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Exogenous application of estradiol to eggs unexpectedly induces male development in two turtle species with temperature-dependent sex determination

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ABSTRACT

Steroid hormones affect sex determination in a variety of vertebrates. The feminizing effects of exposure to estradiol and the masculinizing effects of aromatase inhibition during development are well established in a broad range of vertebrate taxa, but paradoxical findings are occasionally reported. Four independent experiments were conducted on two turtle species with temperature-dependent sex determination (*Chrysemys picta* and *Chelydra serpentina*) to quantify the effects of egg incubation temperature, estradiol, and an aromatase inhibitor on offspring sex ratios. As expected, the warmer incubation temperatures induced female development and the cooler temperatures produced primarily males. However, application of an aromatase inhibitor had no effect on offspring sex ratios, and exogenous applications of estradiol to eggs produced male offspring across all incubation temperatures. These unexpected results were remarkably consistent across all four experiments and both study species. Elevated concentrations of estradiol could interact with androgen receptors or inhibit aromatase expression, which might result in relatively high testosterone concentrations that lead to testis development. These findings add to a short list of studies that report paradoxical effects of steroid hormones, which addresses the need for a more comprehensive understanding of the role of sex steroids in sexual development.

1. Introduction

Whether an embryo develops into a male or female has important consequences on its morphology, physiology, behavior, and ultimately its fitness. Indeed, males and females differ in most aspects of their biology, and this observation has led to numerous questions about the underlying mechanisms that determine the trajectory of sexual development (Mittwoch, 1996, 2000). Research on this topic has revealed a remarkable diversity of sex-determining mechanisms (SDMs), ranging from SDMs completely dependent upon genetic factors that reside on sex chromosomes (genotypic sex determination; GSD) to those that are primarily dependent upon environmental factors experienced during embryogenesis (environmental sex determination; ESD). Even within SDMs, numerous factors can interact to impact the sexual development of an individual (Kozielska et al., 2006; Radder et al., 2009; Warner et al., 2013). For example, in species that exhibit temperature-dependent sex determination (TSD, a form of ESD where temperature during development determines offspring sex), sex steroids can also influence the differentiation of gonads in ways that permanently affect an individual's sex (Wibbels and Crews, 1994; Bowden et al., 2000; Elf, 2004; Warner et al., 2009).

The hormonal environment surrounding embryos affects whether gonads differentiate into testes or ovaries, which has been repeatedly confirmed by experimental manipulation of the hormonal environment of developing embryos (e.g., Wibbels and Crews, 1992; Crews, 1996; Bowden et al., 2000). For example, application of 17β estradiol to eggs almost always feminizes gonads, even at male-producing temperatures under TSD and in species that have sex chromosomes (Freedberg et al., 2006). Although this pattern is broadly consistent across taxa, estrogen-induction of male development has been reported (Hayes, 1998; Janes et al., 2007). In contrast, inducing male development with an application of testosterone to eggs has received less success, particularly when

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eggs are incubated at female-producing temperatures (Wibbels and Crews, 1992). This is likely because female-producing temperatures increase aromatase activity, thereby converting testosterone into estradiol (Wibbels and Crews, 1992; Rhen and Lang, 1994), and this hypothesis is supported by the induction of male sex determination using non-aromatizable androgen treatments (Wibbels et al., 1992). By converting androgens to estrogens, aromatase plays a key role in sex determination. This is exemplified by studies that block aromatase activity, which facilitates male development even under female-producing temperatures (reviewed in Warner, 2011). As a result, sex-reversed males have similar gonad morphology to normal males, exhibit normal male behaviors, and are capable of spermatogenesis as adults (Elbrecht and Smith, 1992; Piferrer et al., 1994; Wennestrom and Crews, 1995; Chardard and Dournon, 1999; Shine et al., 2007; Warner and Shine, 2008; Warner et al., 2010).

These hormonal manipulations not only have provided an important perspective on the role of steroids in sex determination, but also have yielded novel insights into the sex-specific effects of incubation temperature in many reptiles with TSD. For example, because incubation temperature and sex are naturally confounded under TSD, these sex manipulation approaches are useful in decoupling these factors to understand their independent effects on offspring fitness. This approach has been used on the common snapping turtle (*Chelydra serpentina*) and demonstrated that variation in offspring growth is influenced by incubation temperature and is not due to an innate difference between the sexes (Rhen and Lang, 1995). These hormonal manipulations have also been important in experimentally testing the leading model for the adaptive significance of TSD (Charnov and Bull, 1977), which states that incubation temperature has a differential effect on male and female fitness (i.e., TSD enables each sex to develop at its optimal incubation temperature). Indeed, by applying an aromatase inhibitor to eggs of the jacky dragon (*Amphibolurus muricatus*), Warner and Shine (2005, 2008) showed that males produced from eggs incubated at normal male temperatures have greater reproductive success than sex-reversed males produced under female temperatures (and likewise for females).

