Temperature fluctuations and estrone sulfate affect gene expression via different mechanisms to promote female development in a species with temperature-dependent sex determination

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Summary statement

We explore how thermal fluctuations and estrone sulfate affect sex determining genes. We find warm temperature and estrone sulfate induce female development but act on different points of the sex determining pathway.

Abstract

Variation in developmental conditions can affect a variety of embryonic processes and shape a number of phenotypic characteristics that can affect offspring throughout their lives. This is particularly true of oviparous species where development typically occurs outside of the female, and studies have shown that traits such as survival and behavior can be altered by both temperature and exposure to steroid hormones during development. In species with temperature-dependent sex determination (TSD), the fate of gonadal development can be affected by temperature and by maternal estrogens present in the egg at oviposition and there is evidence that these factors can affect gene expression patterns. Here, we explore how thermal fluctuations and exposure to an estrogen metabolite, estrone sulfate, affect the expression of several genes known to be involved in sexual differentiation; *Kdm6b*, *Dmrt1*, *Sox9*, *FoxL2*, and *Cyp19A1*. We found that most of the genes responded to both temperature

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and estrone sulfate exposure, but that the responses to these factors was not identical in that estrone sulfate effects occur downstream of temperature effects. Our findings demonstrate that conjugated hormones such as estrone sulfate are capable of influencing temperature dependent pathways to potentially alter how embryos respond to temperature and highlight the importance of studying the interaction of maternal hormone and temperature effects.

Introduction

Conditions during embryonic development can have strong and lasting effects on offspring phenotype. The degree to which developmental conditions vary depends upon several factors including the local environment, parity mode, and maternal contributions to the embryo. Embryos of most oviparous species complete the majority of their development outside of the female and may be subjected to both abiotic and biotic forces that can alter their developmental trajectories. For example, abiotic factors such as environmental temperature can affect the rate of embryonic development, survival, and sexual differentiation (Crews et al. 1994, Booth et al. 2006, Turriago et al. 2015, Lambert et al. 2018). Biotic factors such as maternal steroids can also affect offspring development and behavior (Carere and Balhazart 2007), including begging behavior, offspring growth and success, immune function, and survival (Groothuis et al. 2005, Ruuskanen 2013, Groothuis et al. 2019, Mouton and Duckworth 2021). Many traits are subject to the effects of both abiotic and biotic factors, for example metabolic rates in birds can be affected by both incubation temperature (DuRant et al. 2012) and testosterone exposure (Nilsson et al. 2011). It remains to be determined whether abiotic and biotic factors can elicit their effects through similar mechanisms or if these effects arise independently. Work in reptiles with temperature-dependent sex determination (TSD) may provide some insights into how abiotic and biotic factors might interact at a mechanistic level to affect offspring phenotype. Because reptiles with TSD have a single phenotypic trait (e.g., sex) that is sensitive to both temperature and steroids, they provide a unique opportunity to investigate the mechanisms by which abiotic and biotic factors act and potentially interact to affect hatchling phenotype.

For many species with TSD, exposure to constant, warm incubation temperatures induce ovary development, and exposure to constant, cool temperatures induce testis development (Bull 1985, Wibbels and Crews 1995, Merchant Larios et al. 1997). In the red eared slider turtle (*Trachemys* scripta), eggs incubated at 26°C for the entirety of development will produce 100% male hatchlings and eggs incubated at 31°C for the entirety of development will produce 100% female hatchlings (Wibbels and Crews 1995). In natural nests, *T. scripta* embryos are regularly exposed to both male- and female-producing temperatures during development (Carter et al. 2017) and recent research demonstrates that transient bouts of heat exposure are sufficient to

induce ovary formation. For example, exposure to 29.5 ± 3 °C for only eight days (from a baseline of 27 ± 3 °C) can induce a 50:50 hatchling sex ratio (Carter et al. 2018). The timing of exposure is critical; sex ratios are not affected by temperatures early in the incubation period before gonads have developed or by temperatures late in the incubation period after gonadal fate becomes committed (Wibbels et al. 1991a, Breitenbach et al. 2020). The stages of development where gonadal fate is affected by incubation temperatures is termed the "temperature sensitive period" (TSP) (Wibbels et al. 1991a). These findings highlight how under more natural incubation conditions, warm temperatures can induce ovary formation during relatively short periods of development.

Investigations into how warm incubation temperatures induce ovary formation led to the discovery that exogenous estradiol can override the effects of male-producing temperatures on developing embryos and result in female biased sex ratios (Fleming and Crews 2001, Murdock and Wibbels 2006, Barske and Capel 2010, Capel et al. 2017, Ge et al. 2017, Ge et al. 2018). Most of these studies were conducted using constant incubation temperatures, with eggs being treated with estradiol at the beginning of the TSP (Wibbels and Crews 1995, Ge et al. 2018). While these studies were critical for identifying a role for estradiol in ovary formation, they did not reflect natural conditions with regards to embryonic exposure to maternal estrogens. Maternally derived steroids, including estrogens, are present in the yolk of many oviparous vertebrates, and these endogenous compounds have been found to affect hatchling phenotype (Bowden et al. 2000, Lovern and Wade 2001, Lovern and Wade 2003, Paitz and Bowden 2013, Carter et al. 2017). In *T. scripta* eggs, maternal estrogens exhibit seasonal variation, where late season eggs have both more estradiol (Bowden et al. 2002, Carter et al. 2017) and more estrone sulfate (Paitz and Bowden 2013). Once development begins, levels of estradiol in the yolk decline rapidly (Paitz and Bowden 2009) as estradiol is metabolized to estrone sulfate, resulting in increasing levels of estrone sulfate in the egg (Paitz and Bowden 2013). This results in eggs having low levels of estradiol and high levels of estrone sulfate after the first third of development, which is when exogenous estradiol manipulations occur in most studies (Wibbels and Crews 1995, Ge et al. 2018). Importantly, when estrone sulfate is applied either immediately after oviposition or after the first third of development, ovary development can be induced (Paitz and Bowden 2013). Further, embryos from late season eggs, which have naturally higher concentrations of maternal estradiol and estrone sulfate, are more likely to develop into female hatchlings (Bowden et al. 2000, Carter et al. 2017). These findings demonstrate that estrone sulfate is capable of inducing ovary formation in a similar manner to estradiol and that both are present as maternally derived compounds.

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While prior work has shown that both warm incubation temperatures and elevated maternal estrogens are capable of inducing ovarian development in embryos, the question of how, mechanistically, temperature and estrogens influence gonadal development remains. Much recent work has focused on a series of conserved genes that have been identified as playing a role in the sex-determining process. At present, we have a greater understanding of how temperature directly induces the male developmental pathway, a process that involves temperature sensitive epigenetic regulation of genes necessary for testis development. *Kdm6b* is a histone demethylase which activates *Dmrt1* and triggers the male sex-determining pathway (Ge et al. 2017, Ge et al. 2018). *Kdm6b* is temperature sensitive as it exhibits temperature-responsive intron retention, where an intron is retained at cool, male-producing temperatures (Deveson et al. 2017, Marroquín-Flores et al. 2021). While the functional consequence of intron retention in *Kdm6b* remains an open question, intron retention occurs at the temperature which promotes its expression, suggesting that intron retention does not impair *Kdm6b* function (Deveson et al. 2017, Marroquín-Flores et al. 2021). The temperature response of *Kdm6b* is likely regulated by CLK kinases that activate RNA binding proteins (via phosphorylation) at cool temperatures to promote intron retention (Haltenhof et al. 2020). We have previously shown that removal of the retained intron of *Kdm6b* occurs rapidly in response to a fluctuating female-producing temperature and results in an overall drop in expression (Marroquín-Flores et al. 2021). Thus, the effect of incubation temperature on the expression of *Kdm6b* appears to result from temperature-sensitive splicing that regulates epigenetic processes like histone demethylation. At warmer, female-producing temperatures, *FoxL2* and *Cyp19A1* exhibit increased expression (Govoroun, et al. 2005, Hudson et al. 2005, Pannetier et al. 2006, Batista et al. 2007, Bowden and Paitz 2021), but whether this is due to a direct effect of warm temperature or simply the absence of antagonistic genes like *Dmrt1* remains unknown. It is known that exogenous estradiol, when applied at the beginning of the TSP, also effects the expression of many of the genes involved in gonadal differentiation (Ramsey and Crews 2007, Matsumoto et al. 2013). Estradiol suppresses *Kdm6b*, *Dmrt1* and *Sox9* expression (Fleming and Crews 2001, Murdock and Wibbels 2006, Barske and Capel 2010, Ge et al. 2018) and induces *FoxL2* and *Cyp19A1* (Ramsey and Crews 2007, Matsumoto et al. 2013). Despite being abundant throughout development and capable of inducing ovary formation, the effect of estrone sulfate on the expression of genes involved in sex-determination has not been explored, nor has the effect of estrone sulfate on *Kdm6b* splicing.

