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The temperature-sensitive period (TSP) during incubation of broad-snouted caiman (*Caiman latirostris*) eggs

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Abstract. All crocodiles studied to date exhibit temperature-dependent sex determination. During the many weeks from egg laying to hatch there is a period of 10 to 15 d in the middle third of incubation (in the American alligator) during which the sex of the embryo is irreversibly fixed, referred to as the temperature-sensitive period or TSP. In this work we investigated the TSP in *Caiman latirostris* eggs incubated at female-inducing and male-inducing temperatures (29°C and 33°C respectively) by switching eggs from 29°C to 33°C and vice versa at timed interval throughout incubation. Compared to *Alligator mississippiensis* the duration of TSP was longer, and the onset of TSP was at an earlier stage of incubation.

Keywords: *Caiman*, Alligatoridae, Crocodylia, Reproduction, Eggs, Incubation, Sex determination.

Introduction

All crocodilians are said to exhibit temperature-dependent sex determination (TSD), but not all species have been fully investigated (Lang and Andrews, 1994; Deeming, 2004). For most crocodilians the incubation period lasts for many weeks, from 63-85 d in the American alligator (*Alligator mississippiensis*) to as long as 90-110 d for *Crocodylus porosus* (Webb et al., 1987; Lang and Andrews, 1994). During the many weeks of incubation there is a period of 10 to 15 d in the middle third of incubation (in the American alligator) during which the sex of the embryo is irreversibly fixed, referred to as the temperature-sensitive period or TSP (Mrosovsky and Pieau, 1991; Lang and Andrews, 1994). Male-inducing or female-inducing temperatures applied outside the TSP have no effect on the sex of the embryo.

The TSP however, has been investigated in only four of the 23 species of extant crocodilians, *A. mississippiensis*, *C. palustris*, *C. porosus* and *C. johnstoni* (Webb et al., 1987; Lang and Andrews, 1994).

The Broad-snouted caiman, *Caiman latirostris*, has a large latitudinal range in South America, from 5° South (Verdade, 2001) to 32° South (Melo, 2002). Because of thermal differences between these latitudinal extremes, it is likely that there are differences among populations in TSD and TSP, as is seen in populations of the Snapping turtle, *Chelydra serpentina* separated by ~29° of latitude (Ewert et al., 2005). Nest (or incubator) temperature affects both the sex of the embryos and the duration of incubation. At 33°C, a temperature that produces 100% males, the duration of incubation is 69.9 ± 5.1 d (mean ± SD); at 29°C, a temperature that produces 100% females, the duration of incubation is 80.9 ± 3.7 d (mean ± SD; Piña et al., 2003).

There have been no studies on latitudinal variation in any of the crocodilian species. In this paper we present data on the TSP in one of the southernmost populations of *C. latirostris*,

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from Santa Fe, Argentina (S31°37′56″ W60°41′ 58″), and compare this with the American alligator, a close relative of the broad snouted caiman, and a species for which there is extensive information.

Materials and methods

In Santa Fe, Argentina, *C. latirostris* lay eggs from late December to mid January. Hatching takes place from late February to late March. Caiman eggs were collected from the breeding stock at Estación Zoológica Experimental in Santa Fe. Nests were checked every morning during the laying period to insure that there were no clutches older than 12 hr post-oviposition. The entire clutch from each nest was transferred to the laboratory and placed in incubators. A total of 375 eggs from 11 nests collected over four consecutive years were used in the experiment.

From each clutch we randomly assigned groups of five eggs to each incubation treatment. Two control groups, to provide 100% male and female hatchlings (29 and 25 eggs respectively), were incubated at constant temperature of 33°C and 29°C for the entire incubation period. Each experimental treatment group was placed at an initial temperature of 33°C or 29°C and then shifted to 29°C or 33°C according to the schedule shown in table 1.

Incubators consisted of plastic containers (80 × 45 × 30 cm) filled to a depth of approximately 10 cm with water. An aquarium heater with a thermostatic control was placed in the water, and above the water a sheet of plastic. Damp nesting material was placed on the plastic sheet into which the eggs and a temperature data logger (Onset Computer Corporation, Pocasset, MA) were placed. The data loggers were programmed to record temperature every thirty minutes and were checked daily. Each incubator was covered with a Styrofoam lid and sealed with plastic wrap to ensure high humidity. Using these incubators temperature was maintained within ±0.5°C, and the relative humidity close to saturation.