Although these hormonal manipulations have provided insights into the underlying physiological mechanisms of TSD and its adaptive significance, the generality of such endocrinological effects across species with TSD is still poorly studied. This is a valid concern because the pathways involved in sex determination under TSD could vary among taxa (Uller et al., 2007; Uller and Helantera, 2011), and therefore the impact of hormone application may diverge depending on a variety of factors (e.g., timing of application, genetic variation). In addition, different doses of chemicals to eggs could even have opposing effects on sexual differentiation (Hayes, 1998; Mori et al., 1998; Parmigiani et al., 2000), resulting in non-intuitive patterns. For example, prenatal exposure to small amounts of estradiol increases prostate size in mice, and excessive androgens can feminize fish (Mori et al., 1998; Devlin and Nagahama, 2002). In the frog *Rana pipiens*, estradiol can induce female development as expected, but relatively high doses result in 100% males (Richards and Nace, 1978; Hayes, 1998).

In this study, the effects of exogenous estradiol and an aromatase inhibitor on sex determination were quantified in two species with TSD, the painted turtle (*Chrysemys picta*) and the common snapping turtle (*C. serpentina*). Although both species have been studied extensively, hormone manipulation experiments have only previously been performed on *C. serpentina* and not on *C. picta*. This study was replicated four times (with independent, but similar experimental designs) due to paradoxical findings in the first experiment, and results demonstrate across all four studies that estradiol consistently induces male gonad development in offspring even at normally female-producing incubation temperatures.

2. Materials and methods

2.1. Study species

This work focused on two common turtle species that have been used extensively for research on TSD. The painted turtle (*C. picta*) ranges across most of the United States and southern Canada. At our study site (Thomson, Illinois), relatively warm constant incubation temperatures (>29 °C) produce female offspring and cool incubation temperatures (<27 °C) produce males. The pivotal temperature that produces a balanced sex ratio is about 28 °C (Paukstis and Janzen, 1990). The effects of exogenous application of estradiol or aromatase inhibitors have not previously been evaluated in this species.

The common snapping turtle (*C. serpentina*) also is distributed throughout much of the United States and southern Canada, as well as Central America and northern parts of South America. The effect of constant incubation temperature on primary sex ratios in *C. serpentina* differs considerably from that of *C. picta*, in that both cool and warm constant temperatures produce female offspring and intermediate temperatures produce males. At the study site in Illinois, constant temperatures <21 °C and >29 °C produce mostly females, and constant incubation at 24.5–26 °C produces males. Pivotal temperatures occur around 21.5 °C and 27.5 °C (Janzen, 2008). For this species, studies of a Minnesota population found that exogenous application of estradiol to eggs induces female development, whereas application of an aromatase inhibitor produces males (Rhen and Lang, 1994, 1995).

2.2. Collection of eggs

Four independent experiments were designed to evaluate the effect of exogenous applications of estradiol and an aromatase inhibitor on sex determination; these studies took place in 2008, 2010, and 2011 for *C. picta*, and in 2009 only for *C. serpentina*. For all years, known nesting areas were regularly searched for actively nesting females. When nesting females were observed, their location was marked and their eggs were retrieved later that day (typically within 3 h of oviposition). However, in 2008, 23 of the 44 *C. picta* clutches were obtained from females (captured during terrestrial nesting forays) by inducing oviposition with an injection of oxytocin; in later years all females were allowed to nest naturally. Nests were carefully excavated and single clutches were put into individual containers with moist sand. Eggs were gently cleaned of soil or sand, and then immediately weighed and individually labeled with a Sharpie marker. All eggs were kept cool prior to transport to Iowa State University where they were placed into incubation temperature treatments within 1–9 days of oviposition.

2.3. Experimental designs

Experimental designs were similar among years, but differed in some key aspects (Table 1). All experiments had a full factorial design to evaluate the effect of egg incubation temperature, chemical treatment (estradiol, aromatase inhibitor, control), and their interaction on offspring sex ratios.

2.3.1. C. picta

In 2008, eggs were randomly assigned to a position in a 4×5 matrix among 24 plastic shoeboxes and were half buried in moist vermiculite (-150 kPa). The shoeboxes were placed in one of three incubators (8 shoeboxes in each incubator) set at (1) a warm temperature (30 °C) known to produce females, (2) an intermediate temperature (28 °C) that produces a balanced sex ratio, and (3) a cool temperature (26 °C) that produces male offspring (Paukstis

Table 1
Details of four experiments designed to assess the impacts of incubation temperature and hormone manipulation on offspring sex ratios in two turtle species with temperature-dependent sex determination. Each experiment consisted of a full factorial design using incubation temperature, chemical application, and their interaction as independent variables. Chemical dosage was included as a third factor in the 2011 experiment.