While estrogens and female-producing temperatures can both induce ovarian development, tissues produced under varying feminizing conditions can differ in gene expression (Ramsey and Crews 2007, Barske and Capel 2010, Canesini et al. 2018) and in morphology (Díaz-Hernández et al. 2013, Canesini et al. 2018), suggesting that developing gonads respond differently to these two stimuli. In order to determine whether gene expression and intron retention events

are affected by female-producing conditions, we use a fluctuating incubation environment where embryos are transiently exposed to both male- and female-producing temperatures throughout development, mimicking conditions embryos may experience in wild populations (Carter et al. 2017, Carter et al. 2018, Breitenbach et al. 2020). In order to determine whether gene expression and intron retention events are affected by estrogens, we treated eggs with exogenous estrone sulfate and incubated them under male-producing temperatures. We hypothesize that the expression of sex-determining genes will differ in response to the feminizing effects of female-producing temperatures and exogenous estrone sulfate. Our approach enables us to decouple the effects of steroid metabolites and naturalistic incubation temperatures on gene expression and hatchling phenotype.

Materials and methods

T. scripta eggs were purchased separately from Concordia Turtle Farm, LLC (Jonesville, LA) in May 2020 and May 2021 for two experiments. In both years, eggs were excavated the day they were laid and shipped overnight to the laboratory. Clutch identity was not available for shipped eggs. Egg collection was approved by the Louisiana Department of Agriculture and Forestry. All hatchling work was carried out in accordance with methods approved by the Illinois State University Institutional Animal and Care Use Committee (IACUC).

Study 1: Effect of warm temperatures and estrone sulfate on gene expression

Egg Collection, Incubation, and Sampling

In 2020, 142 eggs were randomly sorted into one of three treatment groups to control for clutch identity (**Figure 1**). The first treatment was to test for the effect of a heat exposure on gene expression. The second treatment was to test for the effect of estrone sulfate on gene expression. The third treatment was a male-producing condition to serve as a control. Prior to incubation, eggs assigned to the estrone sulfate treatment were dosed topically with a 10 μl bolus of 70% ethanol containing 10 μg of estrone sulfate, all other eggs were given a 10 μl bolus of 70% ethanol as a control. All eggs were incubated for the first 24 days at a male-producing temperature of 25.0 ± 3°C (IPP 110 Plus, Memmert GmbH + Co.KG, Schwabach, Germany). After incubation day 24, the eggs in the heat exposure group were shifted to a female-producing temperature of 29.5 ± 3°C (IPP 400, Memmert GmbH + Co.KG, Schwabach, Germany) and remained at female-producing temperature for the remainder of the study. All other eggs remained at male-producing temperature.

Eggs in all treatment groups were sampled and staged (Greenbaum 2002) at four points between incubation days 24 and 44. Sampling was designed to capture gene expression across the TSP (Greenbaum stages 15-21). Incubation temperature can affect the rate of embryonic

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development (Stubbs and Mitchell 2018) thus, eggs were sampled based on developmental stage, not incubation day. Embryos were staged by a series of stage-specific indicators such as embryo size, eye disk development, and shell ridge development (Stubbs and Mitchell 2018). Embryonic gonads were dissected and placed into 1 ml of Trizol for RNA isolation (Figure 1). Fifty-nine of the eggs not used for embryonic studies continued to incubate until hatching to determine sex ratios. Approximately six weeks post-hatch, hatchlings were euthanized and sexed by macroscopic examination of the gonads.

Quantification of gene expression and intron retention

The expression of several genes involved in testis and ovary development were quantified using qPCR, including specific transcripts of *Kdm6b* that contain or lack an intron (Marroquín-Flores et al. 2021). Embryonic gonads stored in Trizol were homogenized and extracted using 2-propanol (Fisher Chemical) and chloroform (Marroquín-Flores et al. 2021). cDNA was synthesized to a standardized concentration of 40 ng/μl using a Maxima First Strand cDNA Synthesis Kit (Thermo Scientific), following the Manufacturer's protocol. PowerUp SYBR Green Master Mix (Applied Biosystems) was used for RT-qPCR to capture changes in genes expression for *Kdm6b*, *Dmrt1*, *Sox9*, *FoxL2*, and *Cyp19A1*. Three primers were used to capture the expression of *Kdm6b*. One set of primers, *Kdm6b*Ge, was non-discriminating and amplified all *Kdm6b* transcripts (Ge et al. 2018). The other two primer pairs, *Kdm6b*(+IR) and *Kdm6b*(-IR*)*, were designed to discern the intron-containing transcript and the spliced transcript, respectively (Marroquín-Flores et al. 2021). For each primer pair, gene expression was normalized using the housekeeping gene, *Gapdh*, and relative expression was calculated using the ΔΔCT method (Rao et al. 2013). RNA was also extracted from hatchling gonads, as described above, to verify that developed gonads that were morphologically determined to be ovaries or testes were expressing genes consistent with those tissues. cDNA was synthesized for 8 hatchlings from each treatment to a standard concentration of 40 ng/μl and RT-qPCR was used to determine the expression of *Dmrt1* and *FoxL2* in the gonads of hatchlings from each treatment (**Supplemental Figure 1**). All RT-qPCR reactions were performed in triplicate.

Study 2: Physiological doses of estrone sulfate and gene expression (2021)

A follow up study was conducted to examine the effects of more physiologically relevant doses of estrone sulfate on gene expression. In 2021, 87 eggs were randomly sorted into three treatment groups, where eggs were given a 10 μl bolus of 150ng estrone sulfate, 75ng estrone sulfate, or 10 μl of 70% ethanol. Similar to the prior study, eggs were placed in moist vermiculite upon receipt and placed into temperature-controlled incubators within 36 hours of being laid. All eggs were incubated under a male-producing temperature of 25.0 ± 3°C for the duration of the study. Eggs were sampled every three days, starting on incubation day 35 and ending on incubation day 47, to capture changes in gene expression at later stages of

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development (stages 18-21), when downstream testis and ovarian-typical genes become upregulated. Embryos were staged at the time of sampling and gonads were dissected and placed into 1 ml of Trizol for RNA isolation. Tissues were prepared for RT-qPCR to capture changes in *Kdm6b*, *Dmrt1*, *Sox9*, *FoxL2*, and *Cyp19A1,* as described above. No eggs were allowed to hatch in 2021.

Statistical Analysis

All statistical tests were conducted using R (The R Project for Statistical Computing, Vienna, Austria). For study 1, embryos within the four sampling intervals were grouped by stage to establish four stage ranges for analysis. Most studies that establish markers for developmental stages have been conducted at constant temperatures. Under fluctuating temperatures, many embryos exhibit traits associated with two stages, despite being sampled on the same day and under the same incubation conditions. For example, an embryo may exhibit serration of the digital plate, but no obvious digits (indicative of stage 17) while also having distinctive scutes and a lower eyelid (indicative of stage 18) (Greenbaum 2002). Additionally, other embryos sampled on the same day and under the same incubation conditions may exhibit traits consistent with a single developmental stage. To account for these differences, embryos were grouped into the following stage ranges for analysis: stages 15-16, stages 17-18, stage 19, and stages 20-21. Gene expression was analyzed using a generalized linear model, which allowed us to specify distributions for data that were not normally distributed. For all analyses, data were transformed to better fit the assumptions of the model. To determine the effects of the treatment on gene expression, treatment and stage of development were used as fixed effects and estimated marginal means (R Package: emmeans) were used for post hoc comparisons among groups. Two individuals in the estrone sulfate-treated group and two individuals in the female-producing temperature group were underdeveloped at the time of sampling and were removed from the analysis. For study 2, gene expression was analyzed using a generalized linear model and data were transformed to better fit the assumptions of the model. Treatment (male-producing temperature, 75ng estrone sulfate, or 150ng estrone sulfate) and sampling day were used as fixed effects and estimated marginal means were used for post hoc comparisons.