Upon hatching, the caimans were marked on both hind feet using Monel tags (#001; National Band and Tag Co., Newport, KY), and hatch date recorded. The sex of each animal was determined after 10 months when they had reached a length of 60 cm. At this size sex determination is unambiguous by examination of the phallus (Joanen and McNease, 1978; Webb et al., 1984; Allsteadt and Lang, 1995; Piña et al., 2003). The sex of dead embryos ($n = 34$) was determined macroscopically based on shape, texture and color of the gonads, and by presence or absence of Müllerian ducts.

Data on duration of incubation were analyzed by one-way ANOVA, where temperature was the grouping factor and incubation period the response. Tukey's test (HSD) was applied to test for differences among groups (Zar, 1996). The data set was tested for normality with Shapiro-Wilks test's on the residuals of the ANOVA.

Results

Length of incubation

A number of eggs failed to hatch and some of the hatchlings lost their identification tags (compare tables 1 and 2), nevertheless a sufficient sample size was available for statistical analysis. In general, length of incubation was affected by temperature ($F_{22,178} = 35.77$; $P < 0.001$, table 1), but in some cases the combination of temperatures was not additive. The greater the amount of time eggs were at 33°C, the shorter the incubation period. Controls at 33°C and 29°C were not significantly different from number of days previously reported, (mean ± SD) 72.6 ± 2.9 ($n = 14$) vs. 69.9 ± 5.1 ($n = 12$), and 84.7 ± 4.3 ($n = 10$) vs. 80.9 ± 3.7 d ($n = 12$) respectively (Piña et al., 2003). Eggs at 33°C for up to 25 d then shifted to 29°C had an incubation period not significantly different from those at 29°C for the entire time. Eggs at 29°C for ten days and then switched to 33°C had an incubation period no different from eggs at 33°C for the entire incubation (table 1).

Sex determination

All surviving eggs at 33°C ($n = 18$) produced males, and all eggs at 29°C ($n = 13$) produced females, similar to results previously reported (Piña and Donayo, 2000; Piña et al., 2003; Stocker et al., 2003). The results of the temperature-shift treatments are shown graphically in figure 1. In table 2 the numbers of males and females produced at each incubation regime are presented. Switching temperatures up to 18 d, from 29°C to 33°C, or vice versa, had no effect on hatchlings' sex, and likewise shifts after 39 d had no effect. There are, however, several days between 18 and 25 and between 39 and 46 for which we have no data. At an initial temperature of 29°C, the ability to induce the male sex by switching to 33°C is already weak by day 39, only two males and 21 females (table 2), suggesting that by day 40 or 41 the female sex is firmly fixed. In eggs initially incubated at 33°C

Table 1. Number of days (±SE) to hatching at different treatments. In column A comparisons are made between duration of incubation at 29°C for the entire period and those switched from 29° to 33°C; in column B between duration of incubation at 33°C for the entire period and those switched from 29° to 33°C; in column C between duration of incubation at 29° for the entire period and those switched from 33° to 29°C; in column D between duration of incubation at 33° for the entire period with those switched from 33° to 29°C. Asterisks indicate a significant difference at $P < 0.01$ for all comparisons.

Days at initial temp	29°C	*n*	A	B	33°C	*n*	C	D
10	72.5 ± 0.29	4	*		82.2 ± 0.92	5		*
18	76.59 ± 0.51	17	*	*	78.92 ± 0.82	13		*
25	77.87 ± 0.29	15	*	*	75.73 ± 0.49	15	*	
32	80.94 ± 0.38	18		*	75.58 ± 0.59	19	*	
39	80.14 ± 0.64	14		*	75.2 ± 0.49	15	*	
46	83.14 ± 0.77	7		*	72	1	*	
53	84.25 ± 1.11	4		*	73.25 ± 1.11	4	*	
60	87.8 ± 0.37	5		*	75.25 ± 1.03	4	*	
67	87.8 ± 1.07	5		*	74 ± 0	2	*	
74	88 ± 0.77	5		*	75	1	*	
81	90.25 ± 0.48	4		*				
Complete	84.7 ± 1.35	10		*	72.64 ± 0.77	14	*	

