high at stage 17 in the E_2-treated group; however, it decreased throughout the rest of the developmental stages (Fig. 3A). Functional landscape analysis

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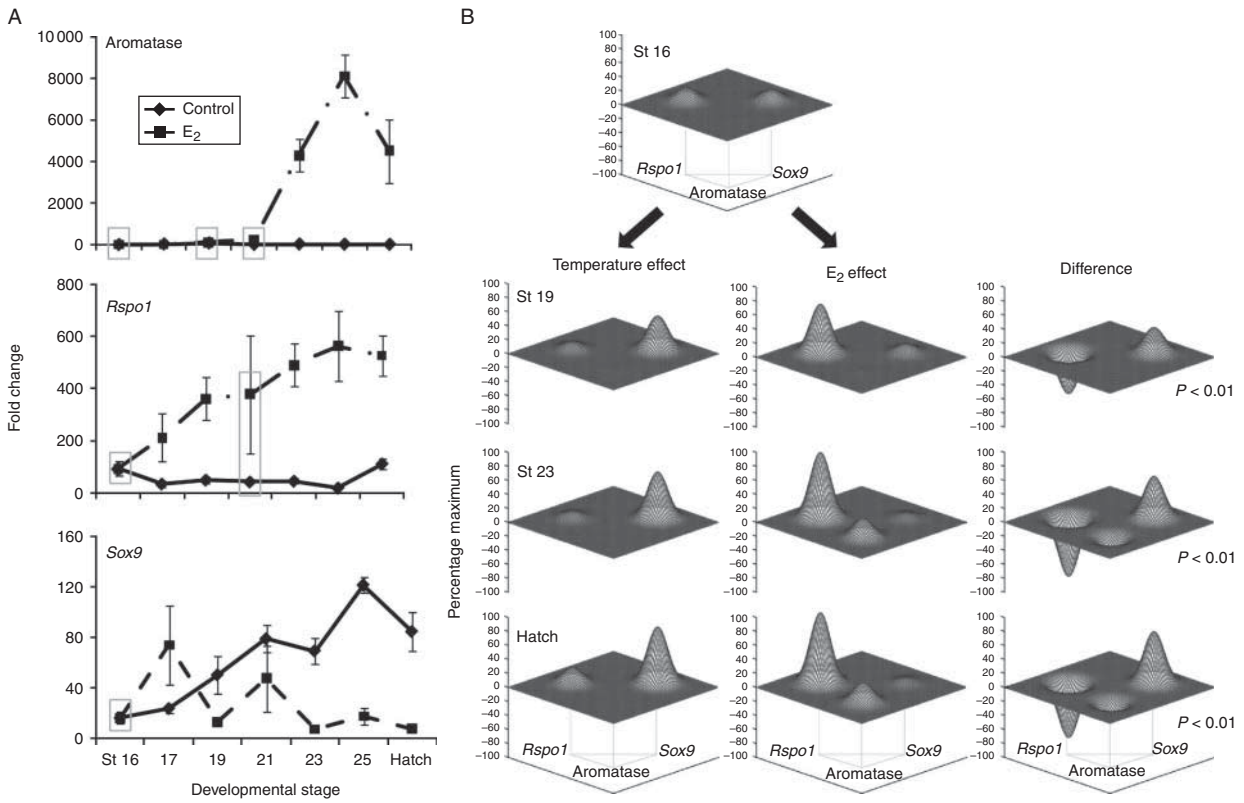

Figure 3

The effect of E_2 on the expression of aromatase, *Rspo1*, and *Sox9*. (A) Changes in gene expression in E_2-treated slider gonads during development *in ovo*. Eggs at the MPT were topically treated with E_2 at stage 16. Gonads were collected at stage (St) 17, 19, 21, 23, 25, and hatching. The relative gene expression levels of aromatase, *Rspo1*, and *Sox9* were measured by qPCR and analyzed using the ΔCT method. All values were normalized to a housekeeping gene, *PP1*, and represented as a fold change. Each time point included $n=6$–8 gonads/treatment/time point. Data are represented as the mean ± S.E.M. Grey boxes indicate no statistical difference between the two treatments within a developmental stage by Wilcoxon rank sum test. Points without the boxes were significantly different. All *P* values from statistical analysis are shown in Supplementary Table 1. (B) Landscape analysis for a functional change in relationship among aromatase, *Rspo1*, and *Sox9*. Values from qPCR were represented as a percentage maximum in each gene and plotted by developmental stages. Difference represents a subtraction of a treatment group (E_2 effect) from a control group (temperature effect) in each stage.