Results

Study 1: Effect of warm temperatures and estrone sulfate on gene expression

Both warm temperatures and estrone sulfate affected patterns of gene expression but their specific effects differed. As previously demonstrated, *Kdm6b* expression was significantly lower when embryos experienced warm temperatures (Marroquín-Flores et al. 2021), but embryos treated with estrone sulfate did not exhibit reduced *Kdm6b* expression. When examining non-

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discriminate *Kdm6b* expression using the primers from Ge et al. 2018, we found that temperature had a significant effect on *Kdm6b* expression (X^2 = 14.3733, DF = 2, p < 0.001, **Figure 2a**). Embryos that experienced the shift to female-producing temperature had significantly lower expression when compared to embryos at male-producing temperature (p < 0.01) and those in the estrone sulfate treatment (p < 0.01, **Figure 2a**). There was also some variability in non-discriminate *Kdm6b* expression between stages (X^2 = 9.3689, DF = 2, p < 0.05, **Figure 2a**), but after correction for multiple contrasts, the pattern was only marginally significant. For the intron-containing transcript of *Kdm6b* (*Kdm6b*(+IR)), we found an interaction effect between the treatment and stage of development (X^2 = 28.263, DF = 6, p < 0.001). *Kdm6b*(+IR) was not responsive to the exogenous estrone sulfate but was responsive to incubation temperature by stages 17-18 (**Figure 2b**). Embryos that experienced the shift to female-producing temperature had significantly lower expression of the *Kdm6b*(+IR) transcript compared to embryos at male-producing temperature (p < 0.001) and embryos in the estrone sulfate treatment (p < 0.001) in stages 17-21 (**Figure 2b**). *Kdm6b*(-IR) did not respond to the treatment (X^2 = 1.4782, DF = 2, p = 0.4774) and did not change in expression across stages of development (X^2 = 6.3443, DF = 3, p = 0.096; **Figure 2c**).

Unlike *Kdm6b*, all other genes involved in gonadal differentiation responded to both the female-producing temperature treatment and treatment with estrone sulfate. In *Dmrt1*, we identified a significant interaction effect between the treatment and stage of development (X^2 = 15.432, DF = 6, p < 0.05, **Figure 3**). Across all stages of development, *Dmrt1* expression was significantly lower in estrone sulfate treated embryos when compared to embryos in the male-producing temperature treatment (p < 0.001) and embryos in the female-producing temperature treatment (p < 0.001). *Dmrt1* expression was significantly reduced in embryos in the female-producing temperature treatment compared to those in the male-producing temperature treatment by stages 20-21 of development (p < 0.001). *Dmrt1* expression also increased in male-producing temperature treated embryos between stages 15-16 and stages 17-18 (p < 0.001). In *Sox9,* we also identified a significant interaction effect between treatment and stage of development (X^2 = 59.618, DF = 6, p < 0.001, **Supplemental Figure 2**). When compared to male-producing temperature, *Sox9* expression was significantly lower in response to female-producing temperature (p < 0.001) by stages 20-21 and in response to the estrone sulfate treatment (p < 0.001) by stages 19-21. *Sox9* expression also increased in embryos in the male-producing temperature treatment between stage 19 and stages 20-21 of development (p < 0.001).

FoxL2 expression was affected by both the treatment and developmental stage (X^2 = 17.596, DF = 6, p < 0.01, **Supplemental Figure 3a**). *FoxL2* expression was significantly higher in response to female-producing temperature (p < 0.001) and treatment with estrone sulfate (p < 0.01) by

stages 20-21 of development, when compared to embryos incubated under male-producing temperature. *FoxL2* expression also increased in embryos in the female-producing temperature treatment between stage 19 and stages 20-21 of development ($p < 0.0001$). *Cyp19A1* was affected by both the treatment and developmental stage ($X^2 = 17.959$, DF = 6, $p < 0.01$, **Supplemental Figure 3b**), where expression was significantly higher in response to female-producing temperature ($p < 0.001$) and treatment with estrone sulfate ($p < 0.001$) by stages 20-21 of development, compared to embryos incubated under male-producing temperature. As evidence that our treatments resulted in the expected sex ratios, 94% of the embryos exposed to warm temperatures developed ovaries (15/16), 100% of the embryos treated with estrone sulfate developed ovaries (18/18), and 0% of the embryos incubated under cool temperatures developed ovaries (0/25).

Study 2: Physiological doses of estrone sulfate and gene expression (2021)
When applied at lower doses meant to mimic levels found in late season eggs, estrone sulfate did not affect gene expression. There was no effect of the treatment on non-discriminate *Kdm6b* expression ($X^2 = 1.41114$, DF = 2, $p = 0.4938$, **Supplemental Figure 4a**). When isolating the intron containing transcript of *Kdm6b*, we found no effect of the treatment on *Kdm6b*(+IR) expression ($X^2 = 0.4893$, DF = 2, $p = 0.783$, **Supplemental Figure 4b**). In the *Kdm6b* transcript lacking the intron, we saw a small effect of the treatment on *Kdm6b*(-IR) expression, but the effect was only marginally significant after correction for multiple contrasts ($X^2 = 8.0162$, DF = 2, $p < 0.05$, **Supplemental Figure 4c**).

Dmrt1 expression was not affected by the treatment ($X^2 = 1.001$, DF = 2, $p = 0.6063$), but did change across the sampling period ($X^2 = 76.363$, DF = 4, $p < 0.001$), where *Dmrt1* expression increased between incubation day 38 and day 41 ($p < 0.001$, **Supplemental Figure 5a**). Similarly, *Sox9* was not affected by the treatment ($X^2 = 1.018$, DF = 2, $p = 0.6012$), but was by sampling period ($X^2 = 53.054$, DF = 4, $p < 0.001$), where *Sox9* expression increased between incubation day 38 and day 41 ($p < 0.001$, **Supplemental Figure 5b**). *FoxL2* expression was also not affected by the treatment ($X^2 = 1.7296$, DF = 2, $p = 0.4211$) or sampling period ($X^2 = 1.8090$, DF = 4, $p = 0.7708$; **Supplemental Figure 6a**). *Cyp19A1* expression was not affected by the treatment ($X^2 = 2.8355$, DF = 2, $p = 0.2423$, **Supplemental Figure 6b**), but was by incubation day ($X^2 = 2.8355$, DF = 4, $p < 0.001$). However, the effect of incubation day was eliminated when corrected for multiple comparisons.

Discussion

In this study, we characterize the expression of sex-determining genes in response to naturalistic incubation temperatures and an abundant estrogen metabolite to decouple the effects of thermal and hormonal stimuli on gene expression and hatchling phenotype. We find

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that patterns of gene expression and intron retention differ in response to the feminizing effects of warm temperatures and exogenous hormones. When applied at doses that mimic maternally derived levels, we find that estrone sulfate, in isolation, does not induce similar changes in expression. Our findings highlight the complexity of processes organizing development and the necessity of using experimental designs that mimic environmental conditions.

Warm temperatures and estrone sulfate have different effects on intron retention

Temperature-sensitive intron retention has recently been identified as an important regulator of gene expression in species with TSD (Deveson et al. 2017, Georges and Holleley 2018). We used multiple primers to characterize changes in *Kdm6b* expression and intron retention in response to temperature and hormone manipulations. We found that both warm temperatures and estrone sulfate induced ovary development, but the mechanisms underlying this induction differ. We show that exposure to female-producing temperatures can largely eliminate intron retention in *Kdm6b* during the stages of development when sex is determined (**Figure 2b**) and that this decrease in intron retention corresponds to downregulation of *Kdm6b* (**Figure 2a**). However, estrone sulfate did not have an effect on *Kdm6b* expression or intron retention (**Figure 2**), which is notable because other work has demonstrated that *Kdm6b* expression is sensitive to the effects of estradiol (Ge et al. 2018). Several factors could contribute to the differences between the effects of estradiol and estrone sulfate on *Kdm6b*. Estrone and estradiol are the products of the aromatizable androgens androstenedione and testosterone, respectively (Crews et al. 1996). Estrone sulfate is an estrone metabolite that can influence sexual development and is abundant in the yolk of *T. scripta* eggs during the thermal sensitive period of development (Paitz and Bowden 2013), but the mechanism by which estrone sulfate induces femininization in *T. scripta* embryos is still unknown. It has been proposed that sulfonation enables uptake of estrogen sulfates by the embryo, which can then be converted back into estrogens to induce developmental changes (Pasqualini et al. 1986, Paitz et al. 2012, Paitz and Bowden 2013, Paitz et al. 2017), but this has not been experimentally tested. Our current and prior findings suggest that *Kdm6b* responds to changes in incubation temperature (Marroquín-Flores et al. 2021). Estrone sulfate, however, does not affect expression or splicing of the *Kdm6b* transcript.

