Pivotal range and thermosensitive period of the pig-nosed turtle, *Carettochelys insculpta* (Testudines: Carettochelydidae), from northern Australia

Jeanne E. Young, Arthur Georges, J. Sean Doody, Peter B. West, and Rachael L. Alderman

Abstract: Understanding temperature-dependent sex determination in nature often depends on knowledge of species-specific attributes that are integrated into the relationship between temperature and sex. We determined two such attributes for the pig-nosed turtle, *Carettochelys insculpta* Ramsay, 1886, in tropical Australia: the pivotal range in temperature that separates the male-producing domain from the female-producing domain, and the thermosensitive period during which the embryonic sex is influenced by temperature. The pivotal range for *C. insculpta* was very narrow, spanning only about 1 °C, and was centered on 32 °C, which is high but consistent with temperatures reported for other tropical species. The thermosensitive period spanned developmental stages 17–21 for temperature influence in the direction of maleness and 18–21 for temperature influence in the direction of femaleness. This period is slightly narrower than that for other reptile species but broadly consistent with the middle third of incubation.

Résumé : La compréhension de la détermination sexuelle reliée à la température en nature exige une connaissance des caractères spécifiques à l'espèce qui sont intégrés dans la relation entre la température et le sexe. Nous avons découvert deux de ces caractères chez la tortue à nez de cochon, *Carettochelys insculpta* Ramsay, 1886, d'Australie tropicale : l'étendue charnière de température qui sépare le domaine de production de mâles de celui de production de femelles, ainsi que la période de thermosensibilité pendant laquelle le sexe de l'embryon est influencé par la température. L'étendue charnière de température chez *C. insculpta* est très étroite, s'étendant sur seulement environ 1 °C et centrée sur 32 °C; il s'agit d'une température élevée, mais semblable à celles qui ont été signalées chez d'autres espèces tropicales. La période de thermosensibilité couvre les stades 17–21 pour les températures qui mènent vers la production de mâles et de 18–21 pour celles qui mènent à la production de femelles. C'est une période plus restreinte que chez d'autres espèces de reptiles, mais elle correspond grosso modo au tiers médian de la période d'incubation.

[Traduit par la Rédaction]

Introduction

Temperature-dependent sex determination (TSD) in reptiles has received considerable attention in recent decades, and we now know much about its modes of expression in various species (Ewert and Nelson 1991; Ewert et al. 1994). However, an evolutionary explanation for TSD remains elusive (Shine 1999; Valenzuela et al. 2003), partly because too few studies have been undertaken in the context in which TSD evolved, that is, under natural conditions.

Temperature varies in natural reptile nests on two temporal scales. There is the periodic diel cycle brought about by the cycles of night and day, and there is the aperiodic variation that results from seasonal trends in temperature and local day-to-day variation in weather. We have a reasonable understanding of the interplay between average incubation temperature and diel temperature variation in determining offspring sex (Georges 1989; Georges et al. 1994, 2004) with good predictive power, at least in the laboratory (Georges et al. 1994), and a growing understanding of the effect of aperiodic variation in temperature on offspring sex, though good predictive power is still elusive (Valenzuela 2001).

Using temperatures of natural nests to predict offspring sex also requires knowledge of two intrinsic attributes of the organism in question. We need to know the pivotal temperature or the pivotal range (Mrosovsky and Yntema 1980; Bull 1980 (threshold temperature); Mrosovky and Pieau 1991), which is the typically narrow range of temperatures within which both sexes are produced and which separates a predominantly male-producing domain of temperature from a

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J.E. Young,[1,2] **A. Georges, J.S. Doody, and R.L. Alderman.** Applied Ecology Research Group and Co-operative Research Centre for Freshwater Ecology, University of Canberra, Canberra, ACT 2601, Australia.
P.B. West.[3] Vertebrate Pest Research Unit, NSW Agriculture, Forest Road, Orange, NSW 2800, Australia.

[1]Corresponding author (e-mail: jeanne.young@cdu.edu.au).
[2]Present address: P.O. Box 237, Melrose Park, South Australia 5039, Australia.