Species	Year	Sample size (clutches, eggs)	Incubation temperatures (°C)	Chemical applications	Chemical dosage
C. picta	2008	44, 446	26 ± 2, 28 ± 2, 30 ± 2	Fad, E2, EtOH	Double
C. serpentina	2009	15, 180	Constant 25, 30	Fad, E2, EtOH	Double
C. picta	2010	48, 505	Constant 26, 28, 30	Fad, E2, EtOH	Double
C. picta	2011	19, 147	Constant 26, 30	Fad, E2, EtOH	Single, double

and Janzen, 1990). Incubators were programmed to fluctuate ±2 °C throughout each day to partially simulate daily thermal fluctuations in natural nests.

To evaluate the role of steroids in sex determination, estradiol and an aromatase inhibitor were applied to eggs in each temperature treatment. A subset of eggs (n = 44–58 eggs per temperature treatment) received 17β estradiol (15 μg of E2 dissolved in 5 μl of 100% EtOH). Another subset of eggs (n = 46–59 eggs per temperature treatment) received Fadrozole (80 μg of Fad dissolved in 5 μl of 100% EtOH). Fadrozole (Ciba-Geigy CGS016949A, Novartis Pharmaceuticals AG, Basel, Switzerland) is an aromatase inhibitor that blocks the conversion of testosterone to estradiol, and should result in male development (Crews et al., 1994). As a control, eggs received 100% EtOH (n = 44–58 eggs per temperature treatment). Five microliters of one solution was applied to the surface of each egg. Prior to application, 1–2 eggs per temperature treatment were sacrificed for embryo staging to ensure that chemicals were first applied around stage 14 (just prior to the initiation of gonad differentiation). Accordingly, chemicals were applied 12, 14, and 15 days after the eggs were placed in incubators for the high, intermediate, and low temperature treatments, respectively. A second dose was applied to each egg seven days after the first dose. That is, all eggs received a second dose of the chemical volume described above for each treatment.

The above experimental design was repeated in 2010, but constant incubation temperatures were used rather than fluctuating thermal regimes, and fresh stocks of Fadrozole and 17β estradiol were obtained from Novartis Pharmaceuticals and Sigma, respectively. In addition, the dose of chemicals applied to eggs increased slightly (E2: 30 μg/5 μl 100% EtOH; Fad: 100 μg/5 μl 100% EtOH). For each treatment, 45–73 eggs were used. For the 2011 study, constant incubation temperatures were used again, but the 28 °C treatment was removed (i.e., only the two extreme temperatures (26 °C vs 30 °C) were used). This experimental design also involved an additional factor to evaluate the effect of chemical dosage on sex determination. Thus, the 2011 experiment used a 2 × 3 × 2 full factorial design (i.e., 2 temperatures [26, 30 °C] × 3 chemicals [E2, Fad, EtOH] × 2 doses [single vs double dose]; 12–13 eggs per treatment). For double dose treatments (same quantities as in 2010), chemicals were applied to eggs 11 and 8 days after eggs were put in the incubator for the 26 °C and 30 °C treatments, respectively. Then another dose was applied seven days later (as in the 2008 and 2010 experiments). For the single dose treatments, eggs were left undisturbed for the remainder of incubation after the first dose was applied.

2.3.2. C. serpentina

Twelve eggs were collected from 15 different *C. serpentina* nests within 3 h of oviposition, and were randomly assigned to a position in a 3 × 5 matrix among 12 shoeboxes. All eggs were half buried in moist vermiculite (−150 kPa). Eggs were incubated at 25 °C and 30 °C, which produce mostly males and females, respectively. The eggs from each temperature treatment were evenly assigned to three groups. One third of eggs received 17β estradiol (15 μg/5 μl EtOH; Rhen and Lang, 1995). Another one third of eggs received Fadrozole (100 μg/5 μl EtOH). The remaining eggs were used as a control, receiving 100% EtOH (5 μl). Chemicals were applied to the surface of each egg when the embryos were approximately 30% through the total incubation period. A second dose was applied to each egg six days after the first dose.