revealed that a functional relationship of three genes at the MPT was completely altered by a treatment with E_2 from the early developmental stages. In E_2-treated gonads, *Rspo1* expression gradually increased while *Sox9* expression was suppressed, as typically observed at the FPT (Fig. 3B). However, unlike a typical landscape at the FPT, it occurred without a spike of *aromatase* expression (Figs 3B compared to 2B).

Exogenous ligands shape ovary, not testis, phenotype differently from incubation temperature

We further examined whether a certain phenotype shaped by exogenous ligands followed the same landscape pathway as the phenotype induced by temperature. Eggs incubated at the FPT and those treated with E_2 at the MPT produce an ovarian phenotype at hatching (Fig. 1). A functional relationship of three candidate sex-determining genes showed no difference between the FPT and the MPT at stage 16 (before the treatment; Fig. 4A). However, the topical treatment of E_2 at the MPT at stage 16 had induced a different landscape pattern from control FPT at the midway of development, stages 19 and 23 (Fig. 4A). The difference in landscape pathway diminished at the hatching (Fig. 4A). As opposed to the ovarian phenotype, the landscape pattern of gene expression in testis induced by AI+T treatment at the FPT exhibited no difference from the testis induced by control MPT throughout development (Fig. 4B). These results agree with a phenotypic difference between temperature- and ligand-induced gonads, especially prominent in ovarian formation, reported in red-eared slider gonads previously (Barske & Capel 2010, Matsumoto & Crews 2012).

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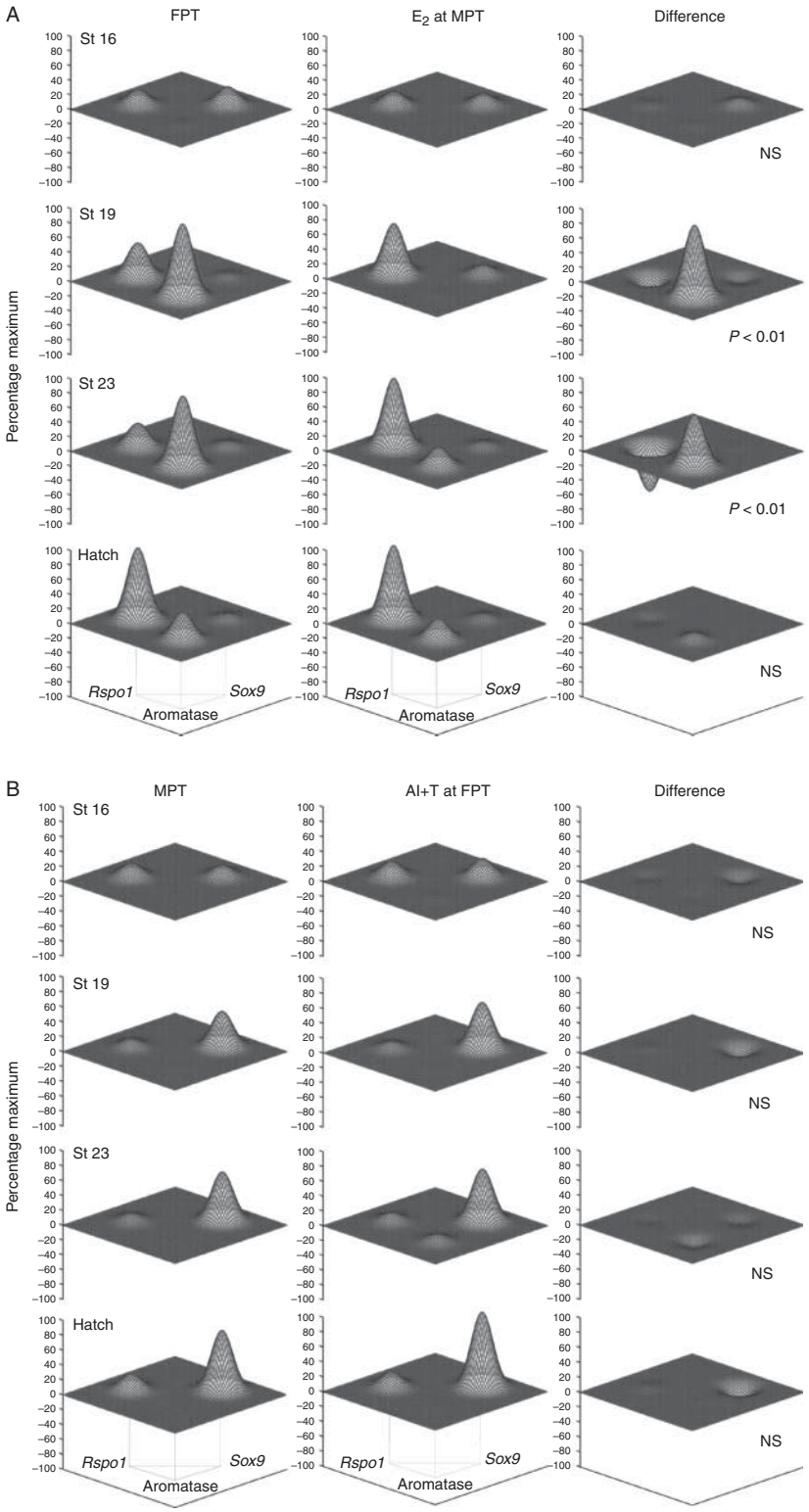