Warm temperatures and estrone sulfate have similar effects on downstream gene expression

Unlike *Kdm6b*, *Dmrt1* and *Sox9* exhibited a similar response to warm temperatures and estrone sulfate. We found that *Dmrt1* was downregulated in response to both estrone sulfate and female-producing temperatures. *Dmrt1* exhibited a particularly strong response to estrone sulfate, where expression was lower in response to estrone sulfate than in response to female-producing temperatures (**Figure 3**). In response to male-producing temperatures, *Dmrt1*

increased in expression at later stages of development, consistent with canalization to the male pathway (**Figure 3**; Shoemaker-Daly et al. 2010, Ge et al. 2017, Breitenbach et al. 2020). *Sox9* exhibited similar responses to the female-producing temperature and estrone sulfate treatments, which supports prior work on the effect of temperature and estrogens on *Sox9* expression (**Supplemental Figure 2**; Shoemaker-Daly et al. 2010, Matsumoto et al. 2013, Breitenbach et al. 2020). When we looked at genes involved in ovarian development, both *FoxL2* and *Cyp19* responded to the estrone sulfate and female-producing temperature treatments (**Supplemental Figure 3**). While prior work has demonstrated that *Foxl2* and *Cyp19* expression is sensitive to the effects of estradiol (Matsumoto and Crews 2012, Matsumoto et al. 2013), we show that both *FoxL2* and *Cyp19* are also sensitive to estrone sulfate. Estrone sulfate induced the expression of *FoxL2* and *Cyp19* in eggs incubated under male-producing temperatures to levels similar to eggs incubated under female-producing temperatures. Similar to prior work, our fluctuating warm temperature treatment also resulted in increased expression of *FoxL2* and *Cyp19* at later stages of development (Willingham et al. 2000, Murdock and Wibbels 2003, Matsumoto and Crews 2012, Breitenbach et al. 2020). These findings suggest that warm temperature exposure and estrone sulfate can both induce ovarian differentiation by impacting downstream gene expression.

Estrone sulfate as a mediator of maternal effects on sex ratios

While our data show that high doses of estrone sulfate can alter gene expression and induce ovarian differentiation, doses that more closely mimic natural concentrations of estrone sulfate did not affect the expression of any of the genes studied. *T. scripta* eggs contain a variety of maternal estrogens, including both estradiol and estrone sulfate (Paitz and Bowden 2010, Paitz and Bowden 2013, Carter et al. 2018). Temperature and estrogens exhibit a synergism, where late season eggs have higher concentrations of estradiol and estrone sulfate and are more likely to produce female hatchlings at intermediate temperatures (Wibbels et al. 1991b, Paitz and Bowden 2010, Paitz and Bowden 2013, Carter et al. 2018). In our second study, we evaluated the effects of estrone sulfate by itself at physiologically relevant concentrations and did not see an effect on gene expression. It is worth noting that the endogenous levels of estrone sulfate in treated eggs were not quantified, and it is possible that the amount of estrone sulfate applied exogenously may represent only a fraction of what the embryo received. It is also possible that lower doses of estrone sulfate may have reduced potency and be less likely to induce changes at the molecular level (Crews et al. 1991, Crews 1996, Sheenen et al. 1999). Additionally, exogenous estrogens have different dosage effects when they are applied individually and in combination, even when used at low concentrations (Bergeron et al. 1999). Applied exogenously, 150ng of estrone can induce female development in 30% of *T. scripta* embryos, but when applied in conjunction with 75ng of estradiol or 10ng of estriol, these estrogens can induce 40% and 50% of embryos to develop as female, respectively (Bergeron et al. 1999). It is

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likely that the seasonal shift in sex ratios are mediated by a synergism between multiple maternally derived estrogens, rather than in response to a single estrogen.

In this study, we show that estrone sulfate can impact embryonic development by effecting the expression of sex-determining genes. These results are further supported by our hatchling data, which show that both female-producing temperatures and estrone sulfate can lead to gonads that are morphologically female and that hatchling gonads exhibit patterns of gene expression consistent with ovarian differentiation (**Supplemental Figure 1**). Few studies have examined gene expression in response to maternal estrogens or in response to naturalistic incubation temperatures, and to the best of our knowledge, this study is the first to characterize the expression of sex-determining genes in response to an estrogen metabolite. In *T*. *scripta*, estradiol is present at the time of oviposition but declines prior to gonadal differentiation and is converted into various estrogen sulfates (Paitz and Bowden 2009, Paitz et al. 2011, Paitz et al. 2012, Paitz et al. 2013). The decline of estradiol early in development corresponds to increases in estrone sulfate during the thermal sensitive period, which leads to development of ovaries (Paitz and Bowden 2009, Paitz et al. 2012, Paitz et al. 2013). While the exact mechanism has not yet been identified, steroid sulfates appear to play an important role in moderating the effects of maternal steroids on embryonic development (Paitz and Bowden 2008, Paitz et al. 2013). Our data further support this by showing that estrone sulfate can elicit its effects on the embryo by impacting the expression of sex-determining genes in ways that are similar to the effects of estradiol.

Taken together, our findings suggest that development can be differentially regulated by biotic and abiotic conditions. We find that *Kdm6b* responds directly to changes in incubation temperature through temperature-responsive intron retention, which enables *Kdm6b to* regulate the expression of *Dmrt1* via epigenetic modification. In both *T. scripta* and the American Alligator (*A. mississippiensis*), *Kdm6b* exhibits temperature-responsive intron retention, but intron retention in *Kdm6b* leads to different phenotypic outcomes in these species (Deveson et al. 2018). Our findings may provide insight into mechanisms regulating gonadal development, given our results that estrogens can also regulate *Dmrt1,* independent of the effects of *Kdm6b*. These results show that *Dmrt1* can be impacted by both changes in incubation temperature and maternal steroids, which may explain seasonal variability in hatchling sex ratios (Bowden et al. 2000, Carter et al 2017). Our findings highlight the value of using environmentally relevant incubation and hormone treatments. While the use of naturalistic laboratory incubations have become more common, TSD is most frequently studied using constant incubation temperatures. However, we know that incubating embryos experience a range of temperatures that regularly fluctuate between male and female conditions during development (Carter et al. 2017). The range of temperatures that embryos

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experience and the duration of exposure can differentially impact phenotypic characteristics such as body size, immune function, gene expression, and sex (Les et al. 2007, Les et al. 2009, Bowden et al. 2014, Carter et al. 2017, Carter et al. 2017, Breitenbach et al. 2020). The interplay between incubation temperature and maternal estrogens (Wibbels et al. 1991b, Crews et al. 1994, Bergeron et al. 1999) underlies the complexity of development in species with TSD. Investigations using treatments which more closely mimic environmental conditions allow us to more accurately characterize the mechanisms organizing gonadal development in species with TSD and should be the focus of future work.

List of symbols and abbreviations

Cyp19A1 - Cytochrome P450 Family 19 Subfamily A Member 1
Dmrt1 - Doublesex and mab-3 related transcription factor 1
FoxL2 - Forkhead box protein L2
Gapdh - glyceraldehyde-3-phosphate dehydrogenase
Kdm6b - Lysine demethylase 6b
Sox9 - SRY-Box Transcription Factor 9
TSP - Temperature-sensitive period

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Competing interests. We declare we have no competing interests.