For all years and both species, shoeboxes were rotated within the incubators twice per week to minimize potential effects of thermal gradients within each incubator. The vermiculite within each shoebox was rehydrated once per week by adding water to restore the original mass of the shoebox. Eggs were checked twice daily for signs of hatching, and each turtle was then housed temporarily in individual plastic cups with a damp paper towel (at room temperature of ∼23 °C). Hatchlings mainly were euthanized with a lethal injection of sodium pentobarbital to enable surgical inspection of gonads for sex identification. Individuals that lacked oviducts, but contained short, smooth, yellowish gonads were classified as males, and individuals with oviducts and long, bumpy, whitish gonads were classified as females. These criteria for sex identification using gross morphology have previously been verified with histology (Schwarzkopt and Brooks, 1985). During gonad inspection any abnormal characteristics were recorded (e.g., cortex around testes, oviducts without ovaries). Gonads from 23 individuals from the 2011 experiment were excised and histology was performed to assess our sex identification based on gross morphology. These 23 individuals were represented by males and females from all treatments, with the exception that only males were used from the estradiol treatments (because no such eggs produced females). See Wibbels et al. (1991a) for histological procedures.

2.4. Statistical analyses

All data were analyzed with SAS software (SAS Institute, 2000). Due to the independence of each experiment and slight differences each year in experimental designs, each annual data set was analyzed separately, but qualitative comparisons were made across species and years.

Logistic regression models were used for all analyses. For experiments conducted in 2008, 2009, and 2010, incubation temperature, chemical treatment, and their interaction were included as independent variables, and offspring sex was the dependent variable. Analyses were the same in 2011 except that chemical dosage and interactions were also included as additional independent variables in the model. Because some temperature by chemical treatment interactions produced all of one sex, the Firth option in proc logistic was used to account for separability (i.e., when all observations in a treatment have the same status). Logistic regression was also employed to assess the effects of incubation temperature and chemical treatment on egg hatching success.

Based on records of gonad abnormalities (e.g., cortex on testes in males, lack of oviducts in females, lack of ovaries in females with oviducts), individuals were classified as having either 'normal' or 'abnormal' gonads (based on gross morphology), and an additional logistic regression analysis was performed to quantify the effects of incubation temperature, chemical treatment, and their interactions on the frequency of abnormal gonads. In 2011, dosage had no effect

on the frequency of abnormalities, and therefore this variable was not included in our final model.

3. Results

Similar results were observed across all four experiments on both *C. picta* (Fig. 1) and *C. serpentina* (Fig. 2) with strong temperature, chemical, and interactive effects on sex ratio (Table 2). For eggs that were not hormonally manipulated (control eggs), the cool treatment (26 °C) induced more male development than did the warm treatment (30 °C) in both species. For *C. picta*, intermediate temperatures (28 °C) in 2008 and 2010 produced an intermediate proportion of males between the two extreme temperature treatments. Overall, slightly more males were produced in 2010 (32.5% male in 2008 versus 47.6% male in 2010).

For eggs treated with Fadrozole, patterns did not differ markedly from those in the control treatments (Figs. 1 and 2), suggesting very little, if any, effect of aromatase inhibition on sexual differentiation. However, slightly more males were produced in 2008 and 2011 across incubation temperatures, but fewer were produced in 2010 relative to their respective control treatments. For *C. serpentina* in 2009, sex ratios did not differ between the Fadrozole-treated eggs and the control treatment (Fig. 2).

Application of estradiol to eggs resulted in nearly all male development in every experiment (Figs. 1 and 2). For *C. serpentina*, remarkably more male production was observed under the female-producing temperature (30 °C) than under the natural male-producing temperature (25 °C). In the 2011 experiment with *C. picta* eggs, chemical dose had no impact on sex ratios (Table 2 and Fig. 1).

Egg hatching success was relatively high overall, and was not influenced by experimental treatments in most cases (Table 3). The only significant finding was in the 2009 experiment on *C. serpentina* eggs, where eggs incubated at 25 °C had higher hatching success than those incubated at 30 °C (87.2% vs 66.3% egg survival).

Based on gross morphology, the overall proportion of abnormal gonads was low, but was influenced by hormone treatment and the interaction between temperature and chemical treatment; these effects were significant only in 2008 and 2010 (Table 3 and Fig. 3). Despite these significant effects, no overarching pattern was observed (e.g., one type of chemical treatment never resulted

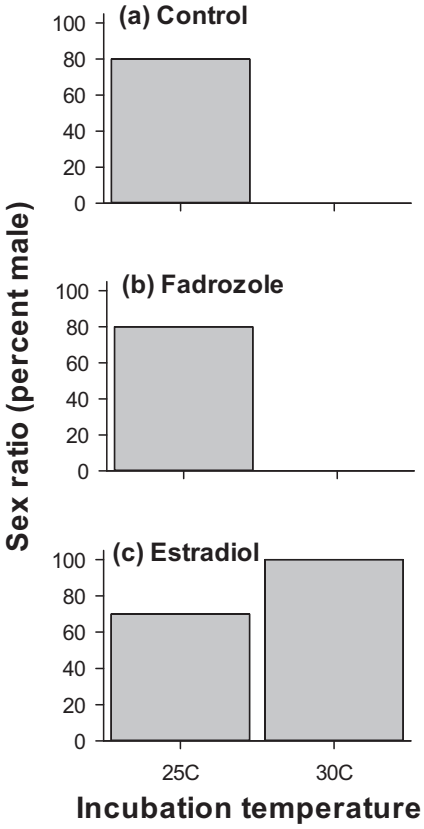

Fig. 2. Effect of incubation temperature and hormone manipulation on hatchling sex ratio of the common snapping turtle (*C. serpentina*). This experiment was performed only in 2009.

