The effects of atrazine and temperature on turtle hatchling size and sex ratios

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Temperature influences some hormone-governed developmental processes, as do environmental contaminants known as endocrine-disrupting compounds. Although many vertebrates exhibit developmental sensitivity to temperature or contaminants, little research has focused on the potential interaction of these two external influences during development. Here, embryos of the red-eared slider turtle (*Trachemys scripta elegans*) are used to model the potential interaction of increased temperature and the herbicide atrazine. The atrazine level selected (0.5 parts per billion) was based on common environmental concentrations and is within the range of federally approved drinking-water levels. Sex ratio was the endpoint of population effect in this study, with an increase in females construed as an effect of either temperature, atrazine, or the two together. Mass, carapace length and width, and plastron length were also assessed as endpoints of individual effects of exposure. Results show that increased temperature or atrazine alone do not affect sex ratio, but that the two interact to significantly increase the female fraction. Plastron length, carapace length and width, and mass were higher in atrazine-exposed turtles kept at the lower temperature, a result with fitness implications.

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Temperature and endocrine-disrupting contaminants may interact as developmental factors. In vertebrates, temperature can affect hormone-governed developmental pathways, including those involved in sex development (Chardard *et al.* 1995; Crews 1996; Hayes 1998), behavior (Rhen and Crews 2000), and metamorphosis (Hayes 1998). Production or activity of steroid hormones is the inferred endpoint of these temperature-induced pathways (Crews 1996). The implication is that changes in temperature alter developmental hormone levels or activity, which in turn affect sex and other parameters. Small temperature changes can cause important changes in organisms; in some reptiles, a 4 °C difference in incubation temperature can shift nest sex ratio from 100% male to 100% female (Crews 1996). Developmental temperature also influences other parameters in reptiles, including growth rate and mass (Tousignant and Crews 1995; Du and Ji 2003).

Many contaminants disrupt the same pathways during vertebrate development, often mimicking or blocking natural steroid hormone activity, typically at very low (parts per billion [ppb]) concentrations (Crisp *et al.* 1998). Exposure to these low doses can result in the same outcome as exposure to increased temperature (Willingham and Crews 2000).

Research shows that small temperature increases can augment the effect of a single low dose of natural steroid during development (Crews 1996), raising the question of whether temperature can interact with contaminants that mimic steroids. The aim of the current research was to investigate whether a low, environmentally relevant dose of the commonly used herbicide atrazine interacts with increasing temperature to exert significant effects on sex development and other parameters in a temperature-sensitive species.

The species used to model these exposure effects in this study is the red-eared slider turtle, *Trachemys scripta elegans* (Figure 1), a species with temperature-dependent sex determination. Lower incubation temperatures (< 29.4 °C) produce a male-biased sex ratio in this species, while higher temperatures increase the female fraction; 31 °C results in 100% females. Estrogen exposure has the same effect as high temperature (ie more females) (Wibbels *et al.* 1994), and application of endocrine-disrupting contaminants produces the same result (Willingham and Crews 1999). The sensitive period for sex determination by exposure to temperature, estrogens, or contaminants is the middle third of development.

Materials and methods

Turtle eggs were purchased commercially (Kliebert Alligator and Turtle Farm, Hammond, LA) and transported to the laboratory. The eggs had all been laid on the same day and were maintained at 29.4 °C for 12 days, until brought to the lab. Eggs were maintained all together at room temperature for a week until embryos were at approximately stage 17 of development (Greenbaum 2002), as confirmed by three independent researchers. We selected 105 viable eggs from an initial group of 2000. The eggs were placed randomly into trays containing 1:1 vermiculite:water and incubated at one of

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Figure 1. *Red-eared slider turtle* (Trachemys scripta elegans).

two temperatures, either 26 °C or 29.2 °C. The supplier had placed the eggs in small containers in groups of 15–20; these containers were arrayed on a lab bench, and one egg from each was serially placed into a given incubation tray to avoid clutch effects. In addition, at the time of treatment, eggs were again shuffled around among the incubation trays. Because eggs were initially kept at 29.4 °C by the supplier, we anticipated a male-biased sex ratio, rather than 100% males. The 29.2 °C temperature is known to yield mixed-sex ratios in the absence of any other manipulation.

Eggs were not weighed or marked to enable linkage of initial egg mass and individual hatchlings. Such an approach had been attempted previously, using grouped eggs for uniform incubation, but turtles tend to emerge from the eggs in groups, making identification of the originating egg problematic. Placing eggs individually into separate containers would have introduced incubation variables involving the containers, location in the incubator, incubation materials, and humidity, any of which could conceivably have confounded our results. In addition, we did not have the opportunity to take the mass of the eggs on the day of laying at the commercial supplier. For these reasons, we did not attempt to link initial egg mass and individual hatchlings.

