Estrogens Induced Male Production at a Female-Producing Temperature in a Reptile (Leopard Gecko, Eublepharis macularius) with Temperature-Dependent Sex Determination

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Abstract.—Estrogens and estrogen mimics can affect offspring sex ratios in a wide variety of animal species including Leopard Geckos, Eublepharis macularius, a species with temperature-dependent sex determination (TSD). Estrogens can disrupt effects of temperature on sex determination by feminizing or sex-reversing embryos incubated at male-producing temperatures. Estrogens may have paradoxical effects at different incubation temperatures and in different concentrations. In this study, E. macularius eggs were exposed to ethanol, estradiol benzoate, or estradiol 17β at the beginning of the thermosensitive period when sex determination occurs. Eggs were treated and incubated at each of three incubation temperatures known to produce varying primary sex ratios. At a male-producing incubation temperature, estrogen-treated groups produced more females than negative control groups. This result has been reported in other TSD reptiles. In our study, we showed that at a female-producing incubation temperature, estrogen-treated groups produced significantly more males than negative control groups. This is the first report of its kind in which a TSD reptile was shown to produce significantly more males at a female-producing temperature. Our results suggest a complex feedback relationship between aromatase and cofactors during the thermosensitive period of egg incubation in E. macularius. Expected effects of exogenous estrogens on contaminant-exposed wildlife populations may differ depending on nest-site temperatures.

In species with temperature-dependent sex determination (TSD), sex is determined by the temperature to which an embryo is exposed during a thermally sensitive period of incubation. The influence of exogenous estrogens on TSD has been widely reported across species with varying patterns of TSD. In TSD species, sex determination appears to be controlled by the embryonic ratio of estrogens to androgens (Pieu, 1974). Temperature may influence this ratio in TSD species like Leopard Geckos, Eublepharis macularius, by affecting the production and/or activity of aromatase, the enzyme that converts androgens to estrogens (Desvages and Pieu, 1992a,b; Crews et al., 1994). Eublepharis macularius follow a FMR pattern of sex determination in which an increased proportion of males is produced at an intermediate incubation temperature (31–33°C), whereas an increased proportion of females is produced at cooler (26–28°C) and warmer (34–35°C) incubation temperatures (Viets et al., 1993; Crews, 2003). Eublepharis macularius eggs are known to hatch in distinguishable and predictable sex ratios from these temperatures (26:0% males; 30:24% males; 32.5:74% males; Viets et al., 1993). This species is ideal for studies of sex-determining response to incubation environment because of their relatively quick generation time, ease of handling and care, availability of fertile adults and detailed study of their TSD mechanism (Viets et al., 1993; Tousignant and Crews, 1994; Janes and Wayne, 2006).

Exogenous steroids alter the expression of steroidogenic enzyme genes that control sex differentiation (Devlin and Nagahama, 2002). In this way, estrogens and estrogen-mimicking compounds disrupt TSD at male-producing incubation temperatures (Bull et al., 1988; Tousignant and Crews, 1994). Typically, estrogens feminize male reproductive tissues or override TSD causing sex-reversal of males (Fry and Toone, 1981; Belaid et al., 2001). Application of estrogenic compounds to eggs of TSD species, such as freshwater turtles and crocodilians, also affects sex differentiation, organizational and activational development, and sex-specific behavior in resulting hatchlings (Jeyasuria et al., 1994; Sheehan et al., 1999; Willingham et al., 2000). The disruptive influence of estrogen has been inordinately studied at male-producing incubation temperatures as opposed to female-producing or intermediate temperatures (Tousignant and Crews, 1994). At different incubation temperatures, TSD embryos produce different concen-
trations of sex steroids, presenting an opportu-
nity to analyze the effects of exogenous estro-
gens at different background concentrations (Elf, 2003). When applied to eggs at female-
producing temperatures, exogenous estrogens combine with high concentrations of naturally
synthesized estrogens. In comparison, when
exogenous estrogens are applied to eggs at
male-producing temperatures, total embryonic
estrogen content will be lower. By comparing
estrogen-treated eggs incubated at male- and
female-producing temperatures, we can com-
pare the effects of high and higher concentra-
tions of estrogen on TSD, which are not always
straightforward.