Author contributions. R.A.M.-F. conceived of the study, carried out the molecular laboratory work and sampling, carried out statistical analyses and drafted the manuscript; R.T.P. and R.M.B participated in the design of the study and critically revised the manuscript. All authors gave final approval for publication and agree to be held accountable for the work performed herein.

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Data availability. The supporting data, materials, and code can be accessed in the Dryad Digital Repository: DOI https://doi.org/10.5061/dryad.4qrfj6qcc.

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Figures

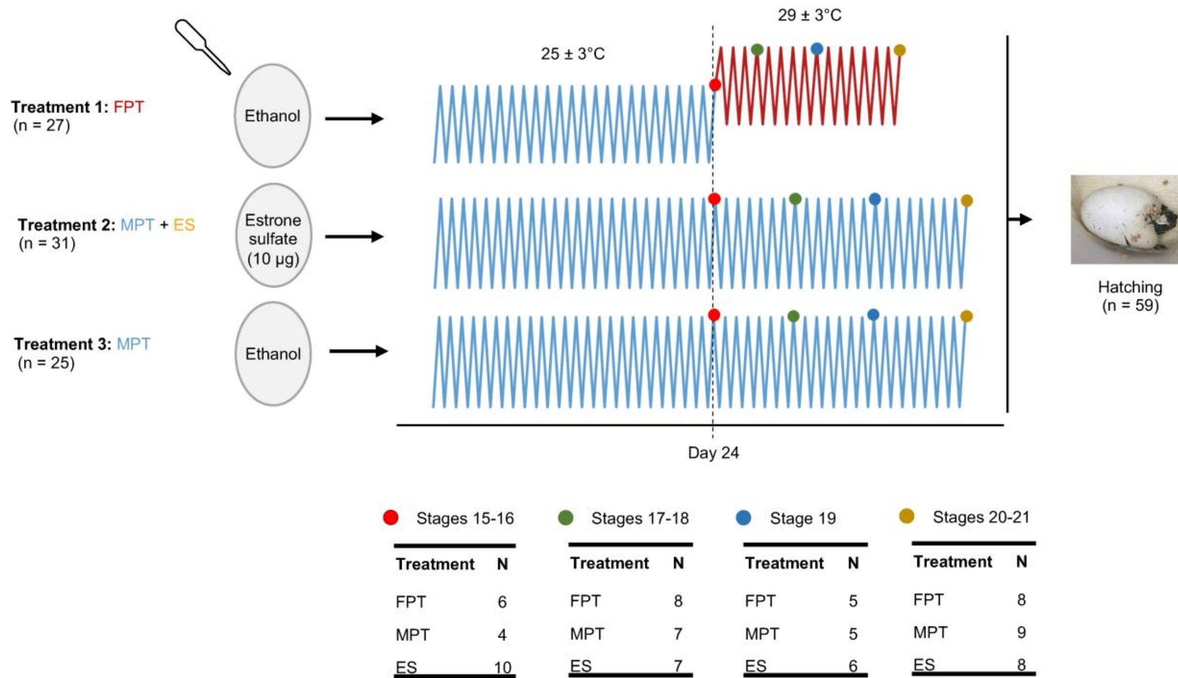

Figure 1. Incubation and hormone treatments applied to eggs for the sex-reversal experiment conducted in 2020. Each replicate represents gonads collected from a single embryo, where the number of embryos (N) per treatment are outlined in the table below each stage of development.

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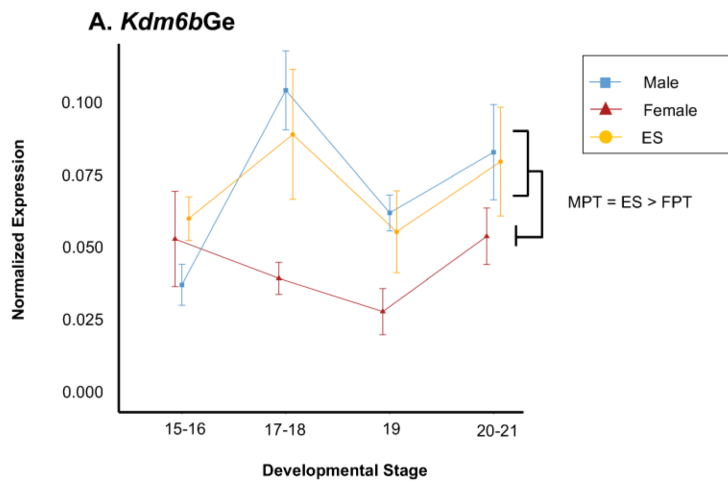

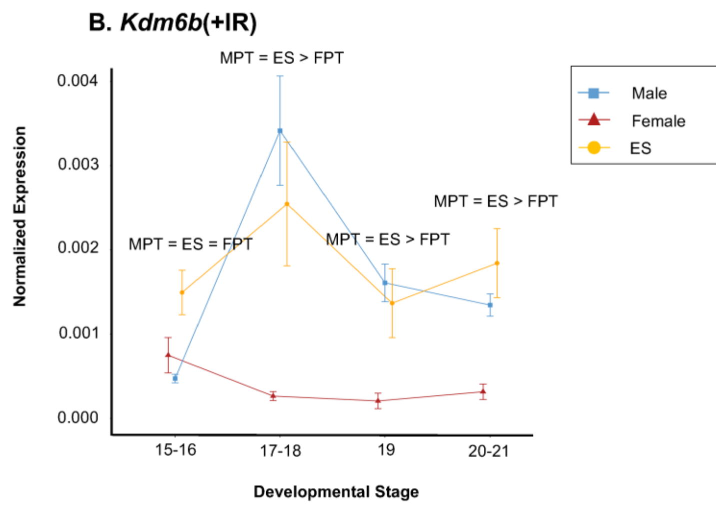

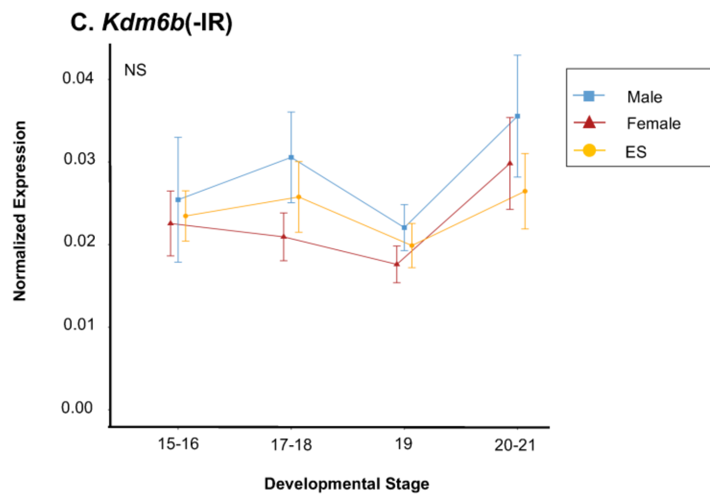

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Figure 2. *Kdm6b* expression (mean ± s.e.m.) in response to the hormone and temperature treatments. *Kdm6b* expression was measured using primers designed to capture all *Kdm6b* transcripts (A), the intron containing *Kdm6b* transcript (B), and the *Kdm6b* transcript lacking the intron (C). Post-hoc comparisons were conducted for significant effects and corrected using the sequential Bonferroni adjustment. The relationship of significant effects between male (MPT), female (FPT), and estrone sulfate (ES) are denoted using greater than (>), less than (<), or equal to (=) symbols.