Eggs were placed in desktop incubators (Brinsea, Titusville, FL), and randomized again into one of three possible treatment groups per temperature: temperature control (no treatment), ethanol (vehicle) control, or atrazine-treated. Treatment with atrazine involved applying 5 µL of ethanol containing 5 ng/10-g egg (0.5 ppb) of atrazine (special preparation, Accustandard) to the eggshell using a pipettor (Wibbels *et al.* 1994). The atrazine dose was based on federal limits of allowable levels in drinking water. In the vehicle control group, 5 µL of ethanol was applied to the eggshell. Incubation continued to hatching; temperatures were monitored daily with the use of the incubator digital reading and an in-incubator thermometer, and egg groups were shifted daily to avoid any positional effects. Turtles hatched at stage 26 (Greenbaum 2002).

On the day of hatching (emergence from the eggshell), mass (0.1 g) was taken, and carapace width and length and plastron length (0.01 mm) were measured, using digital calipers. Carapace measurements were straightline, minimum (notch-to-notch) measurements. Hatchlings were then sacrificed by rapid decapitation and gonadal sex assessed using parameters described previously (Wibbels *et al.* 1994): Briefly, ovaries are long, thin, white, transparent, and not vascularized. Testes are short and round, opaque, and yellowish, and show evidence of seminiferous tubules. Presence or absence of oviducts was also noted. In this species, ovotestes are extremely rare (circa 1:20 000; pers observ) and have a characteristic baseball-bat shape; none were observed.

Two-by-two contingency table sex ratio data were analyzed using Fisher's exact one-tailed tests. One-tailed tests were applied because of the a priori assumption that atrazine would be estrogenic (Hayes *et al.* 2002). Version 2 of JMP (SAS Institute, Cary, NC) for PC was used for all statistical procedures. Length and width data were first analyzed by ANOVA for overall effects of single variables or interaction of variables; if a significant effect was found, further analysis between groups was performed (either ANOVA, student's t-tests, or nonparametric Wilcoxon).

Results

As expected, we obtained a male-biased sex ratio at the 26 °C temperature, and a circa 50:50 ratio at the 29.2 °C temperature (Figure 2). No significant differences in sex ratio or other parameters were found between the vehicle and temperature control groups, and these groups were combined as "temperature" for analysis, as has been done previously in similar work (Rhen *et al.*, 1999; Willingham *et al.* 1999; Willingham 2001). Neither the 0.5 ppb dose of atrazine nor the increased temperature alone significantly altered the female fraction (Figure 2). However, increased temperature combined with atrazine did significantly increase the female fraction compared to the 26 °C group ($P = 0.03$) (Figure 2).

Table 1 shows the results of a series of effects analyses performed for each of the following: mass, carapace length and width, and plastron length. For each, a P value is given. Where a P value of 0.1 or less resulted for all groups combined, further statistical analyses compared group to group (Figure 3a–d).

An analysis of temperature-by-treatment interaction on

mass revealed a significant effect (F = 6.9; df = 1; P = 0.0098); further investigation disclosed an effect of atrazine on mass at 26 °C (ANOVA, F = 5.3; df = 1, 49; P = 0.0252) (Figure 3a). Univariate ANOVA was used because a Shapiro–Wilks test indicated that the atrazine-treated and control groups had a normal distribution (P = 0.18). No other significant effects on mass, including sex and sex interaction with treatment, were observed (Table 1).

For carapace length, the same analyses were performed. The 26 °C–Atr population was not normally distributed and, again, the nonparametric Wilcoxon rank sums analysis was used to compare this group with the 26 °C group. The comparison of 26 °C and 26 °C–Atr groups showed a significant effect of atrazine on carapace length (X^2 = 6.23; df = 1; P = 0.012) (Figure 3c).

