
NEW EVIDENCE CHARACTERIZING TEMPERATURE-DEPENDENT SEX DETERMINATION IN BROAD-SNOUDED CAIMAN, *CAIMAN LATIROSTRIS*

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Abstract.—Temperature-dependent sex determination (TSD) occurs in all three families of the Crocodylia. This study explored the sex ratio of hatchlings under incubation temperatures not previously tested in *Caiman latirostris* to delineate the transitional range of temperature (TRT) between male- and female-producing temperatures. We also estimated the pivotal temperature (T_{piv}) of *C. latirostris* from our study site. We incubated eggs at various temperatures (31°, 32°, 33°, and 34° C). Incubation temperature had a significant effect on sex determination, but had no effect in hatching success. Eggs incubated at 31° C produced 100% females, 32° C produced approximately 70% females, and incubation at 33° C and 34° C produced only males. The TRT was > 31° to < 33° C for female to male transitions, and > 34° C and a temperature > 34.5° C male to female. Results indicate that the limits of T_{piv} should be between 32–33° C (lower T_{piv}), and between 34–34.5° C (upper T_{piv}) for the population of *C. latirostris* in Santa Fe, Argentina. Our analysis support the female-male-female (FMF) pattern, but higher temperature females may be rarely seen in wild because survival of females incubated at such high temperatures is very low.

Key Words.—Crocodylia; incubation; pivotal temperatures; sex determination; transition temperatures

INTRODUCTION

Sex can be determined genetically or environmentally. Temperature-dependent sex determination (TSD) is a type of environmental sex determination in which temperature experienced during embryonic development influence the sex of the offspring. It has been shown to occur in many fish (Conover 2004), most turtles (Ewert et al. 1994), and some lizards (Rhen and Crews 1999). In addition, researchers have demonstrated TSD in the 12 crocodylian species examined, which occur in all three designated families of the Order Crocodylia (Lang and Andrews 1994; Piña et al. 2003; Deeming 2004; Valenzuela 2004).

Within the family Alligatoridae, the American Alligator (*Alligator mississippiensis*) was the first

species documented with TSD (Ferguson and Joanen 1982) in which certain incubation temperatures were shown to generate sexual phenotype ratios of 50%. Mrosovsky and Pieau (1991) defined these certain incubation temperatures as pivotal temperatures within TSD species (T_{piv}). Of note, the pivotal temperature for a particular TSD species resides within a transitional range of temperatures (TRT), or the range of constant incubation temperatures that produce both sexes in variable sex ratios. Broad-snouted Caiman (*Caiman latirostris*) as described by Ewert et al. (1994) exhibits Pattern II TSD (female-male-female), which includes low viable temperatures (29° C and 31° C) producing 100% females, 33° C producing 100% males, and higher viable temperature (34.5° C) producing some females but with a very low hatching success (16%, Piña et al.

2003, 2007; Jungman and Piña 2013). However, T_{piv} and TRT have yet to be determined for this species and our aim was to characterize these defined thermal endpoints within a population of *C. latirostris* from Santa Fe Province, Argentina.

MATERIALS AND METHODS

We collected Broad-snouted Caiman eggs from nine wild nests shortly after oviposition during two breeding seasons, and transported to the Proyecto Yacaré facilities in Santa Fe province, Argentina. We determined individual egg viability by the presence of an opaque eggshell banding and we included only those eggs considered viable ($n = 172$) in the experiment. We determined embryo stage (i.e., clutch age) by opening one representative egg from each clutch following Jungman et al. (2008). At the beginning of the experiment, all clutches had a structural age lower than stage 10 (equivalent to 10 d of incubation at 31° C), which is at least 10 d younger than the beginning of the reported temperature-sensitive period reported for the species (Piña et al. 2007). We evenly distributed eggs from each clutch among established temperature incubation treatment groups, with two replicates for each treatment group (total 10 incubators, four in the first breeding season and six in the second one).

We incubated eggs in constant-temperature incubators (Model SEMEDIC I-290D, INGELAB, Buenos Aires, Argentina) following Piña et al. (2005) and we maintained the eggs at high humidity following Jungman et al. (2008; humidity records not maintained). We incubated eggs at either 32° C or 33° C ($\pm 0.3^\circ$ C) during the first breeding season with two replicates (total four digital incubators), and at 31° C, 33° C, or 34° C ($\pm 0.3^\circ$ C) during the second breeding season with two replicates (total six digital incubators). Digital recording verified that incubation temperatures remained within 1° C of their overall mean throughout the incubation period in both seasons. For example, 100% of recorded temperatures in the most stable incubator varied only $\pm 0.2^\circ$ C (from 30.8° to 31.2° C), while 83% of temperatures in the least stable incubator varied -0.2° C to 0.3° C (from 30.8° to 31.3° C); both incubators produced 100% females. We monitored temperatures with HOBOTM Data Loggers ($\pm 0.1^\circ$ C; Onset Computer Corporation, Pocasset, Massachusetts, USA) placed throughout the plastic trays and checked daily throughout the incubation period.