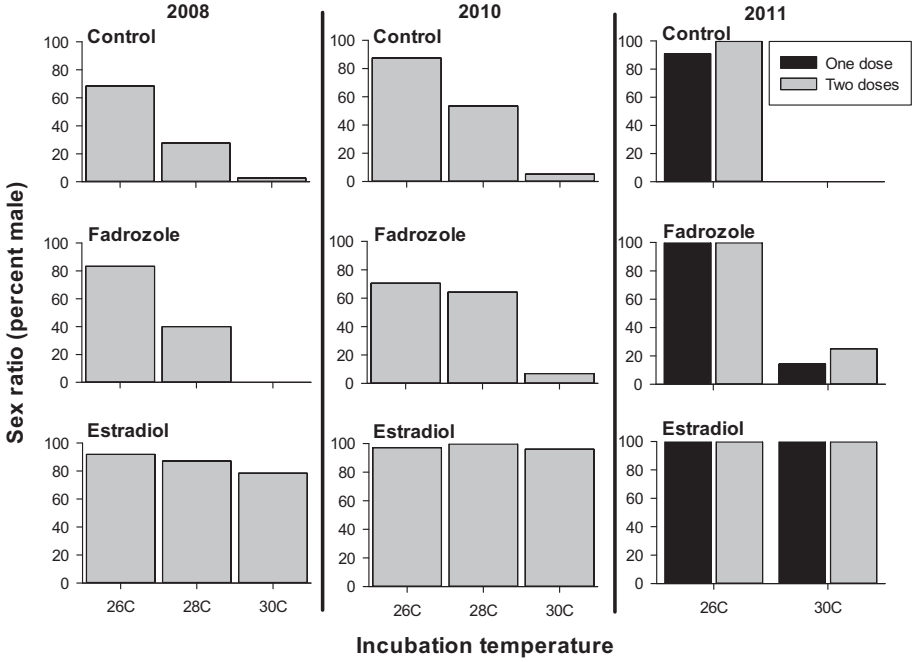

Fig. 1. Effect of incubation temperature and hormone manipulation on hatchling sex ratio of the painted turtle (*C. picta*). The first column shows results from the 2008 experiment, the second column shows results from the 2010 experiment, and the third column shows results from the 2011 experiment. In the 2011 experiment, eggs were incubated only at the temperature extremes and we quantified the effect of dose on offspring sex ratios.

Table 2
Effects of incubation temperature, chemical treatment (i.e., estradiol, Fadrozole, EtOH), and dosage on offspring sex ratios in two turtle species with temperature-dependent sex determination. The effect of dose was only assessed in the experiment in 2011.

Species (year)	Temperature	Chemical treatment	Temp × chemical	Dosage
C. picta (2008)	$\chi^2 = 41.6$, $P < 0.001$	$\chi^2 = 57.5$, $P < 0.001$	$\chi^2 = 13.7$, $P = 0.008$	–
C. serpentina (2009)	$\chi^2 = 4.5$ $P = 0.033$	$\chi^2 = 6.6$, $P = 0.037$	$\chi^2 = 9.1$, $P = 0.011$	–
C. picta (2010)	$\chi^2 = 19.04$, $P < 0.001$	$\chi^2 = 26.4$, $P < 0.001$	$\chi^2 = 7.1$, $P = 0.129$	–
C. picta (2011)	$\chi^2 = 16.61$, $P < 0.001$	$\chi^2 = 11.54$, $P = 0.003$	$\chi^2 = 7.66$, $P = 0.022$	$\chi^2 = 0.14$, $P = 0.710$

Table 3
Effects of incubation temperature, chemical treatment (i.e., estradiol, Fadrozole, EtOH), and their interaction on egg survival and the frequency of abnormal gonads in the painted turtle (*C. picta*) and common snapping turtle (*C. serpentina*), two species with temperature-dependent sex determination. In 2011, chemical dosage had no effect on egg survival or the frequency of abnormal gonads, and therefore this variable was removed from the final model. No abnormal gonads were reported for *C. serpentina* embryos.