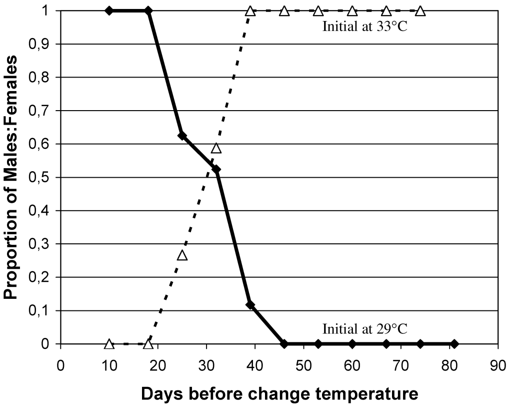

Figure 1. Proportion of male and female *C. latirostris* hatchlings produced during temperature-shift experiments.

Table 2. Sex of *Caiman latirostris* hatchlings from different incubation treatments. *The numbers in each column are the male and female hatchlings produced. For example, 32 days at 29°C before switching to 33°C resulted in 13 males and 14 females. The shaded area represents the period during which sex is determined, the thermo-sensitive period.

Days at	29°C		33°C	
initial temp	Male	Female	Male	Female
10	4*	0	0	5
18	14	0	0	12
25	13	9	4	15
32	13	14	12	9
39	2	21	26	0
46	0	8	1	0
53	0	3	3	0
60	0	5	5	0
67	0	5	2	0
74	0	5	2	0
81	0	4	–	–
No switch	0	13	18	0

the male sex is fixed sometime between 33 and 38 d.

Discussion

Length of incubation

While incubation at 33°C clearly results in a shorter incubation period than at 29°C, an initial incubation temperature of 33°C for the first 18 d, followed by 29°C did not reduce total incubation time. There was no significant difference in duration of incubation between these eggs and those maintained at 29°C throughout incubation. Eggs maintained at 33°C for 32 d and then switched, however, did have a significantly shorter incubation period than eggs at 29°C throughout incubation. By 32 d the higher temperature is having an effect. The duration of incubation in eggs at an initial temperature of 29°C for 10 d, followed by 33°C, was no longer than eggs maintained at 33°C throughout incubation (table 1). These results would suggest that the very early stages of development are not affected by temperature, at least at the two temperatures tested. After 18 d at an initial temperature of 29°C, however, an effect on length of incubation becomes apparent. Eggs at this initial temperature had a significantly longer incubation period than eggs at 33°C throughout incubation (table 1). These results agree with those of Larriera et al. (1996) working on the same species, who suggested that incubation temperature has little effect on the duration of incubation during the first one or two weeks. Similarly, eggs held for 25 d at 33°C and then switched to 29°C until hatch did not have a longer incubation period than eggs at 33°C for the entire incubation period. The cooler temperature during the second half of incubation did not appear to slow development.

One of the principal causes of variability in studies on crocodilian egg incubation is the clutch effect (Lang and Andrews, 1994; Piña et al., 2003). To reduce the clutch effect in this experiment, eggs from different clutches were randomly distributed among treatments. Nevertheless, some of the treatments with very small sample sizes gave results that were difficult to explain. For example, the length of incubation in the one egg that survived 74 d at 33°C (table 1) had a longer incubation period than eggs at 33°C for the entire incubation period (75.0 d vs 72.6 d). Clutch variability is also apparent in the unusually long incubation period in eggs at 29°C for 60 through 81 d before being shifted to 33°C (table 1). What is clear from table 1 is that at an initial temperature of 29°C, the total incubation period, after a switch at 25 d, is significantly longer than eggs switched earlier in incubation. In eggs at an initial temperature of 33°C, the total incubation period is shorter after a switch at 18 d.