Some contaminants have opposing effects on
exposed reproductive tissues at high and low
concentrations (Parmigiani et al., 2000). For
example, excessive androgens can lead to
paradoxical feminization in fish because andro-
gens can interact with estrogen receptors (Mori
et al., 1998; Devlin and Naegahama, 2002). A
similar effect has been reported for exogenous
estrogen treatment. Prenatal exposure to small
amounts of exogenous estradiol or diethyl-
esterol increases prostate size, whereas pre-
natal exposure to larger amounts of these two
compounds decreases prostate size in neonatal mice (vomSaal et al., 1997). Also, Hayes (1998)
reported that in the genotypically sex-deter-
mined (GSD) amphibian, *Rana pipiens*, low
doses of estradiol (< 0.07 μM) did not affect
sex differentiation, whereas high doses (0.07–
0.18 μM) produced 100% females and even
higher doses (≥ 3.69 μM) produced 100% males.

Incubation temperature has immediate and
long-lasting effects on endogenous estrogen
concentrations in TSD species (Desvages and
Pieau, 1992b; Rhen et al., 2005). Eggs incubated
to 26°C (the female-producing temperature) are
expected to have higher endogenous estrogen
concentrations than eggs incubated at 30° or
32.5°C (Desvages and Pieau, 1992a). In con-
 consideration of the reported paradoxical effects of
high and higher concentrations of estrogens in
*R. pipiens*, the most closely related species in
which this experiment has been conducted, we
hypothesized a pattern in *E. macularius* similar
to that seen in *R. pipiens* (Hayes, 1998). We
hypothesized that exposure of *E. macularius*
eggs to exogenous estrogen (estradiol benzoate
[EB] or estradiol 17β[17β]) would create a high
embryonic concentration of estrogens and sig-
nificantly increase production of females at
a male-producing temperature (as previously
reported) and create a higher concentration of
estrogens and increase the production of males
at a female-producing temperature (a potential-
ly novel result). To test this, we compared
resulting sex ratios of *E. macularius* offspring
that were incubated at varying temperatures.
At each temperature, we compared sex ratios
of offspring exposed to estrogens to those of
offspring exposed to no chemical treatment or
to ethanol, a chemical previously identified
as a negative control in endocrine disruption
experiments (Milnes et al., 2004). If incuba-
tion temperature alters the effects of exoge-
nous estrogens on TSD, then the risk of
estrogen-mimicking contaminants on wildlife
populations may vary with climate (Milnes
et al., 2002).

**Materials and Methods**

For this study, *E. macularius* eggs were
obtained from The Gourmet Rodent, a reptile
breeder in Archer, Florida. Two hundred
sixteen eggs were collected < 24 h after ovipo-
sition. Each egg was collected from a different
dam. All eggs were candled to test fertility. Each
fertile egg was placed individually in a 188 mL
cup; six such cups were banded together in
ringed containers. Six-cup containers were
designed to allow individual eggs to incubate
without chemical cues from other eggs yet allow
quick manipulation by researchers. Each cup
contained 6 g perlite, 17.5 mL tap water, and
one egg and was sealed with a tight-fitting lid
punctured with one gas-exchange hole. Al-
though the gas-release holes may have allowed
limited chemical communication among eggs,
they were necessary to prevent accumulation of
metabolic waste. Egg containers were placed in
an environmental chamber and maintained at
26°C, 30°C, or 32.5°C for the duration of the
experiment. These temperatures were selected
because they produce the most (26°C), interme-
diate (30°C), and least (32.5°C) numbers of
female *E. macularius* (Viets et al., 1993). Cham-
ber temperatures were recorded every minute
throughout the experiment with Hobo® tem-
perature loggers. Loggers were calibrated ac-
cording to the manufacturer’s instructions. In-
cubator temperatures fluctuated by 0.5°C
daily. This variation did not hinder our ability
to produce offspring sex ratios similar to those
reported by Viets et al. (1993). Each day, the
chambers were opened and the containers were
removed. Each cup was opened momentarily
to release metabolic gas waste and check for
hatchlings. The position of the containers within
the three chambers was randomized daily.

Experimental treatments were applied once at
the beginning of the middle third of the
incubation period at each of the three tempera-
tures (Table 1). Solutions were pipetted directly
to the shells of eggs in each treatment group (D.
Crews, pers. comm.). Treatment groups were
Table 1. Incubation temperatures, experimental treatments and sample sizes of Leopard Geckos, *Eublepharis macularius*. Sample sizes varied because of < 100% hatching success of experimental animals. All treatments were administered once at the beginning of the middle third of the incubatory period.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Treatment</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>26°C</td>
<td>No treatment</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>5 μl 95% ethanol</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>5 μl estradiol benzoate (5.3 μM)</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>5 μl estradiol 17β (6.1 μM)</td>
<td>13</td>
</tr>
<tr>
<td>30°C</td>
<td>No treatment</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>5 μl 95% ethanol</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>5 μl estradiol benzoate (5.3 μM)</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>5 μl estradiol 17β (6.1 μM)</td>
<td>17</td>
</tr>
<tr>
<td>32.5°C</td>
<td>No treatment</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>5 μl 95% ethanol</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>5 μl estradiol benzoate (5.3 μM)</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>5 μl estradiol 17β (6.1 μM)</td>
<td>15</td>
</tr>
</tbody>
</table>