Figure 4

Landscape analysis compared within a gonad phenotype, ovary (A) and testis (B). The value of qPCR was represented as a percentage maximum in each gene and plotted by developmental stages (St). Difference was calculated by subtraction of a treatment group (E_2 at MPT or AI+T at FPT) from a control temperature (FPT or MPT respectively) and statistical differences were analyzed using MultiDimBio package in R (Scarpino *et al.* 2013). NS, not statistically significant.

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Treatment with steroid hormone antagonists altered the temperature-specific expression patterns of candidate sex-determining genes *in ovo*

Next, we examined the role of endogenous sex steroid hormones in the expression of candidate sex-determining genes at a specific temperature, i.e. a role of E_2 to induce ovaries at the FPT or testosterone to induce testis at the MPT. Eggs were topically treated with E_2 antagonist at the FPT or testosterone antagonist at the MPT at stage 16. These treatments did not significantly alter the gonad sex ratio or phenotype from control group at hatching (Fig. 1). However, the expression levels of several genes were affected during development. The addition of low doses of the E_2 antagonist ICI 182 780 at FPT delayed the peaking of aromatase expression; however, the overall expression pattern of aromatase was similar to the control group (FPT in Fig. 5). High doses of ICI 182 780 significantly lowered the expression level of aromatase throughout development at the FPT. A low dose of ICI 182 780 decreased the expression of *Rspo1* at stage 17 at the FPT; however, the expression patterns during the rest of the developmental stages were not altered. In contrast to our prediction, a high dose of ICI 182 780 increased *Rspo1* expression at stages 17 and 21 at the FPT (FPT in Fig. 5). The expression of *Sox9* significantly decreased with the treatment of low and high doses of ICI 182 780 at stages 21, 23, 25, and hatching and at stages 19 and 25 at the FPT respectively; the overall expression pattern of *Sox9* during gonadal development, however, remained consistent to control at both doses (FPT in Fig. 5). Next, we examined the role of endogenous testosterone in the expression of candidate sex-determining genes at the MPT by topically applying the androgen antagonist flutamide on the eggs at stage 16. A low dose of the androgen antagonist at MPT did not affect aromatase and *Rspo1* expression. With high doses of flutamide at the MPT, there was an increased expression of aromatase at stage 19 and *Rspo1* at stage 17, though these effects diminished in subsequent stages (MPT in Fig. 5). The expression of *Sox9* was increased at stage 17 and decreased at stage 25 and hatching by low doses of flutamide, while high doses led to decreased expression at stages 21, 25, and hatching (MPT in Fig. 5).