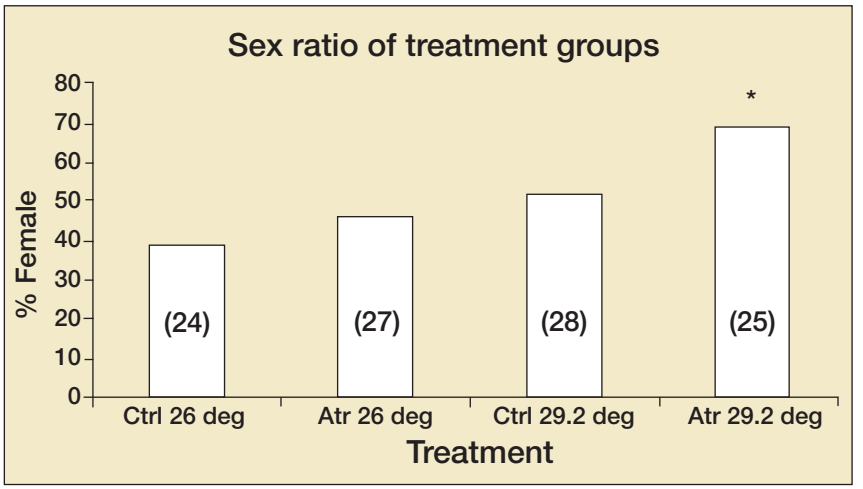

Figure 2. *Percent female hatchlings from each treatment group. N given in ( ) in bars. Asterisk indicates significant difference in female ratio versus 26 °C.*

An effects analysis of temperature-by-treatment interaction on carapace width resulted in further investigation (F = 3.53; df = 1; P = 0.06). A Shapiro–Wilks test for normal distribution indicated that neither the 26 °C nor the 26 °C–Atr populations were normally distributed; thus, a Wilcoxon rank sums nonparametric test was performed. Results indicated a significant difference in carapace width between the two groups (X^2 = 5.45; df = 1; P = 0.0197) (Figure 3b). Also observed was a potential effect of temperature on carapace width (P = 0.0720); however, ANOVA analysis of the 26 °C vs. 29.2 °C hatchlings did not warrant a conclusion of a significant effect (P = 0.066).

An analysis of temperature-by-treatment interaction on plastron length revealed a significant effect (F = 8.2; df = 1; P = 0.005). Both the 26°C and 26°C–Atr groups were normally distributed; further investigation showed an effect of atrazine on plastron length at 26 °C (ANOVA, F = 10.35; df = 1, 49; P = 0.0023) (Figure 3d). A potential temperature-based effect on plastron length was identified in the effects analysis (P = 0.0068; Table 1); however, this effect of temperature is attributable to the 26 °C hatchlings that were exposed to atrazine.

Discussion

The results imply that atrazine, temperature, and temperature and atrazine together, exert endocrine influences and interact with each other. The stepwise increase in the female fraction as temperature is increased, when atrazine is added, and in the presence of both, directly reflects the stepwise response to increased temperature or exogenously applied estrogens in this species (Crews 1996). With the assumption that temperature and/or atrazine indicate an endocrine signal or dose, each graph in Figure 3 exhibits an inverted U-shaped dose response to an endocrine signal that is increasing as a result of temperature or exogenous atrazine, or both. This type of curve is common in endocrine-disruptor studies (Welshons *et al.* 2003).

Endocrine endpoints under the influence of developmental temperature include gonadal sex in some fish (eg Conover and Heins 1987) and in amphibians (eg Chardard *et al.* 1995) and many reptiles (Janzen and Paukstis 1991), including all crocodilians and some turtles. Other such endpoints include growth rate (Rhen and Lang 1999) and behaviors (Rhen and Crews 2000). All of these parameters can influence individual fitness and thus population success. Sex ratios directly relate to population-level effects.

Atrazine is known to disrupt endocrine-governed developmental processes (Hayes *et al.* 2002). Some low-dose contaminants have also been shown to affect fitness parameters, including mass and growth rate, in red-eared slider turtles (Willingham 2002) and also to reverse sex in this species (Willingham and Crews 1999).

In spite of the established influence of temperature or hormone/hormone mimics on development in vertebrates, to my knowledge no work has been published on

Table 1. Effects analyses for mass, carapace length, carapace width, and plastron length (*P* values)

Effect tested	*Temp*	*Atrazine*	*Sex*	*Temp x atr*	*Temp x sex*	*Atr x sex*	*Temp x atr x sex*
Mass	0.6817	0.5292	0.2787	0.0098	0.2799	0.5831	0.1996
Carapace length	0.1593	0.5154	0.7668	0.0616	0.6193	0.4539	0.2363
Carapace width	0.0720	0.2574	0.9257	0.0631	0.2396	0.9152	0.2818
Plastron length	0.0068	0.2214	0.7947	0.0050	0.4416	0.9066	0.8101