Exposed to no treatment, 5 μl 95% ethanol, 5 μl EB (5.3 μM), or 5 μl E2 (6.1 μM). These volumes and concentrations were selected because they fall within the range that has been shown to predictably affect offspring sex in many species of TSD reptiles, including *E. macularius* (Toussignant and Crews, 1994; Willingham and Crews, 1999; Milnes et al., 2002; Elf, 2003). Higher and lower concentrations of EB and E2 have been shown to increase female production at male-producing temperatures in TSD species. Also, 95% ethanol has served as an effective negative control in endocrine-disruption studies of TSD species (Milnes et al., 2004). The middle third of the incubation period was predicted by averaging the incubatory durations at each temperature from a previous experiment (Janes and Wayne, 2006). This period represents the thermosensitive period for sex determination as well as a period when exogenous hormone exposure can alter sex determination (Bull, 1987; Crews, 2003). Each treatment group consisted of 18 eggs (three containers). Resulting sample sizes (number of hatchlings) varied due to variable hatching success (Table 1).

Upon hatching, geckos were euthanized by exposure to halothane (Fluothane: 2-bromo-2-chloro-1,1,1-trifluoroethane). Hatchlings were fixed in Bouin’s fixative and preserved in 75% ethanol. The gonads, opaque white, cylindrical structures on either side of the posterior end of the dorsal artery, were removed from each gecko and prepared for analysis by light microscopy.

For histology, fixed and preserved gonads were dehydrated by increasing concentrations of ethanol, cleared in two changes of Citrosolv, and infiltrated with paraffin (Fisher 55; Fisher Biotech, Orangeburg, NY) under increasing pressure (0.84, 1.06, 1.48, 1.65 kg/cm²) in an Isotemp vacuum oven. The gonads were sectioned at 8 μm and stained with a modified trichrome of Harris (Humason, 1997). Two researchers (DJ and DB) analyzed gonad sections independently. If seminiferous tubules were identified within the sections, the gecko was identified as male (Fig. 1A). If oogonia were observed in the sections, the gecko was identified as female (Fig. 1B). Laparotomies and identification of oviducts, vas deferens, hemipenes or cloacal scales have been used for sex identification of reptiles in previous studies (Bull, 1987; Toussignant and Crews, 1994). However, these methods were validated by their agreement with gonadal histology, the method of sex identification employed in this study (Gutzke and Bull, 1986). We relied on gonadal histology for sex identification because of its demonstrated validity in previous studies (Viets et al., 1993; Toussignant and Crews, 1994) and the unreliable presence of secondary and accessory sex characteristics in day-old hatchlings.

The whole dataset encompassing each of four treatment groups at each of three temperatures was analyzed by ANOVA using JMP (vers. 6, Cary, NC, 2006). At each temperature, the dependent variables, sexes of offspring (1: female; 2: male) from estrogen-treated groups were compared against sexes of negative control treatment groups that were treated with ethanol or no chemical exposure using a least significant difference (LSD) threshold matrix. The LSD matrix provided results of all possible Student’s *t*-tests within the ANOVA model.

Samples consisted of all individuals incubated with either an estrogen treatment or a negative control. Samples were defined by treatment and temperature, not by ringed containers.

**Results**

The overall ANOVA showed a significant difference among all treatment groups (*F*<sub>5,135</sub> =...
Estrogens are pooled as middle terms. (32.5°C; P < 0.05; Fig. 2).

The proportion of males in pooled negative control groups was significantly different between 26°C and 30°C and between 26°C and 32.5°C but not between 30°C and 32.5°C (Table 2). Also, no significant differences were observed among any treatment groups at the intermediate temperature (30°C; P > 0.05; Table 2) or among egg containers within treatments at different temperatures.

Also had a higher proportion of males than the negative control groups at the female-producing incubation temperature (26°C; P < 0.05; Fig. 2). Although the group exposed to no treatment produced fewer males than the group exposed to 95% ethanol at the male-producing temperature, a Student’s t-test found no significant difference (t = 1.979; LSD = 0.09; P > 0.5).