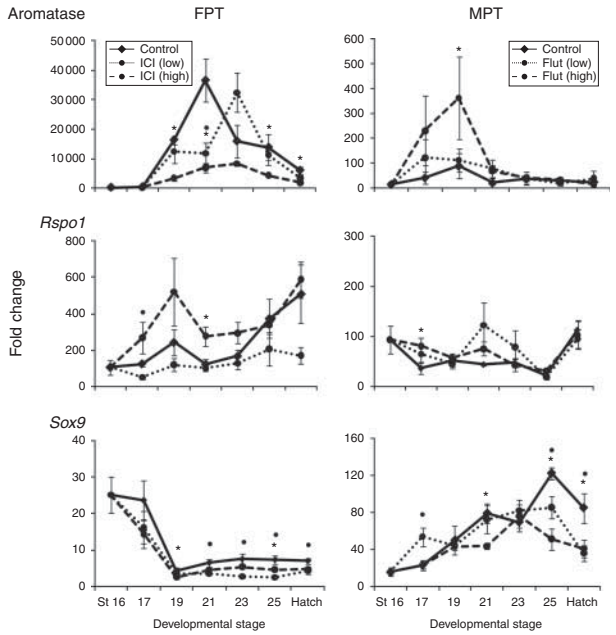

Figure 5
The effect of steroid hormone antagonists on the expression of aromatase, *Rspo1*, and *Sox9* in slider gonads during development. Eggs were topically treated with steroid hormone antagonists (E_2 antagonist at FPT and androgen antagonist at MPT) at stage 16. Gonads were collected at stages 17, 19, 21, 23, 25, and hatching. Relative gene expression levels of aromatase, *Rspo1*, and *Sox9* during development were measured by qPCR and analyzed using the ΔCT method. All values were normalized to a housekeeping gene, *PP1*, and represented as a fold change. The same sample was used for the values at stage 16 in both a control and a treatment group. Each time point included $n=6$–8 gonads/treatment/time point. Data are represented as the mean$\pm$S.E.M. A black circle and an asterisk indicate statistically significant difference(s) in low and high dosages of antagonists respectively from control group analyzed by Wilcoxon rank-sum test. All *P* values are shown in Supplementary Table 1. ICI, E_2 antagonist ICI 182 780; Flut, androgen antagonist flutamide.

The expression pattern of aromatase and *Rspo1* dictated by exogenous ligands and steroid hormone antagonists *in vitro* mimics the expression pattern observed *in ovo*

In previous studies, we showed that the expression pattern of candidate sex-determining genes in isolated, cultured gonads closely mimicked the expression pattern *in ovo* in response to incubation temperatures; this finding suggests that the gonad itself has the ability to dictate its own gene expression pattern according to environmental cues (Shoemaker-Daly *et al.* 2010). In this study, we determined whether the observed gene expression profiles elicited by exogenous ligands and hormone antagonists also occurred in isolated gonads. The expression of the testicular marker *Sox9* was not included in our data analysis because it showed a significant change in expression by the control vehicle treatment (0.03% EtOH) when compared with non-EtOH treatment (data not shown). The expression levels of the two ovarian markers were not affected by the control vehicle treatment and were subsequently analyzed. Treatment with AI+T repressed aromatase expression at days 1, 12, and 20 at the FPT *in vitro* (FPT