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predominantly female-producing domain of temperature. Some species have two pivotal ranges (Yntema 1979; Gutzke and Paukstis 1984). We also need to know the thermosensitive period, which is the period within which temperature exerts its influence on sex. It is typically defined in terms of embryonic stage, and at constant temperatures corresponds approximately to the middle third of incubation in most species that have been studied (Yntema 1979; Bull and Vogt 1981; Pieau and Dorizzi 1981; Souza and Vogt 1994). Under the more complex thermal regimes of natural nests, it is thought to correspond to the same stages of embryonic development, but its temporal duration and location will shift dramatically depending on the thermal regime experienced. Both the pivotal range and the thermosensitive period vary among species (Yntema 1979; Bull and Vogt 1981; Mrosovsky and Pieau 1991; Souza and Vogt 1994).

Knowledge of the interaction between the complex thermal environment of natural nests and offspring sex, and knowledge of the pivotal range and thermosensitive period are critical to field-based studies of maternal nest site selection (Doody et al. 2003), to studies of the potential for maternal manipulation of offspring sex ratios (Roosenberg 1996; Olsson and Shine 2001), to the formulation of evolutionary explanations for TSD (Shine 1999; Valenzuela et al. 2003), and for addressing other important questions about the microevolution of TSD systems. Such knowledge is also important for understanding the likely effect of climate change on species with TSD.

The objectives of this study were to experimentally determine the pivotal range and thermosensitive period of the pig-nosed turtle, *Carettochelys insculpta* Ramsay, 1886, from tropical Australia. We chose *C. insculpta* because it is the only freshwater species in Australia known to have TSD (Webb et al. 1986) and because it lays its eggs in shallow nests that are subject to wide daily temperature fluctuations (Georges 1992). We used constant-temperature incubation experiments to determine the pivotal range, and switching experiments (between constant-temperature incubation conditions) to determine the thermosensitive period.

Materials and methods

Egg collection and transport

Eggs of *C. insculpta* were collected during the dry season from the Daly River, Northern Territory, between Claravale Crossing (14°20′S, 131°34′E) and the inflow of Jinduccin Creek (14°07′S, 131°17′E), and transported by boat to Oolloo Crossing. Eggs for experiments on pivotal range were transported by road to Darwin Airport and couriered by air to a research laboratory at the University of Canberra. Those for the switching experiments of 1998 were transported by road to Douglas Daly Research Farm, 35 km from Oolloo Crossing. Eggs allocated to the supplementary switch experiments in 2000 were transported by road to the Northern Territory University, Darwin. Maximum and minimum egg diameters were measured with vernier callipers (±0.1 mm), and the eggs were weighed on an electronic balance (±0.1 g). Embryos were aged using the procedures outlined by Beggs et al. (2000) together with observations made on the age of nesting crawls and other signs at the nesting site.

Table 1. Hatchling sex ratios produced at a range of constant incubation temperatures for *Carettochelys insculpta* from the Daly River, Northern Territory.

Nominal temperature (°C)	Actual temperature (°C; mean ± SD)	No. of males	No. of females	Mortality
28.0	27.96±0.07	7	0	0
30.0	30.02±0.04	11	0	0
30.5	30.51±0.02	13	0	2
31.0	30.98±0.04	14	0	1
31.5	31.52±0.05	24	0	6
32.0	31.96±0.08	14	10	2
32.5	32.50±0.01	0	14	1
33.0	33.02±0.05	0	10	5
34.0	34.00±0.04	0	15	2
36.0	35.98±0.06	0	0	15

Note: Data for 31.5, 32.0, and 34.0 °C are combined data from experiments conducted in 1995 and 1996. No hatchlings survived at a constant temperature of 36 °C.

Pivotal range

All eggs used in the experiments to determine the pivotal range were ≤8 days old, equivalent to Yntema (1979) stage 6 or younger, at the time of transportation to experiment locations. Eggs that failed to begin development, as indicated by failure to develop an opaque patch (Thompson 1985; Beggs et al. 2000), were not included in experimental allocations.