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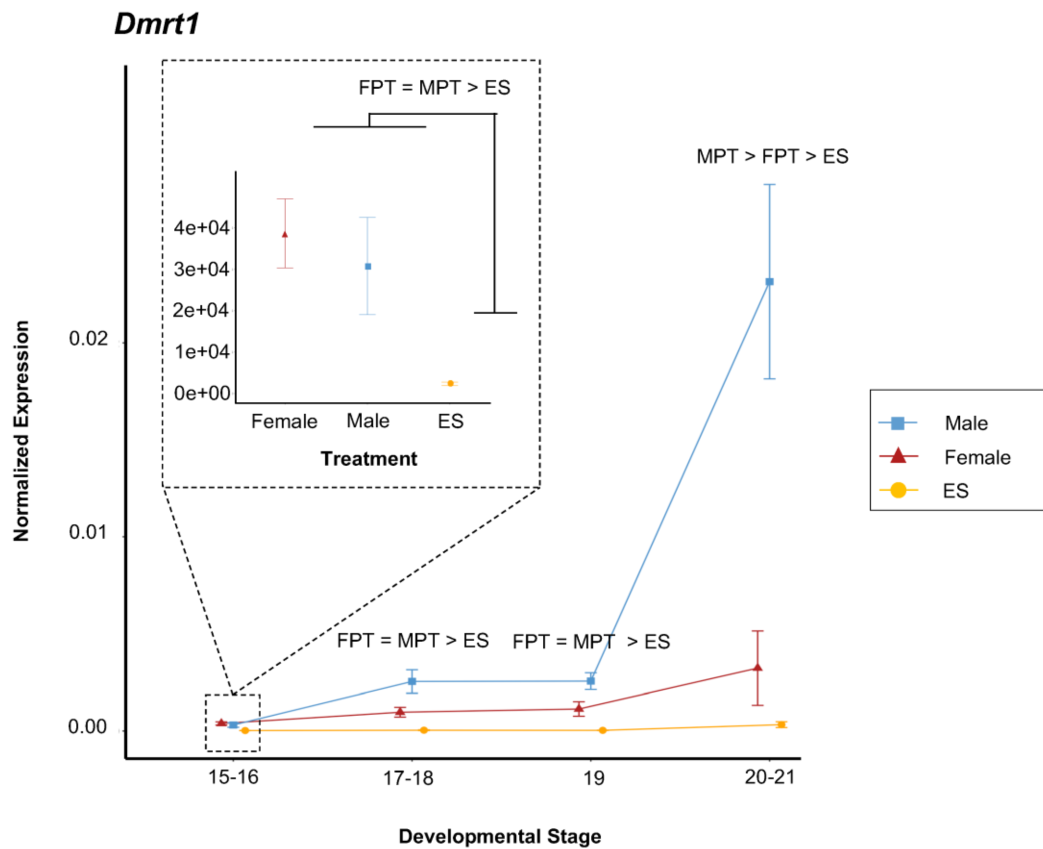

Figure 3. The expression of *Dmrt1* (mean ± s.e.m.) in response to the temperature and hormone treatments. Post-hoc comparisons were conducted for significant effects and corrected using the sequential Bonferroni adjustment. The relationship of significant effects between male (MPT), female (FPT), and estrone sulfate (ES) are denoted using greater than (>), less than (<), or equal to (=) symbols.

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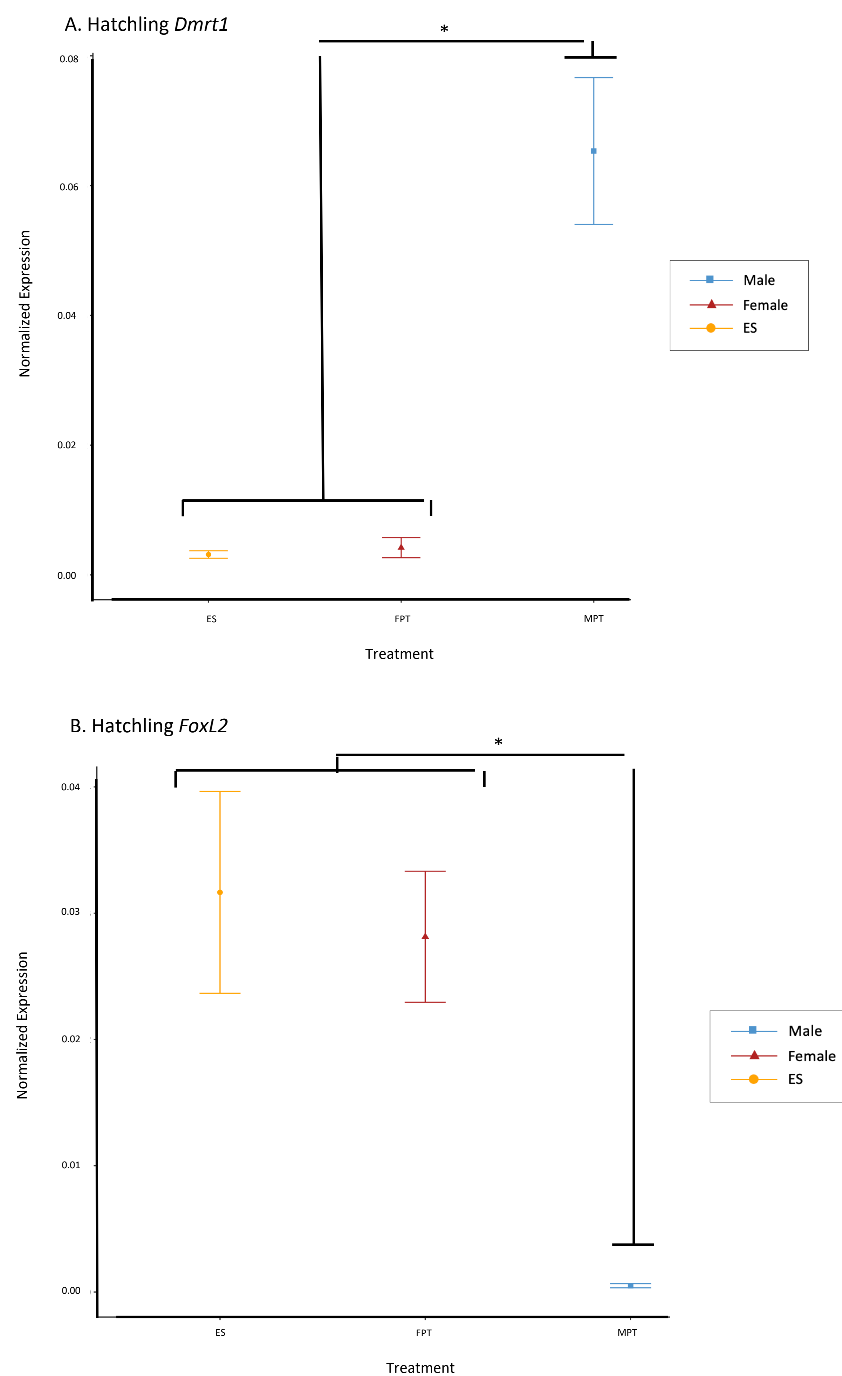

Fig. S1. The expression (mean ± s.e.m.) of *Dmrt1* (A) and *FoxL2* (B) in embryos allowed to hatch. In hatchlings incubated under female-producing temperature and exposed to estrone sulfate, the expression of *FoxL2* (X^2 = 177.49, DF = 2, $p < 0.001$) and *Dmrt1* (X^2 = 77.404, DF = 2, $p < 0.001$) was significantly different than for hatchings incubated under male-producing temperature. Hatchlings treated with estrone sulfate ($p < 0.001$) and incubated under female-producing temperature ($p < 0.001$) had significantly higher *FoxL2* expression compared to eggs incubated under male-producing temperature. Similarly, hatchlings treated with estrone sulfate ($p < 0.001$) and incubated under female-producing temperature ($p < 0.001$) had significantly lower *Dmtrt1* expression compared to eggs incubated under male-producing temperature.