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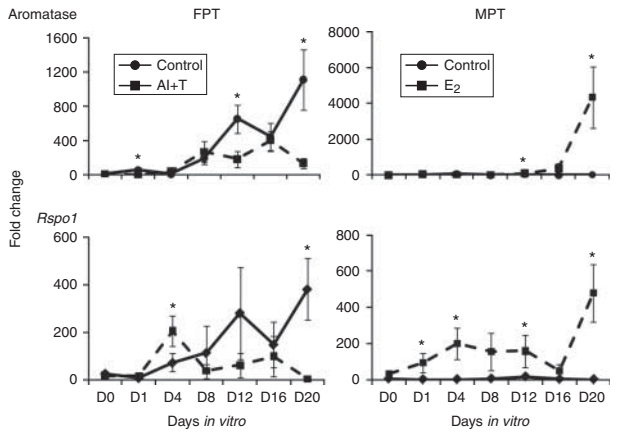

Figure 6
The effect of exogenous ligands on the expression of aromatase and *Rspo1* in cultured slider gonads during development. Embryonic gonads at the FPT and MPT were isolated at stage 16, treated with exogenous ligands, and continuously cultured at the same temperature for up to 20 days. The control vehicle group was treated with a final concentration of 0.03% EtOH. Gonads were collected on days (D) 1, 4, 8, 12, 16, and 20. Relative gene expression levels of aromatase and *Rspo1* during development were measured by qPCR and analyzed using the ΔCT method. All values were normalized to a housekeeping gene, *PP1*, and represented as a fold change. The same sample was used for the values at stage 16 in both a control and treatment group. Each time point included $n=8$–10 cultured gonads/treatment/time point. Data are represented as the mean ± S.E.M. An asterisk indicates statistically significant difference (s) from control group analyzed by Wilcoxon rank-sum test. All *P* values are shown in Supplementary Table 1.

in Fig. 6). The expression of *Rspo1* was decreased by AI+T treatment at day 20 at the FPT, but its expression was not affected at any other time points (FPT in Fig. 6). E_2 treatment increased the aromatase expression at days 12 and 20 and *Rspo1* expression at days 1, 4, 12, and 20 respectively at the MPT (MPT in Fig. 6). As observed *in ovo*, the treatment with E_2 antagonist ICI 182 780 repressed aromatase expression at days 8, 12, and 20 at the FPT *in vitro* (FPT in Fig. 7). The expression of *Rspo1* was also decreased by ICI 182 780 treatment at days 8 and 20 at the FPT (FPT in Fig. 7). The androgen antagonist flutamide altered the expression of aromatase at days 1 and 16 and *Rspo1* at days 4, 8, and 12 at the MPT (MPT in Fig. 7).

Discussion

Sex determination in vertebrates takes place as a gradual process and can be categorized into three phases, onset by genetic or environmental triggers, differentiation into testis or ovary, and maintenance by male- or female-specific molecules. At which phase of the sex determination process the sex steroid hormones come into play remains elusive in nonmammalian vertebrates. However, studies demonstrate that disturbance of sex steroid hormone balance during gonadogenesis often results in redirecting the gonad development against genetic or environmental triggers in nonmammalian vertebrates (Elbrecht & Smith 1992, Crews & Bergeron 1994, Kitano *et al.* 2000). In this study, we investigated the effect of exogenous ligands, i.e. sex steroid hormones and a steroid metabolism enzyme inhibitor, and steroid hormone antagonists on the process of gonad sex determination by examining the patterns of sex-determining gene expression in the red-eared slider turtle. It is known that exogenous ligands can modify the trajectory of gonadal sex determination set by ambient temperature if applied during development in the red-eared slider and other species (Bull & Vogt 1981, Wibbels *et al.* 1991, Crews *et al.* 1996). In the hatchlings with AI+T treatment, the typical developmental expression pattern of ovarian markers aromatase and *Rspo1* was suppressed and the testicular marker *Sox9* was increased (Fig. 2). By contrast, E_2

