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## THERMOSENSITIVE PERIOD FOR SEX DETERMINATION IN THE TUATARA

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**Abstract.**—Understanding the timing of the thermosensitive period, when the gonad is irreversibly committed to either a testis or ovary, in reptiles with temperature-dependent sex determination, is important for evaluation of effects of global warming. We investigated the thermosensitive period of the Tuatara (*Sphenodon punctatus*) using laboratory switching experiments, where incubation temperatures above 22° C produce male hatchlings. Sex is determined by 28% of the full incubation period at 23° C, the male-producing temperature (week 7 of an average 21 week incubation period), and 39% of the full incubation period at 20° C, the female-producing temperature (week 11 of an average 31 week incubation period). The thermosensitive period for the tuatara corresponds broadly to that of other reptiles, at the beginning of the middle third of development, although clearly temperatures prior to this period may also influence sex determination. Refining knowledge of the timing of the thermosensitive period is invaluable for interpreting the effects of nest temperatures on sex determination of hatchling tuatara, as well as predicting sex ratios under climate change scenarios.

**Key Words.**—reptile; *Sphenodon*; temperature-dependent sex determination; thermosensitive period; TSD; Tuatara

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### INTRODUCTION

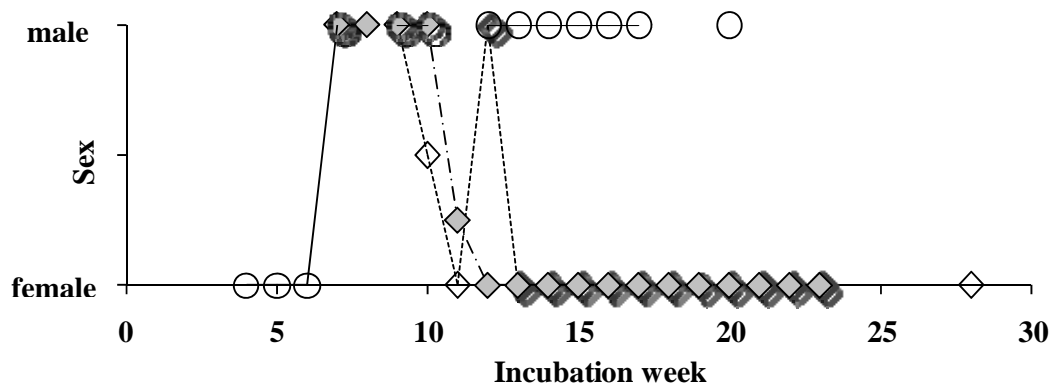
The window of time when temperature exerts its effect on the sex of developing embryos in species with temperature-dependent sex determination (TSD) is important for understanding how environmental factors affect sex determination (Valenzuela and Lance 2004; Shine et al. 2007). The thermosensitive period (TSP), when the undifferentiated gonad is irreversibly committed to either testis or ovarian development, occurs during the middle third of development (see reviews by Andrews 2004; Georges et al. 2004). However, temperatures in natural nests fluctuate daily and seasonally, and are influenced by other events such as rainfall, making the relationship between incubation temperature and its effect on developmental rate difficult to align with incubation time (Georges et al. 2004). For species where eggs are laid early in development, for example, tuatara, crocodylians, and turtles (Dendy 1899; Shine 1983; Bull 1987), the middle third of development may commence more than a month after laying (e.g. in tuatara, Mitchell et al. 2008; in crocodylians, Lang and Andrews 1994) and occur over a period of days or even weeks (Bull 1987; Andrews 2004; Shine et al. 2007). It is only after the TSP has been identified that sex ratios can be predicted and consequent effects on population viability evaluated with respect to increasing temperatures and more variable weather with global climate change (Mitchell et al. 2008; Schwanz and Janzen 2008).