In a 1995 pilot study, eggs from 10 clutches were allocated systematically to temperatures of 30.5, 31.0, and 31.5 °C to eliminate any among-clutch variation and to yield sample sizes of 15 eggs per treatment. These temperatures were chosen because 30 °C is known to produce 100% males and 32 °C is known to produce 100% females (Webb et al. 1986). Remaining eggs from each clutch were evenly divided between 30 and 32 °C to yield sample sizes of 11 eggs per treatment. Eggs from a second batch of five clutches, surplus to eggs for other experiments, were allocated to 28 and 34 °C to yield sample sizes of 7 eggs per treatment. In 1996, eggs from 15 clutches were allocated systematically over temperatures of 31.5, 32.0, 32.5, 33.0, 34.0, and 36.0 °C to yield sample sizes of 15 eggs per treatment except the 34.0 °C treatment, to which only 10 eggs were allocated (building upon the 5 eggs that survived this treatment in the pilot study). The resulting sample sizes for the combined data set are shown in Table 1.

All eggs were incubated in 2-L plastic tubs containing vermiculite (4 g water per 3 g vermiculite) and housed in refrigerated incubators (Thermoline Refrigerated Incubator, Model RI 170). Eggs were buried in the vermiculite, and the moisture content of the substrate was monitored at weekly intervals and held constant. Humidity in the containers was high but was not measured. Temperatures were measured using mercury thermometers (±0.1 °C) calibrated against a thermometer certified by the Australian National Authority of Testing Agencies. Thermometer bulbs were in close proximity to the eggs, and temperatures were recorded twice daily.

Table 2. Summary of results (percentage of *C. insculpta* that were male) from single-switch experiments.

Shift stage	Developmental stages[a]	Direction[b]	% Males[c]	N[d]	Mortality
	8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26				
12		↓	100	10(3)	0
		↑	0	10(3)	1
13		↓	100	9(2)	0
		↑	0	9(2)	0
14		↓	100	9(2)	1
		↑	0	6(2)	1
15		↓	100	8(3)	1
		↑	0	9(3)	1
17		↓	100	7(2)	0
		↑	0	7(2)	0
18		↓	100	7(2)	0
		↑	0	8(2)	0
21		↓	0	8(4)	0
		↑	100	9(4)	0
22		↓	0	7(2)	0
		↑	100	6(2)	0

[a]Based on Beggs et al. (2000); shaded regions represent incubation at the initial temperature and open regions represent the incubation pulse at the alternate temperature.
[b]Arrows denote direction of temperature shift: ↓, 34 to 30 to 34 °C; ↑, 30 to 34 to 30 °C.
[c]Mean percentage of males per clutch.
[d]Number of eggs (number of clutches in parentheses).

After sufficient incubation to bring the eggs to full term (Beggs et al. 2000), the hatchlings were stimulated to hatch by immersion in water (Webb et al. 1986). They were weighed (±0.1 g) and killed by intracranial injection of pentobarbitone. Hatchlings were dissected and the urinogenital system was examined under a dissecting microscope. In particular, the presence of a well-developed (female) or degenerate (male) paramesonephric duct (Mullerian duct) was noted. The right gonad, kidney, and associated ducts were removed, fixed in 10% buffered formalin, embedded in wax, sectioned, and stained with Harris's haematoxylin and eosin (Clark 1981). The sex of each gonad was determined by examination under a light microscope according to the criteria of Webb et al. (1986).

Thermosensitive period

The end points of the thermosensitive period were determined by using a series of single- and double-switch experiments in which eggs were shifted between nominated male- and female-producing temperatures of 30 and 34 °C, respectively. These temperatures were chosen based on the results of the pivotal range experiments and because they are common average temperatures in natural nests (Georges 1992).