Sex determination

Two differences between *C. latirostris* and *A. mississippiensis* stand out: the earlier onset of the TSP at 29°C and 33°C in the caiman as compared to the alligator, and the longer period of thermal sensitivity in the caiman (fig. 2). These results also differ markedly from what has been published on *C. porosus*, *C. johnstoni* and *C. palustris* (Webb et al., 1987; Lang and Andrews, 1994). When initial incubation temperature is 33°C the TSP occurs between d 19 and d 38, a total of 19 d. When initial incubation temperature is 29°C the TSP is between d 19 and 46, a total of 26 d. It is possible that the onset of the TSP is two or four d later in incubation (table 2). Further research will give a more accurate timing of the TSP. Despite this uncertainty, the TSP in *C. latirostris* is clearly different from other crocodilians. The TSP recorded for *A. mississippiensis*, *C. palustris* and *C. porosus* is 15 d, and for *C. johnstoni* only seven d (Lang and Andrews, 1994). As can be seen in figure 2, the TSP in *C. latirostris* occurs much earlier in incubation than in *A. mississippiensis* despite having a similar thermal regime that produces males and females and a total incubation period at 33°C that is actually longer than the alligator. Curiously, at 29°C the incubation periods for the alligator and caiman are identical (Lang and Andrews, 1994). At 29°C the TSP occurs in *C. latirostris* during the first third of the incubation period, whereas in *A. mississippiensis* at the higher temperature of 31°C, the TSP occurs after more than 40% of the incubation period. Incubation of American alligator eggs at a constant temperature of 33°C takes 63-65 d (Lang and Andrews, 1994), whereas incubation of the broad-snouted caiman eggs at 33°C, takes at least 10 d longer (table 1). These results for duration of incubation at a constant temperature at 33°C differ slightly from those of Stoker et al. (2003) who reported 66.7 ± 0.5 and 65.7 ± 0.7 for three and five clutches of *C. latirostris* eggs from Santa Fe. These differences are the result of an unusually wide variation in incubation length among clutches of *C. latirostris* as previously reported by Piña et al. (2003). In other crocodilian species investigated the range of incubation period at different temperatures is less variable (Lang and Andrews, 1994).

A detailed study of development in *C. latirostris* (Iungman, 2006) has compared embryonic stages of *A. mississippiensis* (Ferguson,

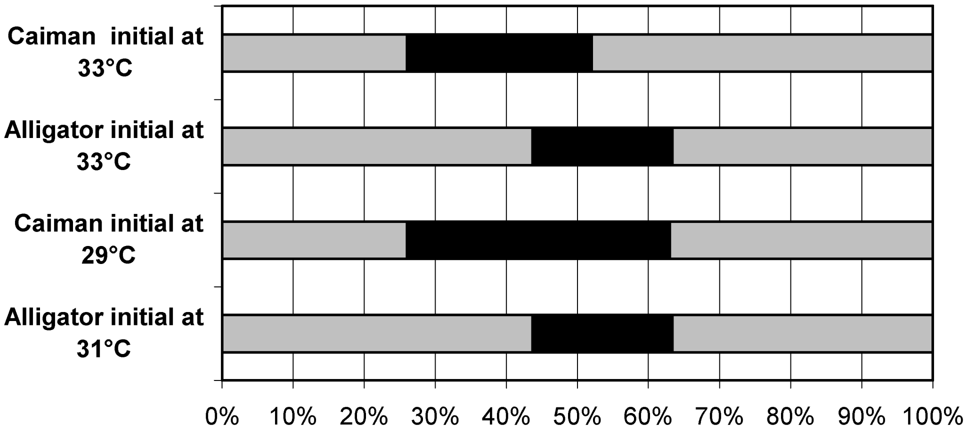

Figure 2. Comparison of the thermosensitive periods (black portions of the horizontal bars) in *C. latirostris* and *A. mississippiensis* as a percentage of total incubation. Direct comparisons at the lower temperatures are not possible as there is no information on the alligator at 29°C.

1985) with those of *C. latirostris*, and shown that Stage 21 (the onset of TSP) in the caiman occurs earlier during incubation than in the alligator. For the first 10 d of incubation at 31°C, the stages of *C. latirostris* are identical to that of *A. mississippiensis*, but by d 15 they start to diverge.

It is not clear at present if the TSP described here for *C. latirostris* is peculiar to the southernmost population from Santa Fe, Argentina, or is typical of the species as a whole. Further research on the TSP and TSD of the more northerly populations and a more precise delineation of the TSP of *C. latirostris* is currently underway.

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