We investigated the TSP of the Tuatara (*Sphenodon punctatus*), using laboratory switching experiments to refine prediction of sex ratios of hatchlings in natural nests. In tuatara, incubation may take 11-16 months (McIntyre 1997) and the TSP is currently an

estimation based on correlation (Mitchell et al. 2006). Sex is determined by the type 1b or female-male pattern of TSD in tuatara, where 100% males are produced from incubation temperatures above 22° C, with a transitional range of less than 1° C (Mitchell et al. 2006). The type II, or female-male-female pattern, has not been confirmed for tuatara (Mitchell et al. 2006), despite the re-classification of most type 1b species as having type II TSD after further experimentation (e.g. Lang and Andrews 1994; Harlow 2004).

Tuatara construct natural nests at depths between 10–230 mm, resulting in highly variable thermal regimes and nesting success. During summer, embryos in successful warm nests are exposed to temperatures over several hours within the range expected to result in mortality of adults (> 30° C; Heatwole 1982), and temperatures may vary 15° C over 24 h due to the relatively shallow construction. In winter, successful nests may be exposed to temperatures as low as 1.6° C (Nelson et al. 2004c). The TSP is estimated to occur between 25 and 55% of development (Mitchell et al. 2006) based on correlation between incubation temperatures from natural nests and sex of the resulting hatchlings (Nelson et al. 2004a). Little is known about the TSP with respect to gonad differentiation in tuatara, as gaining access to eggs for lethal experiments is difficult due to their threat classification and novelty status as the only living representatives of the reptilian order Rhynchocephalia (also known as Sphenodontia) with a limited distribution on offshore islands of New Zealand (Dendy 1899; Gaze 2001).

High hatching success (over 90%) of tuatara eggs has been achieved by incubation at constant temperatures between 18 and 23° C (Nelson et al.



**FIGURE 1.** The number of weeks to sex determination of tuatara hatchlings based on incubation temperatures. Sex is determined by week 7 of an average 21 week incubation period at 23° C and week 12 of an average 31 week incubation period at 20° C. Open circles represent individual eggs shifted from 23 to 20° C; open diamonds (2006) and grey diamonds (2007) represent individual eggs shifted from 20 to 23° C. The sex of two tuatara juveniles was ambiguous (0.25 and 0.5 male). The 0.25 male indicates the average sex of two eggs switched at week 11: one female and one ambiguous (female?).

2004a, 2004b; Mitchell et al. 2006). Hatching success in natural nests varies between 0–100%, with on average only 48% of eggs alive after 12 months (Thompson et al. 1996). Female tuatara on Stephens Island (Takapourewa), the densest population of tuatara (Gaze 2001), lay a clutch of nine eggs (mean size; range 1–19) every 2–5 years (Cree 1994). Eggs are laid at the very early stage of gastrulae, similar to turtles (Moffat 1985). We aimed to define the TSP in tuatara under controlled artificial incubation conditions.

#### MATERIALS AND METHODS

We collected 79 tuatara eggs from 10 clutches from Stephens Island (Takapourewa), Cook Strait, New Zealand for incubation experiments at Victoria University of Wellington (VUW) in November 2006 and October 2007. In 2006, eggs were sourced within 24 h of laying in natural nests, held at < 20° C on Stephens Island, and placed in constant temperature incubation at either 20 or 23° C within five days of laying. Two clutches were laid 4 November and incubated from 8 November, one clutch was laid 8 November and incubated from 13 November, and one clutch was laid 15 November and incubated from 20 November. In 2007, we induced six gravid females to lay eggs (Thompson et al. 1991) between 15–17 October. We held eggs at < 20° C on Stephens Island and incubation began at VUW 19 October.