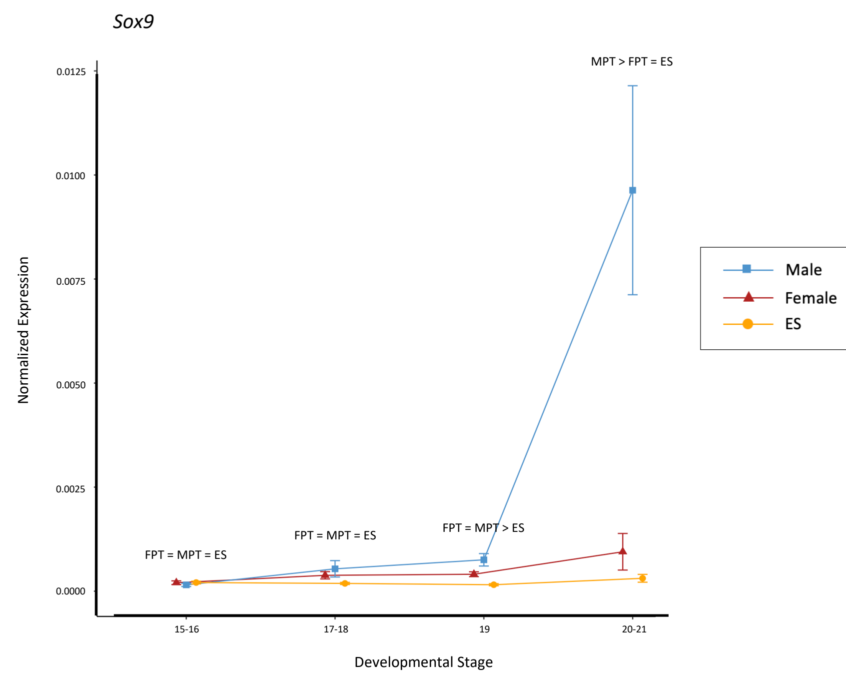

Fig. S2. The expression (mean ± s.e.m.) of *Sox9* in response to temperature and hormone treatments. Post-hoc comparisons were conducted for significant effects and corrected using the sequential Bonferroni adjustment. The relationship of significant effects between male (MPT), female (FPT), and estrone sulfate (ES) are denoted using greater than (>), less than (<), or equal to (=) symbols.

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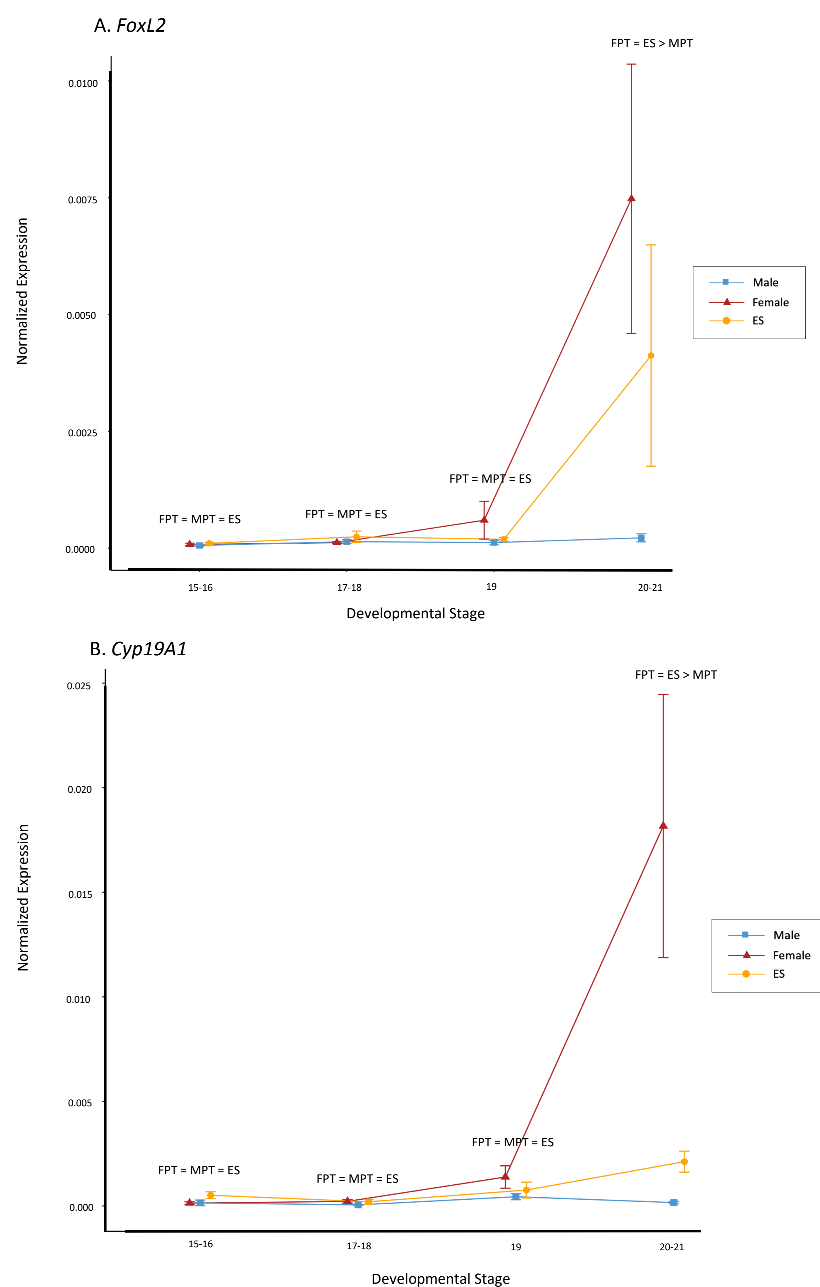

Fig. S3. The expression (mean ± s.e.m.) of the female-produce genes *FoxL2* (A) and *Cyp19A1* (B) in response to the temperature and hormone treatments. Post-hoc comparisons were conducted for significant effects and corrected using the sequential Bonferroni adjustment. The relationship of significant effects between male (MPT), female (FPT), and estrone sulfate (ES) are denoted using greater than (>), less than (<), or equal to (=) symbols.

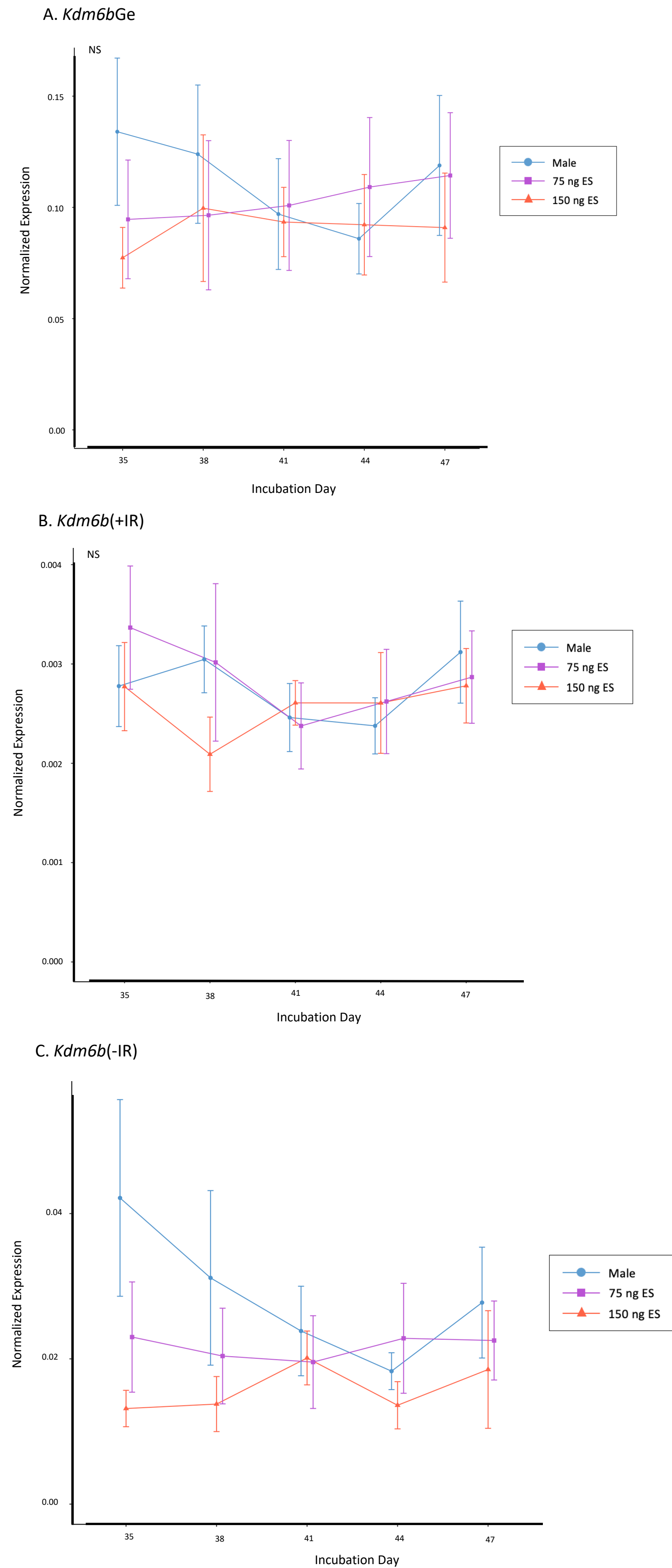

Fig. S4. *Kdm6b* expression (mean ± s.e.m.) in response to the hormone temperature treatments. Expression was captured using primers to measure overall *Kdm6b* expression (A), the intron containing *Kdm6b* transcript (B), and the *Kdm6b* transcript lacking the intron.