Fifty-two clutches of eggs (mean, 11 eggs per clutch; range, 6–16) were collected for use in a total of 19 switch experiments (Tables 2, 3). Eggs were candled before allocation to ensure that they were no older than stage 8 (as described in Beggs et al. 2000). Eggs for each experiment were placed in labeled containers of moist vermiculite in constant-temperature incubators (Thermoline Refrigerated Incubator, Model RI 170) following the protocols outlined above for the pivotal range experiments. Full clutches were allocated to each experimental treatment, with eggs from each clutch split evenly between the two alternate switch directions.

In single-switch experiments, eggs were incubated at the initial temperature (either 30 or 34 °C) until a predetermined developmental stage was achieved, and then the eggs were shifted to the alternate temperature for the remainder of development (Fig. 1*a*). Switches were thus in two directions: 30 to 34 °C (male to female) and 34 to 30 °C (female to male). In double-switch experiments, eggs were incubated at the initial temperature (either 30 or 34 °C) until a predetermined developmental stage was achieved, and then shifted to the alternate temperature for a prescribed number of developmental stages (2, 4, or 6) before being returned to the initial temperature (Fig. 1*b*). Switches were again in two directions: 30 to 34 to 30 °C, and 34 to 30 to 34 °C.

All eggs were candled every second day to assess development (size of embryo, haemodisc and allantois, position and curvature of embryo, sensu Beggs et al. 2000) to determine when eggs had reached the required stage for switching. Stage of development was verified by opening one egg from each clutch for each switch. Early-stage embryos were

Table 3. Summary of results (percentage of *C. insculpta* that were male) from double-switch experiments.

Shift stages[a]	Developmental stages[b]	Direction[c]	Duration[d]	% Males[e]	N[f]	Mortality
	11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26					
16-18		↓	6	0	7(2)	1
		↑	4	100	4(2)	1
17-19		↓	6	0	8(2)	0
		↑	5	100	8(2)	0
18-20		↓	5	0	8(2)	0
		↑	4	100	6(2)	2
19-21[g]		↓	7	0	4(2)	2
		↑	6	100	5(2)	0
20-22		↓	10	0	8(3)	1
		↑	8	100	6(3)	3
16-20[h]		↓	9	17	8(3)	1
		↑	7	100	8(3)	1
17-21[h]		↓	11	33	8(2)	0
		↑	9	100	4(2)	4
18-22[h]		↓	12	50	12(4)	0
		↑	9	56	13(4)	0
19-23[g]		↓	17	75	10(3)	1
		↑	13	33	9(3)	0
20-24		↓	22	0	11(3)	1
		↑	12	100	13(3)	1
16-22[g]		↓	15	100	4(2)	0
		↑	13	0	3(2)	0

[a]Eggs were shifted to the alternate temperature at the start of the first indicated stage and returned to the initial temperature at the start of the last indicated stage.
[b]Based on Beggs et al. (2000); shaded regions represent incubation at the initial temperature and open regions represent the incubation pulse at the alternate temperature.
[c]Arrows denote direction of temperature shift: ↓, 34 to 30 to 34 °C; ↑, 30 to 34 to 30 °C.
[d]Mean number of days for incubation pulse.
[e]Mean percentage of males per clutch.
[f]Number of hatchlings for which sex was determined (number of clutches in parentheses).
[g,h]Occurrence of single-sex clutch (*g*) (not included in "% Males" calculations) and intraclutch variation (*h*).

killed by chilling the eggs below 5 °C for a minimum of 24 h. Late-stage embryos were killed by intracranial injection of pentobarbitone. At the end of each experiment, hatchlings were killed and the sex was determined both visually and histologically as in the pivotal range experiments.