Year (species) Dependent variable	Temperature	Chemical treatment	Temp × chemical	Overall egg survival and frequency of abnormal gonads
2008 (C. picta)				
Egg survival	$\chi^2 = 4.9$, $P = 0.087$	$\chi^2 = 0.5$, $P = 0.798$	$\chi^2 = 1.2$, $P = 0.875$	86.7%
Abnormal gonads	$\chi^2 = 3.5$, $P = 0.173$	$\chi^2 = 7.2$, $P = 0.028$	$\chi^2 = 16.8$, $P = 0.002$	16.3%
2009 (C. serpentina)				
Egg survival	$\chi^2 = 9.0$, $P = 0.003$	$\chi^2 = 1.6$, $P = 0.444$	$\chi^2 < 0.1$, $P = 0.997$	76.7%
2010 (C. picta)				
Egg survival	$\chi^2 = 0.2$, $P = 0.903$	$\chi^2 = 2.4$, $P = 0.299$	$\chi^2 = 2.9$, $P = 0.569$	91.7%
Abnormal gonads	$\chi^2 = 3.3$, $P = 0.191$	$\chi^2 = 6.5$, $P = 0.039$	$\chi^2 = 10.7$, $P = 0.031$	23.1%
2011 (C. picta)				
Egg survival	$\chi^2 = 0.6$, $P = 0.446$	$\chi^2 = 5.6$, $P = 0.061$	$\chi^2 = 0.2$, $P = 0.905$	94.4%
Abnormal gonads	$\chi^2 = 0.03$, $P = 0.854$	$\chi^2 = 2.4$, $P = 0.295$	$\chi^2 = 2.5$, $P = 0.286$	9.7%

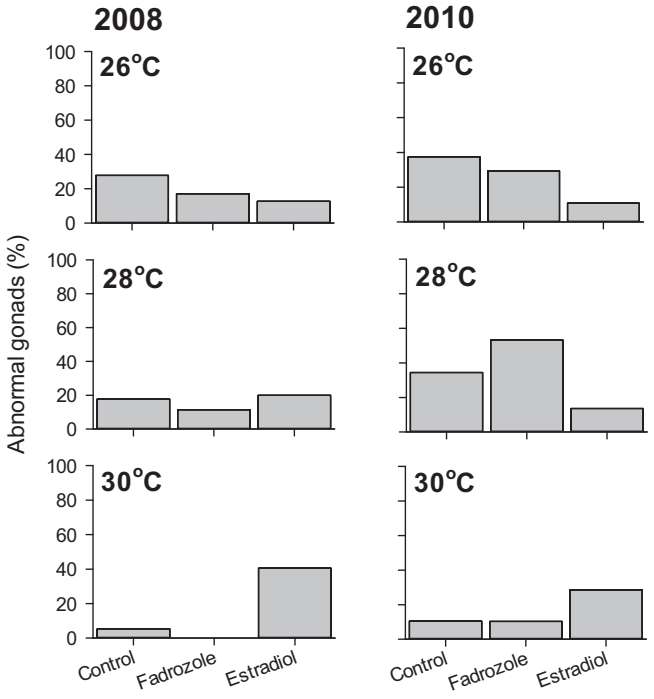

Fig. 3. Effect of incubation temperature and hormone manipulation on the frequency of gonadal abnormalities (based on gross morphology) in hatchling painted turtles (*C. picta*). Gonad and/or reproductive ducts that exhibited any type of abnormality (e.g., reported as lack of oviducts in females, rudimentary oviducts, reduced oviducts, faint oviducts, lack of ovaries in females with oviducts, oviducts without ovaries, questionable ovaries, cortex around testes, unregressed mullerian ducts, gonad problems, etc.) were grouped together into a single category. The first column shows results from the 2008 experiment, the second column shows results from the 2010 experiment. The frequency of gonadal abnormalities was not affected by incubation temperature or hormone manipulation in the 2009 or 2011 experiment (see Table 3).

in increased abnormal gonads across years). In fact, in 2010, the greatest proportion of abnormal gonads occurred in the control treatment.

Based on histological examination of gonads in 2011, thirteen of the 23 gonads exhibited a clear oviduct, ovary, or testis, and these matched nearly perfectly with our sex identification that was based on gross morphology. Fig. 4 provides histological photographs from six representative individuals, and illustrates testes from males produced under 26 °C incubation, and an ovary or oviducts from females produced under 30 °C from the control and Fadrozole treatments. For the estradiol-treated individuals, gonads were reduced in size (e.g., Fig. 4c) or not found (or at least not obvious) on histological slides, although gonads were identified as testes based on gross morphology prior to histological preparation.