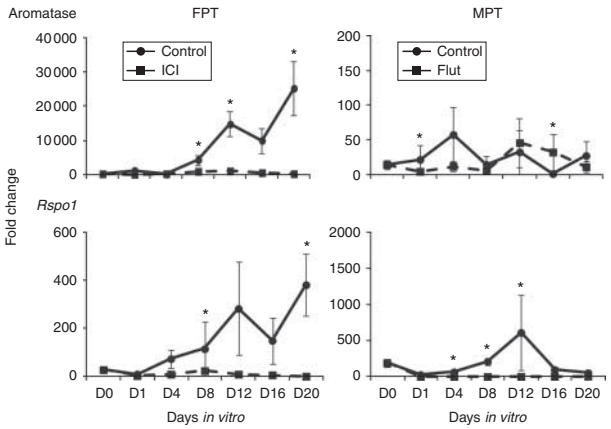

Figure 7
The effect of steroid hormone antagonists on the expression of aromatase and *Rspo1* in cultured slider gonads during development. Embryonic gonads at the FPT and MPT were isolated at stage 16, treated with steroid hormone antagonists (E_2 antagonist at the FPT and androgen antagonist at the MPT), and continuously cultured at the same temperature for up to 20 days. The control vehicle group was treated with a final concentration of 0.03% EtOH. Gonads were collected on days (D) 1, 4, 8, 12, 16, and 20. Relative gene expression levels of aromatase and *Rspo1* during development were measured by qPCR and analyzed using the ΔCT method. All values were normalized to a housekeeping gene, *PP1*, and represented as a fold change. The same sample was used for the values at stage 16 in both a control and a treatment group. Each time point included $n=8$–10 cultured gonads/treatment/time point. Data are represented as the mean ± S.E.M. An asterisk indicates statistically significant difference (s) from control group analyzed by Wilcoxon rank-sum test. All *P* values are shown in Supplementary Table 1. ICI, E_2 antagonist ICI 182 780; Flut, androgen antagonist flutamide.

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treatment at the MPT during TSP increased aromatase and *Rspo1* and decreased *Sox9* expression, which resulted in the production of all female hatchlings (Figs 1 and 2). E_2-treated gonads at the MPT exhibited a missing peak of aromatase expression while typical *Rspo1* and *Sox9* expression patterns at the MPT were observed. It suggests that *Rspo1* activation is a key signal to initiate ovarian differentiation, and exogenous E_2 plays a role in triggering this process when aromatase expression is absent at the MPT. Previous studies also showed that exogenous ligands modify the expression of candidate sex-determining genes, *Rspo1* and *Sox9*. For example, embryonic exposure to DHT increases *Sox9* and suppresses *Rspo1* mRNA levels in developing mouse prostates (Schaeffer *et al*. 2008). In the red-eared slider, multiple treatments of eggs with E_2 completely suppress the expression of *Sox9* by embryonic stage 19 (Barske & Capel 2010). Moreover, *Sox9* mRNA is increased in adult ovaries of mice lacking the presence of estrogen receptors (Couse *et al*. 1999, Dupont *et al*. 2003). In chickens, the inhibition of estrogen synthesis by aromatase inhibitors results in a decrease in *RSPO1* expression in embryonic gonads of genetic females (Smith *et al*. 2008). Although the hierarchy of genetic cascade of these genes during sex determination has not been established in TSD species, we applied a new analytic method to observe relationship of three genes, a snapshot of genetic interaction at a particular developmental time point. In the functional landscape analysis, the expression pattern of candidate sex-determining genes within an individual is considered as one network or landscape and compared to the networks/landscapes induced by various environmental cues (Scarpino *et al*. 2013). Interestingly, when the landscape is compared within a phenotype, E_2-induced ovarian development exhibits a different developmental pattern of the landscape network from FPT-induced ovarian development (Fig. 4A). This observation confirms that the gonads at the FPT and E_2-treated gonads at the MPT follow different gonad differentiation pathways. In testicular development, however, the difference in the gene network between the MPT and AI+T treatments was not observed (Fig. 4B). It is worth noting that the phenotype of ovary induced by E_2 was slightly different from the ovary induced by the FPT, smaller in size with less evidence of primordial germ cells as noted in several turtle species (Merchant-Larios *et al*. 1997, Barske & Capel 2010, Matsumoto & Crews 2012). The phenotypic difference in testis, however, is less noticeable between the two triggers, the MPT and AI+T (Matsumoto & Crews 2012). This observation may be attributed to a different pattern of landscape pathway during a mid period of development in the current study.