Estrogen-treated groups and negative controls were pooled because the LSD matrix showed no significant differences between the two estrogen-treated groups or between the two negative controls at any temperature. Pooling increased sample sizes for the ANOVA without confounding the effects of different estrogens or different negative controls.

5.12, 7 = 0.0003). Pooled estradiol treatment groups (EB and E2) had a significantly lower proportion of males than the pooled negative control groups (no treatment or 95% ethanol) at the male-producing incubation temperature (32.5°C; P < 0.05). Estradiol treatment groups

**Table 2.** Least significant difference (LSD) threshold matrix for Leopard Geckos, *Eublepharis macularius* (t = 1.978; α = 0.05). Results of the t-test within the LSD matrix indicate differences between pairs of means within the overall ANOVA model. Positive values and asterisks show pairs of means that are significantly different.

<table>
<thead>
<tr>
<th></th>
<th>Negative controls (26°C)</th>
<th>Estrogens (26°C)</th>
<th>Negative controls (30°C)</th>
<th>Estrogens (30°C)</th>
<th>Negative controls (32.5°C)</th>
<th>Estrogens (32.5°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative controls</td>
<td>−0.264</td>
<td>0.114*</td>
<td>0.147*</td>
<td>0.05*</td>
<td>0.285*</td>
<td>0.215</td>
</tr>
<tr>
<td>Estrogens (26°C)</td>
<td>0.114*</td>
<td>−0.305</td>
<td>−0.250</td>
<td>−0.153</td>
<td>−0.135</td>
<td>0.058*</td>
</tr>
<tr>
<td>Negative controls</td>
<td>0.147*</td>
<td>−0.290</td>
<td>−0.212</td>
<td>−0.115</td>
<td>−0.077</td>
<td>0.090*</td>
</tr>
<tr>
<td>Estrogens (30°C)</td>
<td>0.05*</td>
<td>−0.153</td>
<td>−0.115</td>
<td>−0.212</td>
<td>0.019*</td>
<td>−0.006</td>
</tr>
<tr>
<td>Negative controls</td>
<td>0.285*</td>
<td>−0.135</td>
<td>−0.077</td>
<td>0.019*</td>
<td>−0.264</td>
<td>0.229*</td>
</tr>
<tr>
<td>Estrogens (32.5°C)</td>
<td>−0.215</td>
<td>0.058*</td>
<td>0.090*</td>
<td>−0.006</td>
<td>0.229*</td>
<td>−0.271</td>
</tr>
</tbody>
</table>

**Fig. 2.** Sex ratios of Leopard Geckos. Treatments were administered to eggs once at the beginning of the middle third of incubation. Error bars represent the standard error of each treatment group across temperatures. Error is represented across all individuals incubated with the same treatment and temperature, regardless of reared container. Departures of pooled estrogen-treated samples (5 µl EB [5.3 µM] and 5 µl E2 [6.1 µM] treatments) from pooled negative controls (no treatment and 95% ethanol treatments) are indicated by: *: P < 0.05. EB and E2 treatments were suspended in 95% ethanol. Note the Y-intercept is ≤ 0.

The novel observation obtained from this study is that exogenous estrogens increased production of males at a female-producing incubation temperature in a TSD reptile. This is the first report of paradoxical function of estrogen at different concentrations in a TSD vertebrate. Clearly, incubation temperature predominantly affects sex determination in TSD species such as *E. macularius* (Viets et al., 1993; Crews, 2003). However, the precise timing and physiological pathways that translate temperature into offspring sex are unknown. As a result, the evolution of TSD and the effects of nest site variables such as exogenous hormone content remain enigmatic. At higher concentrations, estrogens may interact with androgen receptors as androgens interact with estrogen receptors in fish (Mori et al., 1998), or we hypothesize an
inhibitory effect of estrogens on expression of aromatase or another steroidogenic enzyme gene.

Wibbels et al. (1991) suggested that higher doses of estrogen would be required to sex-reverse males incubated at temperatures nearer to male-producing incubation temperatures compared to female-producing incubation temperatures. However, very low doses of E2 have been shown to sex-reverse turtle embryos as effectively as high doses (Sheehan et al., 1999). Threshold dose concentrations may not have the same significance for the effect of exogenous estrogens on sex determination in some or all TSD species (for discussion, see Sheehan et al. 1999).