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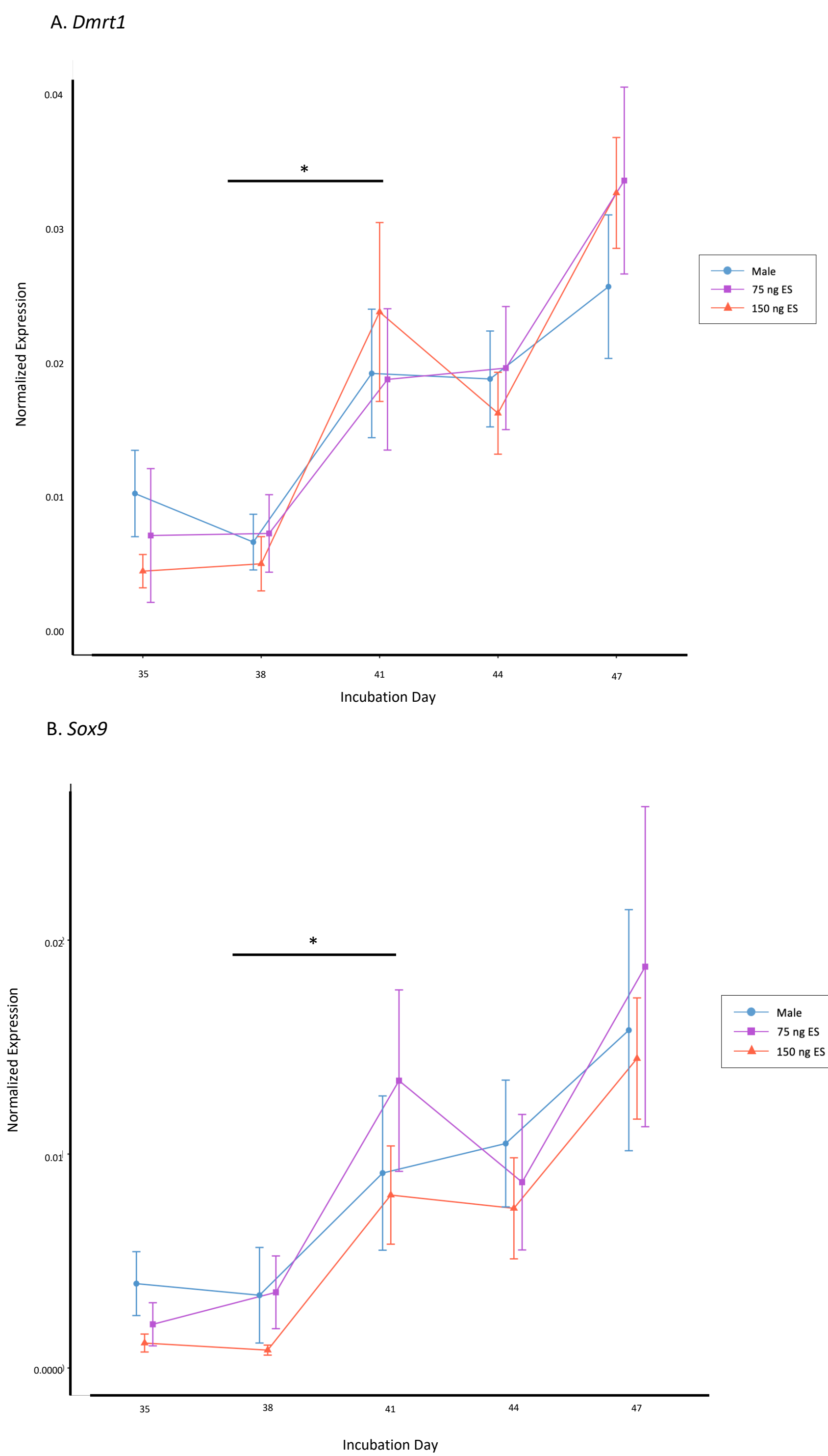

Fig. S5. The expression (mean ± s.e.m.) of the male-produce genes *Dmrt1* (A) and *Sox9* (B) in response to the temperature treatment and treatment with low concentrations of estrone sulfate. Post-hoc comparisons were conducted for significant effects and corrected using the sequential Bonferroni adjustment. Significant effects are denoted with an asterisk (*).

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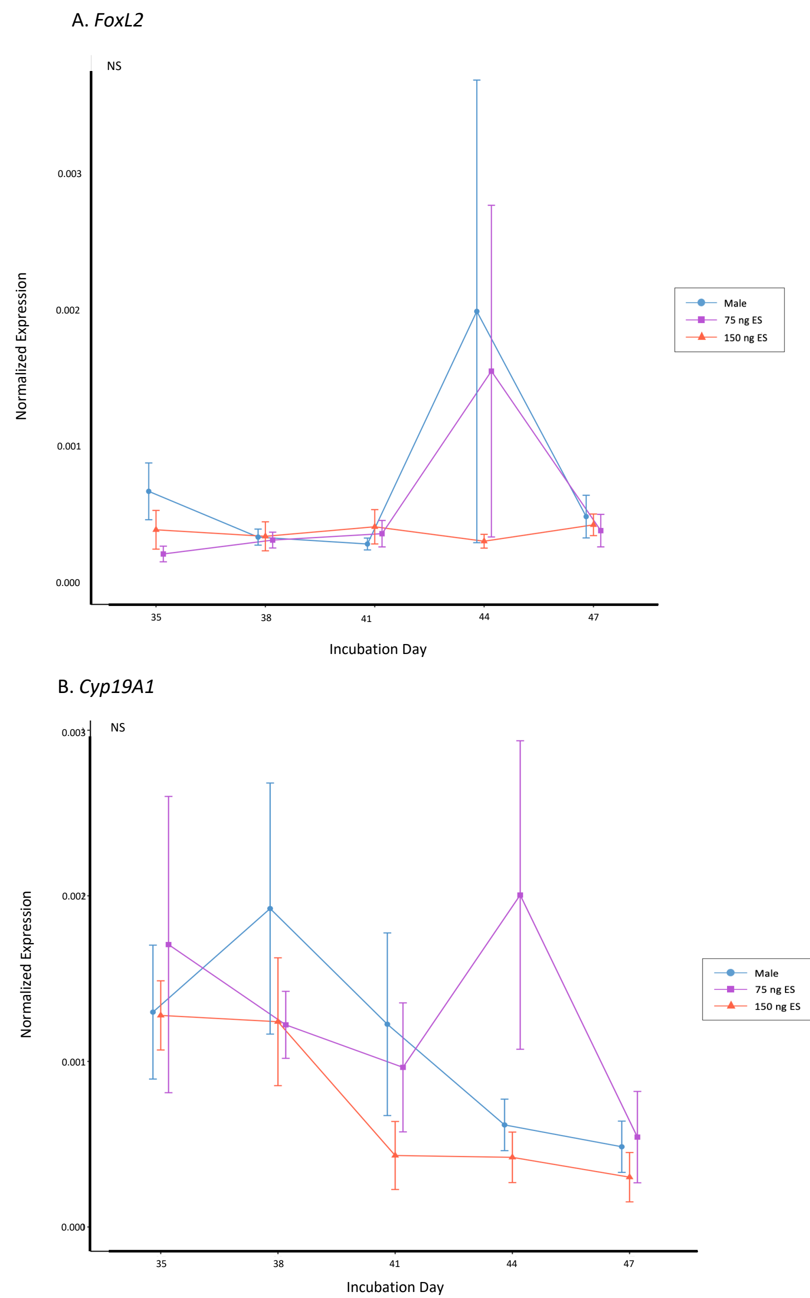

Fig. S6. The expression (mean ± s.e.m.) of the female-produce genes *FoxL2* (A) and *Cyp19A1* (B) in response to the temperature treatment and treatment with low concentrations of estrone sulfate.