The experiment with steroid hormone antagonists revealed hormone–gene interactions, specifically a role of endogenous steroid hormones in the TSD system. Previous studies have shown that steroid hormone antagonists interrupt normal gonadal development, occasionally producing abnormal or intersex gonads in other species (Kang *et al*. 2006, Katoh *et al*. 2006). In our study, although steroid hormone antagonists resulted in little change in sex ratio and gonad morphology at hatching, the expression of candidate sex-determining genes has changed. For example, blocking endogenous E_2 with a low dosage of E_2 antagonist delayed the expression of aromatase at the FPT and a high dosage of E_2 antagonist significantly suppressed aromatase (Fig. 5). This indicates that endogenous E_2 may be responsible for the manifestation of aromatase expression by a positive feedback mechanism at the FPT. Interestingly, despite the low levels of aromatase expression during development in the E_2 antagonist-treated group, the ovaries at hatching did not exhibit any morphological or histological differences from control ovaries at FPT (data not shown). In reptiles and birds, aromatase is thought to be a key enzyme in determining the activation of male vs female pathways by balancing the production of estrogen (Elbrecht & Smith 1992, Jeyasuria *et al*. 1994). Aromatase is still a candidate of the upstream factor to determine gonad sex in TSD species; however, our data suggest that the expression of aromatase may not be a sole determinant for an ovarian development. Similarly, the lack of an observable effect of the androgen antagonist flutamide on aromatase and *Rspo1* gene expression at the MPT suggests that endogenous androgen may not be a primary factor for the suppression of typical ovarian markers at this temperature. Furthermore, *Sox9* expression was not prominently affected by antagonist treatments either. These observations, along with hatching phenotypes, lead to the hypothesis that endogenous hormones may not participate in onset of the gene network of the sex-determining genes directed by temperature, but rather are involved in the gonad differentiation that follows. We cannot rule out the possibility that the dosage and timing of the application of antagonist in this study may not have been sufficient to completely block the endogenous steroid hormones. A previous study shows that blocking endogenous E_2 with multiple treatments of ICI 182 780 leads to a delay in *Sox9* downregulation in *slider* gonad at the FPT (Barske & Capel 2010), which suggests that

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endogenous E_2 has, at least to some extent, a suppressive effect on *Sox9* expression.

We extended our investigations to elucidate whether the extraembryonic environment was involved in ligand-induced patterns of gene expression. Previous studies have shown that isolated embryonic gonads had the ability to sense and respond to the ambient temperature in TSD species (Moreno-Mendoza *et al*. 2001, Pieau & Dorizzi 2004, Shoemaker-Daly *et al*. 2010). In the current study, the isolated gonads also exhibited the expression pattern of the candidate sex-determining genes similar to the one observed *in ovo* when treated with exogenous ligands and steroid hormone antagonists (Figs 6 and 7). However, the timing of the response of gene expression occurred late in the developmental stage with less intensity. For example, *Rspo1* expression at FPT is not suppressed by AI+T treatment until 20 days after treatment (Fig. 6). In E_2-treated gonads, both aromatase and *Rspo1* expression increased, but with less intensity than the increase observed *in ovo*. One possible explanation is that isolated gonads lack the supporting cells normally migrated from underlying mesonephric tissue, resulting in a slow development and a lack of a morphological structure (Yao *et al*. 2004, Shoemaker-Daly *et al*. 2010). The antagonist treatments *in vitro* also followed the observation found *in ovo*: the E_2 antagonist ICI 182 780 suppressed the ovarian markers at the FPT, whereas the androgen antagonist flutamide lacked the consistent effect on the expression of these genes at the MPT (Fig. 7). It confirms the speculation *in ovo* that endogenous E_2 positively affects ovarian markers at the FPT while endogenous androgen is not responsible for suppression of these genes at the MPT.