Results

Pivotal range

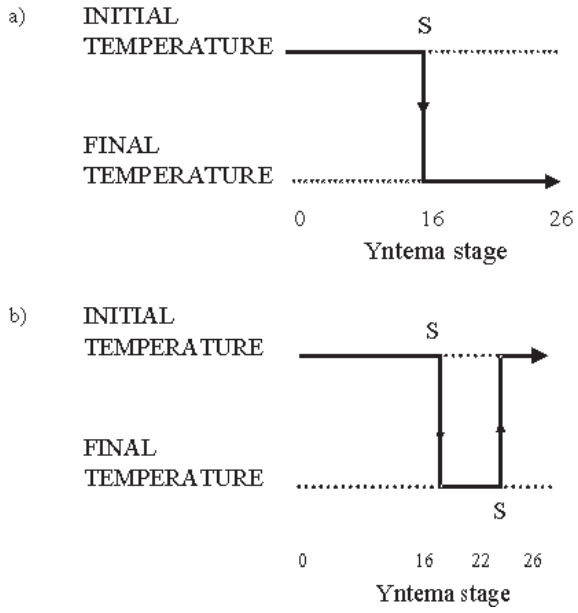
In the pilot study, all eggs at temperatures of 28–32 °C produced male hatchlings, and all eggs at 34 °C produced female hatchlings. In the 1996 study, 100% males were produced at 31.5 °C, both sexes were produced at 32.0 °C (5 males : 10 females), and 100% females were produced at 32.5–34.0 °C. No eggs survived the 36 °C treatment. The pivotal range for *C. insculpta* is clearly centred on 32 °C, with a remarkably narrow range of temperatures producing both sexes (Table 1).

Thermosensitive period

Single-switch experiments

Hatchling sex was determined by the post-switch incubation temperature, regardless of shift direction, in all single-switch experiments in which the temperature shift occurred at stage 18 or earlier (Table 2). In contrast, hatchling sex was determined by the pre-switch incubation temperature in

Fig. 1. Conceptual diagrams of (*a*) single- and (*b*) double-switch experiments in which *Carettochelys insculpta* eggs were shifted between male- and female-producing temperatures of 30 and 34 °C, respectively. "S" indicates a switch and a sacrifice of an embryo for confirmation of developmental stage.



all single-switch experiments where the temperature switch occurred at stage 21 or later (Table 2).

Double-switch experiments

Hatchling sex was unaffected by a switch pulse of only two developmental stages, regardless of the direction or timing of the switch (initial shift occurring at stages 16, 17, 18, 19, or 20 in separate experiments) (Table 3).

When eggs were shifted from 34 °C (female-producing) to 30 °C (male-producing) for a four-stage pulse, the pulse had a masculinizing effect in experiments when the pulse started at stages 16 to 19 but not when the pulse commenced at stage 20. The percentage of hatchlings that were male increased as commencement of the pulse moved from stage 16 to stage 19 (Table 3, Fig. 2).

In the complementary experiments where eggs were shifted from the male-producing temperature to the female-producing temperature for a four-stage pulse, the pulse had a feminizing effect only when it commenced at stages 18 and 19 (Table 3, Fig. 2). No females were produced in response to a pulse that commenced at stages 16, 17, or 20.

For a pulse of six stages (stages 16–22), the temperature that prevailed during the pulse determined the sexual outcome of the hatchlings for shifts in both directions (Table 3).

In 4 of the 5 four-stage double-switch experiments, both male and female offspring were produced from eggs of the same clutch incubated under the same experimental conditions (Tables 2 and 3). Subsequent review of the recorded developmental history of each egg from the clutches with intraclutch variation revealed no apparent developmental abnormalities in any instance. We also observed examples in the two-, four-, and six-stage double-switch experiments of clutches that produced only one sex, regardless of the experimental conditions (Tables 2 and 3). The data from these clutches were excluded from further analysis.

Paramesonephric duct

In the pivotal range experiments and the single-switch experiments, the paramesonephric duct was degenerate in all male hatchlings and well developed in all female hatchlings. For the double-switch experiments, the condition of the paramesonephric duct was more variable. All females had fully developed ducts, but degeneration of the duct in male hatchlings varied from full degeneration (experiments with an initial switch at stage 20) to partial degeneration becoming progressively more pronounced posteriorly (most other double-switch experiments). Histological sections of the paramesonephric duct corroborated the visual diagnosis, and additional serial sections of the gonads of individuals with partial duct development confirmed that these hatchlings were males rather than intersexes (see Pieau and Dorizzi 1981).