Incubation at 20° C results in female hatchlings and incubation at 23° C results in male hatchlings (Nelson et al. 2004b, Mitchell et al. 2006). We switched eggs weekly from their starting incubation temperature to the alternative temperature from approximately 25% to 75% of the full incubation period (Fig. 1). We only switched eggs once. In 2006, we switched eggs either from the female-producing temperature to the male-producing temperature, or vice versa. We randomly

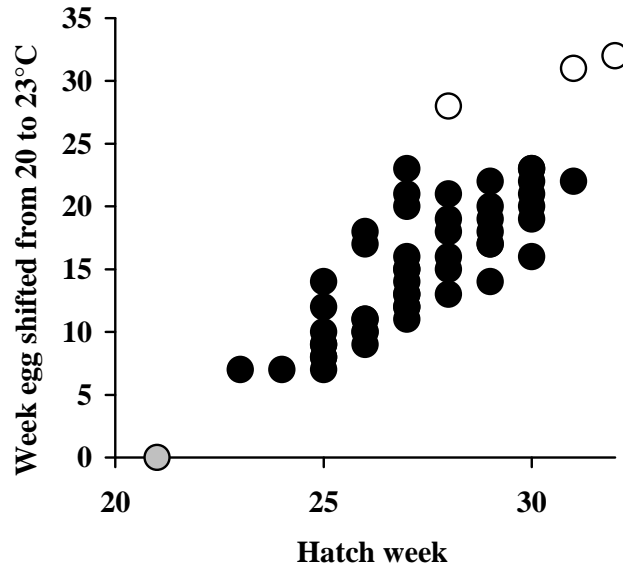
assigned incubation temperatures and switch weeks to eggs, while allowing for equal spread of clutches in incubation treatments and through time. In 2007, all eggs started incubation at the female-producing temperature, and we switched two randomly selected eggs each week to the male-producing temperature. At least one egg was retained at each incubation temperature as a control to confirm the pattern of sex determination. We reared hatchlings in captivity and determined sex by laparoscopy of the right side gonad at approximately one year of age (Cree et al. 1991; Nelson et al. 2004a).

Incubation periods are variable for tuatara (Nelson et al. 2004b) even under identical incubation conditions. Hence, the full incubation period for each temperature was defined as the mean incubation time (in weeks) of the control eggs. We calculated percentage development as the number of weeks since laying (as temperatures were constant) divided by mean incubation period (in weeks).

#### RESULTS

We found that sex is determined by week 7 of an average 21 week incubation period at the male-producing temperature of 23° C (28% of full incubation period) and by week 12 of an average 31 week incubation period at the female-producing temperature of 20° C (39% of full incubation period; Fig. 1). The duration of the TSP is not known. Incubation duration is temperature-dependent, whereby eggs developed faster and hatched earlier if they spent proportionally more time at 23° C (Fig. 2).

The sex of two hatchlings was ambiguous (Fig. 1; labeled 0.25 and 0.5 male). In one, the male-shaped gonad was clearly visible with no evidence of the normally observed seminiferous tubules. In the second, the mullerian duct was clearly visible, but we could not confirm the sex of the gonad (?female)



**FIGURE 2.** The incubation period of tuatara eggs compared to hatch period. Incubation periods were shorter for eggs that spent proportionally more time at 23°C ( $R^2 = 0.72$ ), demonstrating higher incubation temperature increases the rate of development. The control eggs remaining at 20°C hatched after 28-32 weeks incubation (open circles). The control egg remaining at 23°C hatched after 21 weeks (grey circle).

within the time frame allocated for each surgery. We shifted both of these eggs at the end of the thermosensitive period (week 11) from the female to the male-producing temperature. The 0.25 male indicates the average sex of two eggs switched at week 11: one female and one ambiguous (female?). The sex of one juvenile does not conform to the proposed thermosensitive period (Fig. 1; open diamond at 12 weeks).

### DISCUSSION

The thermosensitive period (TSP) occurs by 28% of embryonic development at male-producing incubation temperatures and 39% of embryonic development at female-producing incubation temperatures for tuatara, and is supported by concurrent experiments involving switching eggs from female to male-producing incubation temperatures, and vice versa. Although the end of the TSP appears to occur earlier at male-producing temperatures, this difference is likely to be amplified by our method of switching eggs weekly for both treatments. One week at male-producing temperatures accounts for proportionally more development than at female-producing temperatures, and consequently we have fewer data points that represent coarser measurements for the male-producing temperatures. In addition, we only have one year of data for embryos shifted from male to female-producing temperatures.