4. Discussion

Application of exogenous estrogenic compounds typically feminizes reproductive tissues, and overrides the influence of incubation temperature on sex determination under TSD (Bull et al., 1988; Wibbels et al., 1991b; Rhen and Lang, 1994; Devlin and Nagahama, 2002; Elf, 2004). In addition, Fadrozole has previously been shown to induce male development by blocking the aromatization of androgens to estrogens in many taxa (Piferrer et al., 1994; Wibbels and Crews, 1994; Chardard and Dournon, 1999; Warner and Shine, 2005). Intriguingly, our results in two turtle species across four independent experiments are inconsistent with these well-established findings; the application of Fadrozole had no impact on offspring sex ratios in the present study, and applying estradiol to eggs produced nearly all male offspring, a pattern opposite of expectations.

The lack of an effect of Fadrozole on offspring sex ratios is difficult to explain considering the wealth of studies that demonstrate its masculinizing effect on gonads (e.g., Piferrer et al., 1994; Wibbels and Crews, 1994). In the case of one previous study using Fadrozole with the turtle *Trachemys scripta*, a near pivotal incubation temperature was used in an attempt to increase the sensitivity of the embryos to Fadrozole (Wibbels and Crews, 1994), so it is possible that the higher incubation temperature (30 °C) used in the current study obscured the effect. However, an effect at the intermediate temperatures (28 °C) would be expected, but was

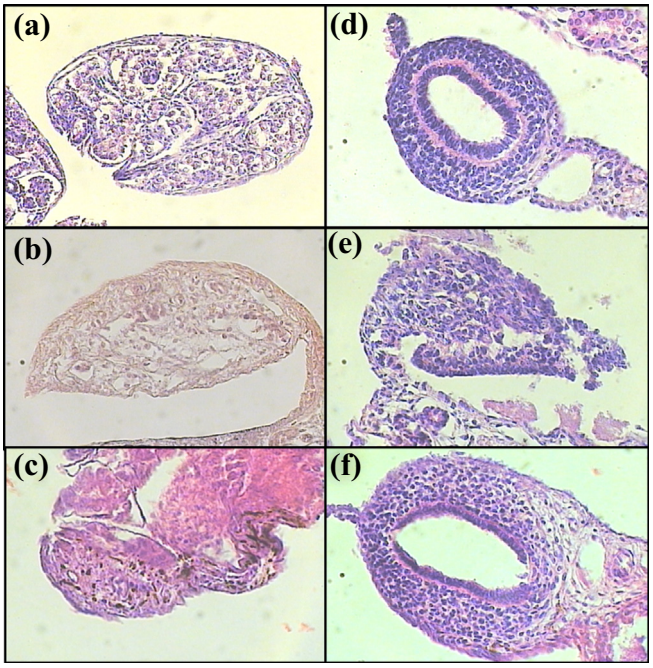

Fig. 4. Gonad histology for hatchling painted turtles (*C. picta*). These histological photographs represent gonads produced under different temperature and chemical treatments in the 2011 experiment. The first column illustrates gonads produce under 26 °C: (a) testis of male from the control treatment, (b) testis of male from the Fadrozole treatment, (c) reduced gonad of male from the Estradiol treatment. The second column illustrates gonads produced under 30 °C: (d) oviduct of female from the control treatment, (e) ovary of female from the control treatment, (f) oviduct of female from the Fadrozole treatment. Ovaries were not obvious in the histological sections for most females, but many individuals had clear oviducts. Histological cross sections of gonads of hatchlings treated with estradiol and produced under 30 °C were not obvious for any individual. However, all individuals from estradiol-treated eggs were identified as male based on gross morphology (i.e., testes were observed prior to histological preparation).

not evident. Another possibility is that the chemical did not properly absorb through the egg shell or was disrupted by other materials on the surface of the shell (although soil/sand was brushed off, the egg surfaces were not immaculate), thereby hindering any effect on sexual differentiation. Although this non-significant result was unexpected, another study of a reptile with TSD (*Alligator mississippiensis*) shows that aromatase inhibition does not facilitate masculinization of the gonad, but still disrupts ovarian development. The authors of that study argue that inhibiting estrogen synthesis alone is not enough to cause masculinization (Lance and Bogart, 1992). Similar mechanisms may be operating in *C. picta*.