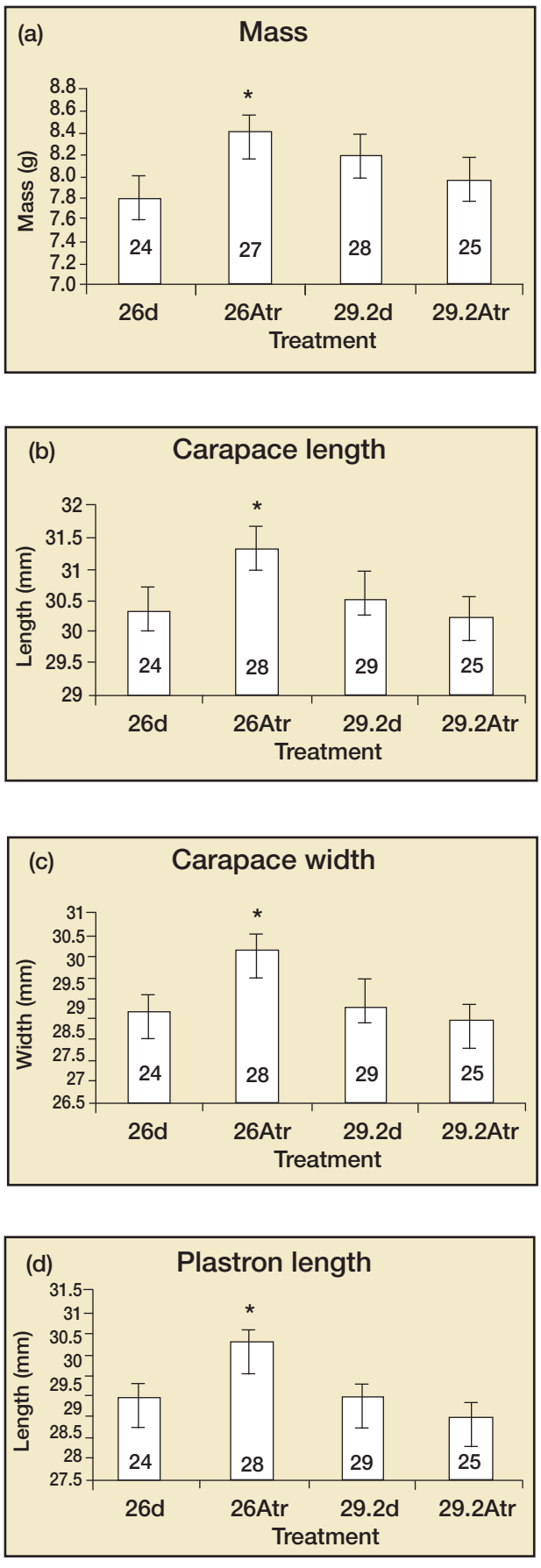

Figure 3. *(a) Mass, (b) carapace length, (c) carapace width, and (d) plastron length of hatchlings from each treatment group. Asterisk indicates a significant ($P < 0.05$) difference in the parameter compared to the 26 °C group. The population size for each group is given inside the bars.*

the potential for a hormone mimic such as atrazine to interact with temperature. The current work demonstrates for the first time that increased temperature alters the effects of an endocrine disruptor, with consequences at the population level.

Also, atrazine alters the expected size/mass of a hatchling to resemble that obtained at a mid-range temperature, with implications for individual fitness. A low, environmentally relevant dose of atrazine resulted in a significant increase in plastron length in turtles incubated at 26 °C. The 26 °C atrazine-exposed turtles were no different from the 29.2 °C groups in mass or carapace width or length. Thus, at a low temperature, atrazine produced the same result as the mid-range, higher temperature alone, namely bigger turtles. Size at hatching has been implicated as a fitness factor in reptiles, including turtles (Rhen and Lang 1999). Some research indicates that mid-range incubation temperatures may reduce fitness (Janzen 1995).

Atrazine may not have increased size or mass at 29.2 °C because hatchlings reached the limits of the species reaction norms for size or mass at that temperature. Research examining the effect of temperature from the high end of the incubation spectrum in this species indicates that that is the case (Willingham 2004).

The fact that we did not assess initial egg mass in the context of the hatchling size parameters leaves open the alternative explanation that initial egg mass was larger among the eggs in the atrazine-treated group and thus produced larger hatchlings. However, eggs were selected and placed in groups with uniformity in mind. In addition, atrazine at 26 °C had an effect on mass that is similar to that obtained with increased temperature (to 29.2 °C) in this study and in another study (Willingham 2004), an outcome that might be predicted if atrazine has estrogen-like effects (as per Hayes *et al.* 2002) and estrogen is also the endpoint of increased temperature.

It is possible that temperature and compounds such as atrazine, which interacted in this study, interact in other species that are influenced by developmental temperature. Although the current work used the turtle as a model for exposure, in a natural setting embryos would be exposed via contaminants in the yolk derived from the mother (eg Heinz *et al.* 1991) and would therefore be exposed from a much earlier period and more persistently.

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