After hatching, we maintained caiman ($n = 141$) under farming conditions (Piña and Larriera 2002). At four months of age, we determined sex in the majority of animals ($n = 132$) by histological methods ($n = 100$, 76%) and visual examination of the clitero-penis ($n = 24$, 18%; Nuñez Otaño et al. 2010). It was not possible

to determine sex for the remaining nine individuals (6% of total).

We analyzed data using Info-Stat® (Di Rienzo et al. 2008). We expressed all values as mean \pm standard deviation (SD) with statistical significance set at $P \leq 0.05$. We tested variables for normality with the Kolmogorov-Smirnov test, and homogeneity of variances between groups verified with the Levene test. Data were normal and homoscedastic. We compared proportions of females to males at each nest temperature using Chi-square. To compare hatching success among incubation temperatures, we used a one-way ANOVA with temperature treatment as the grouping factor and hatching success (# of hatchlings / # of eggs) of every nest as dependent variable. We reported the TRT bounded by two limits: the limit of maximum masculinization (100% individuals) and the limit of maximum feminization (Mrosovsky and Pieau 1991). We determined TRT from a prior study of TSD in *C. latirostris* (Piña et al. 2003), and with the sex ratios determined under the new incubation temperatures used in this study. To estimate T_{piv} (Mrosovsky and Pieau 1991), we plotted the percentage of females against incubation temperature and identified the two consecutive incubation temperatures that include the temperature that should produce a 50% sex ratio. We obtained supplementary data for the graph (eggs incubated at 29° C and 34.5° C) from Piña et al. (2003), a study made in the same caiman population.

RESULTS

Incubation temperature affected sex determination in *C. latirostris*. We obtained 100% females at 31° C, 71.6% females at 32° C, and 0% females at 33° C and 34° C (Fig. 1). At 32° C the proportion of females varied significantly among nests (17–100% females, $\chi^2 = 11.98$, $df = 4$, $P = 0.017$). We determined lower TRT to be above 31° C and under 33° C, while the upper TRT has the lower limit above 34° C, and the higher limit at a temperature higher than 34.5° C, because we do not yet know the upper limit for maximum feminization and embryo viability. Lower T_{piv} should be between 32° and 33° C (closer to 32° C), and higher T_{piv} should be between 34° and 34.5° C (closer to 34.5° C; Fig. 1). We did not find a significant difference in hatching success among treatments ($F_{3,18} = 0.57$, $P = 0.643$) at 31° C ($91 \pm 19\%$), 32° C ($85 \pm 14\%$), 33° C ($78 \pm 15\%$), and 34° C ($81 \pm 24\%$). Average hatching success was $83 \pm 5.5\%$.

DISCUSSION

We verified that incubation temperature has a significant effect on sex determination in *C. latirostris*. Similar to previous reports, a 33° C incubation

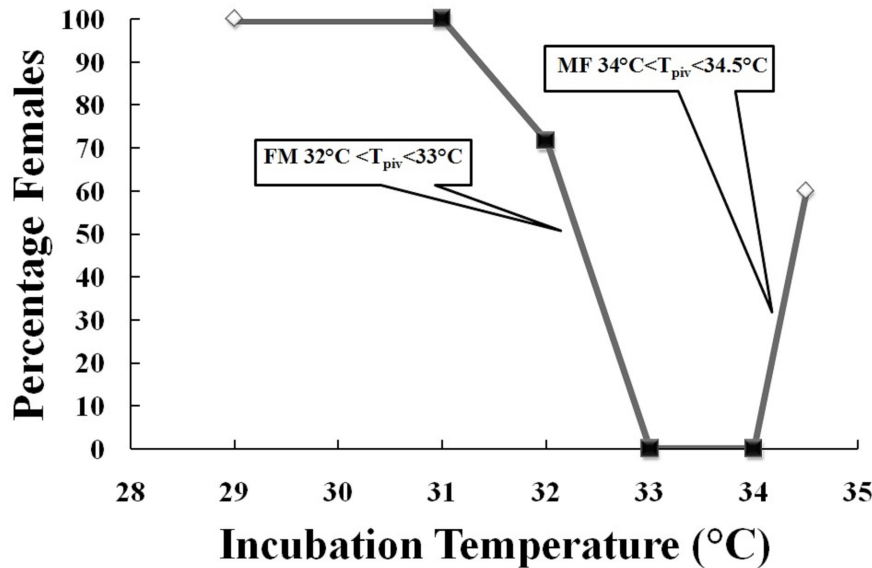


FIGURE 1. Percentage of female Broad-snouted Caimans (*Caiman latirostris*) resulting from each incubation temperature in this study (black squares). The figure also shows the range of temperatures that include pivotal temperature (T_{piv}) in both transitions (Female-Male: $32^{\circ}\text{C} < T_{piv} < 33^{\circ}\text{C}$, and Male-Female: $34^{\circ}\text{C} < T_{piv} < 34.5^{\circ}\text{C}$). Data points at 29°C and 34.5°C (white diamonds) are from Piña et al. (2003).