The TSP, based on correlation between effective incubation temperatures (constant temperature equivalents, or CTE's; Georges et al. 2004) in natural nests and sex of the resulting hatchlings (Nelson et al.

2004a), was estimated to occur between 25 and 55% of development (Mitchell et al. 2006). However, we predict the effective temperatures have occurred prior to that period as embryo sex is set by 28–39% development when eggs are incubated at constant temperatures. Temperatures were only recorded in one location of nests that may include up to three layers of eggs, in soil depths where temperatures fluctuate daily and seasonally (Nelson et al. 2004c). Therefore, although models used to estimate CTE's of nests may predict sex ratios of cool or warm nests that are likely to be 100% female or male (respectively), they cannot be expected to accurately predict sex of all hatchlings from nests experiencing conditions near the pivotal temperature for sex determination.

Switching experiments primarily involved moving only one or two eggs between each experimental temperature once a week, and no back switching, for example 20-23-20° C, due to the difficulty in obtaining tuatara eggs for these types of studies. Nevertheless, the pattern of sex determination was clear, with only two hatchlings of ambiguous sex that were shifted at the end of the TSP. The only hatchling that did not conform to the proposed pattern was from the clutch that stayed on Stephens Island longest and was exposed to temperatures < 20° C for a longer period, and therefore experienced slower development during the first week post-laying.

The timing of the TSP for tuatara corresponds to that of other oviparous reptiles with TSD, at the beginning of the middle third of development, although clearly temperatures prior to this period may influence sex determination of developing embryos (Deeming 2004; Ewert et al. 2004; Harlow 2004). The TSP in

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viviparous lizards is also comparable; thermal choice by gravid mothers during the middle third of embryonic development influences sex of offspring (Robert and Thompson 2001). For tuatara and turtles, the TSP also corresponds to the beginning of the middle third of the incubation duration at constant temperatures due to the early stage of laying (gastrulae). This pattern differs for the skink *Bassiana duperreyi*, for example, where the TSP also occurs at about 30% development but the timing is immediately following laying at the onset of incubation due to prolonged uterine retention of developing embryos by mothers (Shine et al. 2007). Back-switching experiments will be required to establish the duration of the TSP, and histological and molecular studies will be needed to confirm that the TSP occurs during the same period as gonadogenesis. Knowledge of the timing of the TSP will be invaluable for interpreting the effects of temperatures in natural nests on sex determination of hatchlings, where current information suggests that the TSP occurs during the hottest period of the year (Mitchell et al. 2006), an unfortunate pattern given global warming.

*Acknowledgments.*—Thanks to Kim Miller, Jon DeVries, Clare Allen, Jeanine Refsnider and Nicki Mitchell for assistance in the field, with egg weighing, and comments on the manuscript, and Peter Gaze and Ngati Koata Trust for permits to access Takapourewa for research purposes. Research was carried out under Department of Conservation permit number NM-16723-RES and Victoria University of Wellington animal ethics permit number 2006R12. Funding for this research was provided by the Allan Wilson Centre for Molecular Ecology and Evolution, the Zoological Society of San Diego, Education New Zealand, and The Company of Biologists. Thanks to Mike Thompson and Scott Parker for organizing the symposium and this conference volume. This manuscript was written in memory of Raj Radder.

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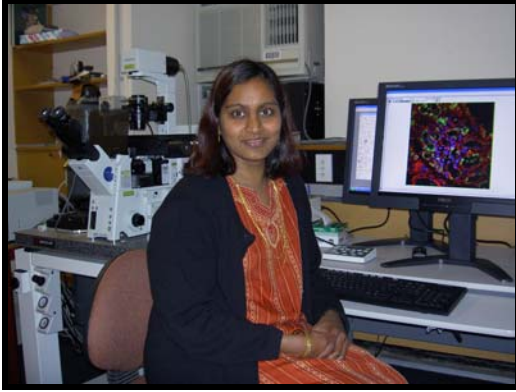


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