Other surprising results were lower hatching success at the female-producing temperature and decreased number of males produced in the group exposed to no treatment at the male-producing temperature (Fig. 2). Eublepharis macularius eggs incubated at lower temperatures have a longer incubation period. Longer exposure to humidity may make eggs more susceptible to pathogens in the substrate. Also, the previously reported significant difference between male production at 30°C and 32.5°C (Viets et al., 1993) was not seen in this study. Minor temperature fluctuations (< 0.5°C) may have caused greater similarity in incubation temperature between the two groups than was reported by Viets et al. (1993).

Unlike previous studies (Fry and Toone, 1981; Crews et al., 1994; Matter et al., 1998; Willingham and Crews, 1999), a masculinizing effect of exogenous estrogens at the female-producing incubation temperature was observed in this study. The increased male production at 26°C suggests negative feedback inhibition on aromatase. Aromatase converts testosterone to estrogen. One hypothesis is that a surplus of estrogen earlier in development inhibits gene expression for aromatase or the action of aromatase, thereby causing decreased production of estrogen later in the thermosensitive period when it is required for feminization of differentiating gonads. For negative feedback inhibition on aromatase to work, the high estrogen concentration must dissipate after aromatase has been inhibited. If estrogens are not cleared from the embryo before sex determination, they would replace estrogens that would be synthesized by aromatase. Clearly, more light must be shed on the sustainability of estrogens in the embryo before and during the thermosensitive period.

Estrogens are responsible for the regression of the medullary region of the developing testis and the proliferation of the cortical region of the bipotential gonad (Crews et al., 1991). Previous studies with reptiles, specifically turtles and alligators with TSD, have not reported increased production of males with any dose of estrogen, androgen, antiestrogen, antiandrogen or aromatase inhibitor (for review see Guillette and Crain, 1996). TSD embryos exposed prior to sex determination to pharmaceutical agents that inhibit aromatase activity exhibit a variety of responses. Trachemys scripta embryos develop according to the temperature at which they are incubated; their sex determination is unaffected by the aromatase inhibitor (Wibbels and Crews, 1992). In contrast, Alligator mississippiensis embryos treated in a similar manner are either female or exhibit an ambiguous gonad regardless of incubation temperature (Lance and Bogart, 1992). Species exhibiting strict genotypic sex determination also have been studied to determine whether ovarian differentiation is sensitive to aromatase inhibition. Genotypically female larvae of the newt, Pleurodeles waltl, differentiate into functional males following treatment with an aromatase inhibitor (Chardard and Dournon, 1999). Thus, in P. waltl, an estrogen deficit triggered by aromatase inhibition resulted in male sex differentiation, regardless of genotype.

Hayes (1998) reported both increased production of females and males in response to high concentration of estradiol in an amphibian. Species variation in response to an altered estrogen profile during embryonic development indicates that several mechanisms could be involved that are dependent on mode of sex determination (strongly TSD versus GSD) or the compound used (either an estrogen, antiestrogen or aromatase inhibitor). High estrogen concentrations in the gonad or brain could alter the release of stimulatory factors from the brain or the gonad by altering gene expression patterns (Devlin and Nagahama, 2002). Direct alteration by exogenous estrogens of the expression of various genes in the gonad, including aromatase or the estrogen receptors, could also explain our result (see Gabriel et al., 2001; Milnes et al., 2002; Katsu et al., 2004).

Future experimentation will measure the expression of steroidogenic enzyme genes in various interactions of temperature and exogenous steroid treatments. Candidate gene (Sox9, wT1, sf1, dmrT1, and others; Modi and Crews, 2005) expression that governs sex determination in TSD species could be upregulated or downregulated directly or indirectly by varying concentrations of exogenous estrogen. Future experimentation should also confirm a similar effect of estrogens on offspring sex ratios at warmer female-producing temperatures (e.g., 34–35°C) in E. macularius. These temperatures were not tested in this study.
because we chose to focus on the effects of estrogens in a range of thermal extremes; temperatures at which the most and fewest females are produced. Embryonic estrogens should be standardized across incubation temperatures to test for other effects of temperature in addition to controlling estrogen concentrations. The similar response to high and higher concentrations of estrogen between E. macularius (a TSD reptile) and Rana pipiens (a GSD amphibian) suggests a shared mechanism between species with different sex-determining mechanisms. Characterizing a similarity between TSD and GSD leads to novel hypotheses on how the mechanisms differ. Studies of the evolution and physiology of sex-determining mechanisms will benefit from recognition of shared patterns between TSD and GSD species. Also, novel conclusions regarding TSD and the effects of estrogen contaminants will help characterize the risk posed by the interaction of contamination and climate change on wildlife populations (Milnes et al., 2002).

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LITERATURE CITED


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