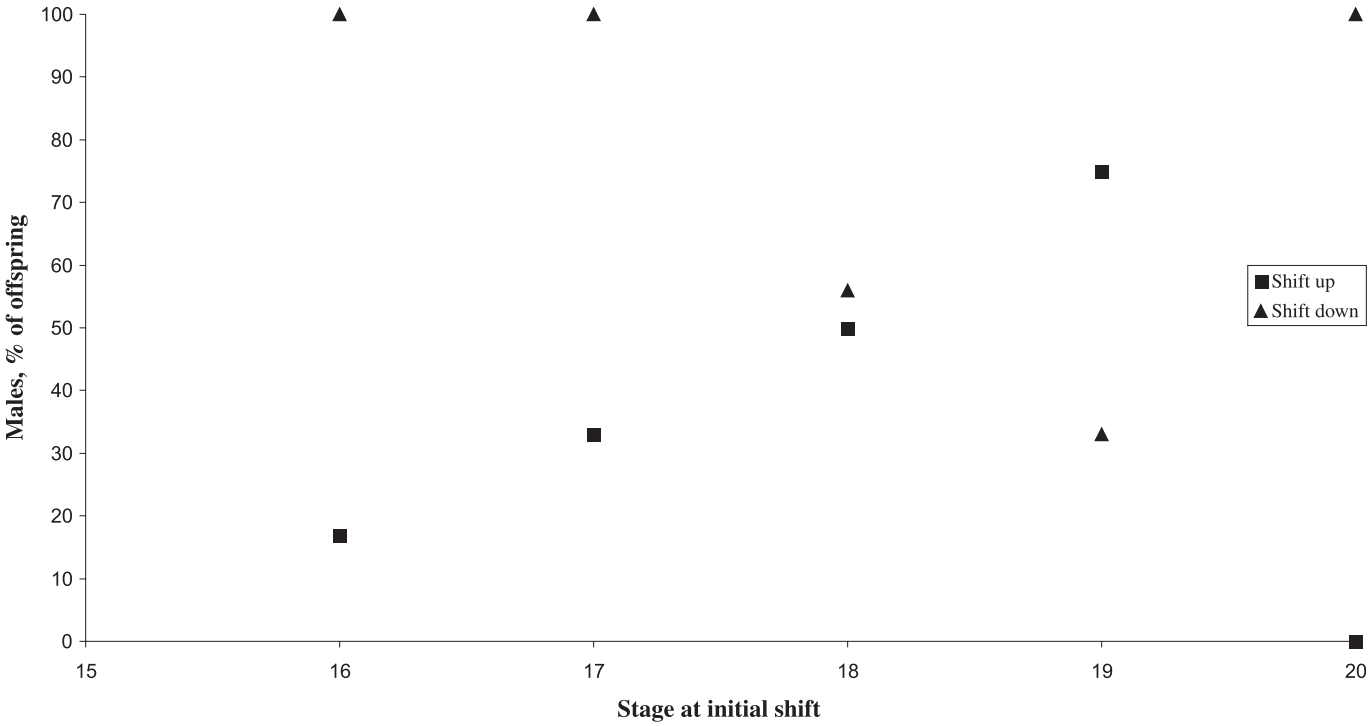
Discussion

The pivotal range of 32 ± 0.5 °C is one of the highest of any turtle species (Souza and Vogt 1994; Valenzuela 2001) and is consistent with the observation that tropical freshwater species tend to have higher pivotal ranges than temperate-zone species (Ewert and Nelson 1991). The pivotal range can be very narrow, as in *C. insculpta*, or quite broad, as in *Caretta caretta* (L., 1758) (Georges et al. 1994). A broad pivotal range may arise as a variant in the mechanism that links sex determination to temperature or because of intraclutch variation in the response to temperature, which smears the pivotal range when data from many clutches are combined. The very narrow range of temperatures that make up the pivotal range of *C. insculpta* indicates that intraclutch variation in the response to a constant temperature regime is slight. However, we did observe intraclutch variation in the response to the double-switch experiments, which may indicate that the necessary duration of exposure to a temperature that influences sex varies among embryos.