Overall, the current study shows that exogenous ligands modify the gonad phenotype by redirecting (suppress or activate) the expression of candidate sex-determining genes. *In vitro* data further suggest that exogenous ligands, as wells as temperature, affect the expression of candidate sex-determining genes in gonads independently of other embryonic organs during development. Although the factors responsible for the regulation of these candidate sex-determining genes yet remain elusive, this study demonstrates further insights into the role of endogenous/exogenous steroid hormones in the process of sex determination in the TSD system.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-12-0260.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Y M designed and performed the study. R Y and C T assisted animal dissection and organ cultures. D C contributed to the research design and supervised the project and Y M wrote the manuscript.

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Supplementary table Statistical analysis: *P* values

Figure number		**Stage**	**Genes**		
Figure 2			*Aromatase*	*Rspo1*	Sox9
	AI+T at FPT				
		16	N/A	N/A	N/A
		17	0.0460	0.0209	0.2936
		19	0.0008	0.0046	0.0008
		21	0.0012	0.0078	0.0012
		23	0.0011	0.0209	0.0008
		25	0.0039	0.0039	0.0039
		Hatch	0.0019	0.0201	0.0039
Figure 3					
	E2 at MPT				
		16	N/A	N/A	N/A
		17	0.0176	0.0381	0.0410
		19	0.4080	0.0005	0.0102
		21	0.9393	0.7897	0.0147
		23	0.0045	0.0005	0.0004
		25	0.0002	0.0003	0.0002
		Hatch	0.0008	0.0008	0.0008
Figure 5					
	ICI (low) at FPT				
		16	N/A	N/A	N/A
		17	0.3446	0.0023	0.1722
		19	0.1722	0.1722	0.5286
		21	0.0109	0.4875	0.0109
		23	0.1152	0.4622	0.0033
		25	0.8312	0.2002	0.0105
		Hatch	0.1152	0.0528	0.0389
	ICI (high) at FPT				
		16	N/A	N/A	N/A
		17	0.3446	0.4008	0.2076
		19	0.0008	0.4008	0.0209
		21	0.0012	0.0491	0.203
		23	0.0933	0.079	0.1556
		25	0.0104	0.8728	0.025
		Hatch	0.0046	0.4008	0.0528
	Flut (low) at MPT				
		16	N/A	N/A	N/A
		17	0.5217	0.0756	0.0043
		19	0.0593	0.6501	0.4059
		21	0.3643	0.2265	0.3258

		23	0.1826	0.5940	0.3743
		25	0.8946	0.3099	0.0193
		Hatch	0.2936	0.5286	0.0063
	Flut (high)at MPT				
		16	N/A	N/A	N/A
		17	0.3229	0.0143	0.9349
		19	0.0329	0.8206	0.3643
		21	0.1986	0.0696	0.0015
		23	0.3359	0.2898	0.5637
		25	0.4142	0.3272	0.0015
		Hatch	0.3017	0.6985	0.0201
Figure 6					
	AI+T at FPT				
		Day 0	N/A	N/A	
		Day 1	0.0111	0.5022	
		Day 4	0.0821	0.0919	
		Day 8	0.6052	0.4690	
		Day 12	0.0164	0.3499	
		Day 16	1.0000	0.6230	
		Day 20	0.0013	0.0003	
	E2 at MPT				
		Day 0	N/A	N/A	
		Day 1	0.5377	0.0373	
		Day 4	0.9451	0.0428	
		Day 8	0.8908	0.1417	
		Day 12	0.0413	0.0082	
		Day 16	0.1256	0.5526	
		Day 20	0.0006	0.0013	
Figure 7					
	ICI at FPT	Day 0	N/A	N/A	
		Day 1	0.6489	0.0638	
		Day 4	0.1455	0.5283	
		Day 8	0.0282	0.0310	
		Day 12	0.0026	1.0000	
		Day 16	0.1017	0.1573	
		Day 20	0.0005	0.0001	
	Flut at MPT	Day 0	N/A	N/A	
		Day 1	0.0215	0.0645	
		Day 4	0.3932	0.0012	
		Day 8	0.4690	0.0004	
		Day 12	0.0601	0.0478	
		Day 16	0.0306	0.0756	
		Day 20	0.8997	0.0877	