Although our results concerning the effect of estradiol were unexpected, estradiol is known to have masculinizing effects on several aspects of normal male development. For example, this steroid is critical in development of the male brain, and masculinizes adult sexual behaviors (Amateau and McCarthy, 2004; Konkle and McCarthy, 2011; but see Adkins (1979)). In fact, estradiol is as effective as testosterone in masculinizing the regulation of gonadotropic hormone release in rats (Christensen and Gorski, 1978). Elevated levels of estradiol also facilitate prostate development in fetal mice (vomSaal et al., 1997), but this pattern reverses when even higher doses are used. Likewise, dose-dependent effects of estradiol on male development of the frog *R. pipiens* have also been demonstrated (Richards and Nace, 1978). Numerous other studies of frogs show that estradiol can engender male development (reviewed by Hayes, 1998). Estradiol can both upregulate androgen receptor expression and bind to androgen receptors themselves, albeit with much lower affinity than androgens (Yeh et al., 1998; Heinlein and Change, 2002; Richter et al., 2007; Poulin et al., 1998). Conversely, high levels of androgens can facilitate feminization in fish by interacting with estrogen receptors (Mori et al., 1998; Devlin and Nagahama, 2002). In *Eublepharis macularius*, a lizard with TSD, estrogens induce male development, but only at female-producing incubation temperatures (Janes et al., 2007). The results of the present study also demonstrate a masculinizing effect of estradiol on gonads. Importantly, however, the lack of obvious gonads from the histological examinations of estradiol-treated individuals suggests that exogenous estradiol is having an abnormal impact on proper gonad development and individuals from the estradiol treatment may not be functional males. Estradiol has previously been shown to affect the integrity of the gonad (Wibbels, unpubl. data), and could have inhibited successful histological preparation for these individuals in this study. Nevertheless, based on gross morphology of gonads, this study adds to a growing list of masculinizing effects of estradiol.

Despite several studies that demonstrate masculinizing effects of estradiol, our results still greatly contradict previous research on other reptiles with TSD, and notably contrast with previous findings from similar research on *C. serpentina* (Rhen and Lang, 1994, 1995). Given these contradictory results, what might explain the patterns found in the current study? Because the effect of estradiol on sex ratios reflects the expectations for Fadrozole (all males produced), stock labels may have been accidentally switched. However, the fact that these results were replicated four times provides convincing evidence that this was not the case. Second, the lack of a Fadrozole effect on sex ratios may have been due to an expired stock. Indeed, in 2008 the Fadrozole used was obtained five years earlier and worked effectively at that time (see Warner and Shine, 2005). Because of this concern, fresh Fadrozole was obtained in 2010, and still produced the same results (i.e., no effect of Fadrozole on sex determination). Similarly for estradiol, new stocks were obtained in 2008 and in 2010 and results were consistent both times. Third, masculinizing effects of high doses of estradiol during development have been reported in frogs (Hayes, 1998), and the experiments in 2008, 2009, and 2010 used a higher dose (e.g., chemicals were applied twice during incubation) than that used in previous studies (Rhen and Lang, 1995; Crews 1996). Therefore in 2011, the influence of dose was assessed, but no dosage effect was detected. Fourth, relatively high egg survival and our ability to sex many dead embryos rule out the possibility that differential mortality between male and female embryos, rather than our manipulations, generated the sex ratio patterns. Lastly, because the predicted sex ratios were produced at each temperature in the control treatments (albeit sex ratios were slightly obscured in the fluctuating thermal regimes in 2008, as would be expected; Georges et al., 2005; Warner and Shine, 2011), it is unlikely that hatchling sex was misidentified based on gross morphology.

The production of male offspring with exogenous estradiol could have been due to an unnaturally high concentration of estradiol surrounding the embryo. Indeed, naturally synthesized estradiol coupled with exogenous application likely raised the total concentration above physiological levels, particularly in eggs incubated at female-producing temperatures (Rhen et al., 2005). These elevated estradiol concentrations could interact with androgen receptors (Mori et al., 1998), or inhibit aromatase expression, thereby decreasing estradiol production during the thermo-sensitive period of sex determination. If this is the case, then the elevated concentration of estradiol must dissipate after aromatase activity has been inhibited, which would result in relatively high concentration of testosterone, leading to testis development. In addition, exogenous estrogen could alter expression of aromatase or estrogen receptor genes in the gonad (Katsu et al., 2004). These scenarios have been suggested previously in another reptile with TSD (Janes et al., 2007) and might explain the paradoxical results of the present

study as well. Additional research that addresses these possibilities is warranted.

Our results also call attention to the issue of publication bias in the scientific literature. If paradoxical results like those described here are left unreported, then such findings might not be as unusual as one would expect. Indeed, we would not have pursued publication of our 2008 results had we not repeated the experiment four times and consistently produced the same outcome. In addition, we are aware of unpublished reports of estradiol failing to feminize gonads in the turtle *Graptemys pseudogeographica kohnii* under male-producing incubation temperatures. Unfortunately, this work was discontinued soon after these unexpected results became apparent (Freedberg, pers. comm.). If studies with similar results are left unpublished, then our overall understanding of the impacts of sex steroids is skewed. We urge scientists to report and/or repeat experiments that either have negative or paradoxical findings. Making these results accessible to the scientific community will advance our knowledge of the biological phenomena that we are trying to understand.

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