Single-switch experiments identify the stage of development after which incubation temperatures will no longer have an influence on hatchling sex. In the present study, when temperatures were initially in the female-producing domain (34 °C) and were subsequently switched to the male-producing domain (30 °C), irreversibility had not been achieved by stage 18 but was achieved by stage 21 (Table 2). The four-stage double-switch experiment supplemented the single-switch experiments and indicated that irreversibility was achieved by at least stage 20 (experiment 20-24, Table 3). A similar outcome was obtained when temperatures were initially male-producing and were subsequently switched to female-producing.

The double-switch experiments, using a pulse of sufficient duration to influence sex, provided additional information on the thermosensitive period of *C. insculpta*. Two-stage pulses did not influence sex in any experiment, but four-stage pulses did affect sexual outcomes. Analysis of the four-stage pulse experiments indicated that any four-stage pulse from the female- to the male-producing temperatures that included stage 19 influenced sexual outcomes, which suggests a thermosensitive period somewhere within stages 16–22. A

Fig. 2. Mean percentage of *C. insculpta* offspring that were male from double-switch temperature experiments that had a pulse duration of four developmental stages. The initial shifts were made at the start of the stage indicated on the *x* axis.



pulse that included stages 20–23 had no influence on sexual outcomes, yet stages 20 and 21 were influential in combination with stage 19. This suggests that stages 22 and beyond are not influential. The trend in offspring sex ratios evident in Table 2 indicates that a pulse from stages 15–18 would be unlikely to have an effect on offspring sex. Hence, stage 16 in combination with stages 17 and 18 is unlikely to be influential, yet stages 17 and 18 are influential in combination with stage 19. This suggests that stage 16 is not influential. We conclude that the thermosensitive period for the influence of the male-producing temperature (30 °C) is defined by stages 17–21.

We can repeat this reasoning for four-stage pulses from the male- to the female-producing domain. Four-stage pulses from stages 18–21 and 19–22 influenced sexual outcomes, which suggests a thermosensitive period somewhere within stages 18–22. However, a pulse including stages 20–23 had no influence on sexual outcomes, yet stages 20 and 21 (at least) were influential in combination with stage 19. This again suggests that stage 22 is not influential. We conclude that the thermosensitive period for the influence of the female-producing temperature (34 °C) is defined by stages 18–21.

Our concept of the thermosensitive period requires some clarification. We define it as including any stage that, alone or in combination with adjacent stages, has an influence on hatchling sex ratios. For example, a pulse comprising stage 19 in combination with one adjacent stage, and presumably a pulse consisting of stage 19 on its own, has no influence on hatchling sex. An effect is apparent when the pulse includes, at a minimum, stage 19 and any two adjacent stages. It is the combination of all three stages that leads to the influence and thus we regard the thermosensitive period as comprising all three stages. The combined effect may result from an accumulated influence (Bull and Vogt 1981) or some other form of interaction whereby a combination of three adjacent stages is influential, yet each stage alone or a pair of stages is not. Our thermosensitive period is thus equivalent to the secondary thermosensitive period of Bull and Vogt (1981), in that it is the period of gonadal sensitivity to temperature rather than their period of irreversible sex determination (achieved by stage 20 in both directional switches in *C. insculpta*). Even so, the thermosensitive period for *C. insculpta* is more tightly constrained than for some other species, where it corresponds to the stages of embryonic development in the middle third of development under constant conditions (stages 16–22).

Determination of the pivotal range and thermosensitive period of *C. insculpta* provides two key parameters used in modeling sexual outcomes in natural nests (Georges et al. 2005), thus facilitating studies aimed at better understanding the relationship between nest temperatures and offspring sex, the way in which mothers might manipulate offspring sex (Roosenberg 1996), the significance of TSD in evolutionary terms, and the potential responses of species with TSD to climate change.

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