temperature produced 100% males, and 31°C produced 100% females (Piña et al. 2003, 2007; Iungman and Piña 2013; Iungman et al. 2015; Parachú Marcó et al. 2015). Our information indicates that 32°C incubation temperature produces approximately 70% females and 34°C incubation temperature produces 100% males in *C. latirostris*. Prior studies additionally reported that low incubation temperatures (29 and 31°C) produce 100% females, and incubation at 34.5°C produces 60% females (Piña et al. 2003).

Inter-clutch variability in sex ratios was reported in different species when incubation temperature is within the TRT (Mrosovsky 1988; Rhen and Lang 1998; Pieau et al. 1999; Godfrey et al. 1999; Ewert and Nelson 2003). Here we showed that incubation at 32°C led to 17%, 60% and 80% females in three clutches, and 100% females in two others. Though our results are based on a relatively small sample size (25 hatchlings, four to six hatchlings per clutch), they demonstrate that factors other than mean incubation temperature can affect sex ratios: yolk estrogen levels (Elf et al. 2002; Elf 2003; Uller et al. 2009), stress level of females while follicles develop (Lovern and Wade 2003), or genetic influence (Mrosovsky and Pieau 1991; Holleley et al. 2015). Developmental rate (Webb et al. 1987), acid-base balance (Etchberger et al. 1992), and humidity (Packard and Packard 1987) also have been shown to influence TSD. We did not measure these parameters, but we think it is reasonable to assume that they were homogeneous within our experimental design.

The relationship between our controlled laboratory results and TSD in wild incubated nests is uncertain. A recent study examined the effects of climatic conditions on sex ratios produced by caimans in wild nests and determined that the mean temperature inside *C. latirostris* nests affected sex determination of hatchlings, where the mean internal temperature was close to 31°C and produced around 68% females (Simoncini et al. 2014). This sex ratio approximated the 70% of females found in the current study when incubated at 32°C under constant temperature, and it is different than 100% females produced when incubated at a constant temperature of 31°C . This is likely the result of daily and weekly variation in wild incubation temperatures compared to stable lab conditions, though the exact explanatory mechanism is yet unknown.

In this work for the first time, we tested an incubation temperature that produced both sexes within the female to male TRT for *Caiman latirostris*, and this could help to understand possible effects of future climate change. Recent climate change models predict that ambient temperatures could increase between 1.5° and 2°C by the end of the 21st Century (Intergovernmental Panel on Climate Change 2014). These proposed increases in ambient temperatures have potentially significant physiological (hatching success; Piña et al. 2007) and/or ecological (skewed population dynamics) impacts for crocodylians throughout their respective ranges (Godley et al. 2001; Mrosovsky et al. 2002; Simoncini et al. 2014). Increasing air temperatures such as this could

not only result in decreased hatching success, but may also result in sex ratio bias in TSD species (Godley et al. 2001; Mrosovsky et al. 2002; Simoncini et al. 2014). Even a 1.5° C increase on incubation temperature could produce a difference in sex ratios in wild caiman populations. If 31° C in the wild produces a sex ratio close to 32° C constant incubation treatment, and if only a 1° C increase changes the sex ratio from 70% females to 100% males, even the lowest estimated increase of 1.5° C would affect natural sex ratio toward males influencing reproduction of wild populations. There could be a modification on female behavior because of this temperature increase, but we assume that a modification of the construction of the nest is unlikely. We know that temperature during the previous week triggers nesting (Simoncini et al. 2013), so females may adjust to increasing temperature by laying eggs earlier on the season. The sex ratios of *Alligator mississippiensis* and *Caiman crocodilus yacare* hatchlings can vary from site-to-site and year-to-year depending on local climatic conditions (Campos 1993; Rhodes and Lang 1996), but they usually tend to overproduce females, and as a result, more female than male hatchlings enter the population (Lance et al. 2000). However, data indicate that some populations of juvenile and/or adult alligator (Nichols and Chabreck 1980; Lance et al. 2000) and crocodilian (Thorbjarnarson 1997) were significantly male biased. This suggests a pattern of differential mortality of females compared to males during the first years of life. In this way, an increase of temperature due to climate change could be reflected in more male hatchlings entering the population and proliferation of juveniles and adults male ratio, with a consequently decrease in reproduction.

We report a delimitation of TRT between male- and female-producing temperatures that characterizes the *C. latirostris* population at our study site. Based on the results of this experiment, and the information cited from previous work (Piña et al. 2003), we were able to report a range of temperatures that would include the T_{piv} in *C. latirostris*. It is interesting to note that a small temperature difference (< 0.5° C) may be sufficient to reach MF pivotal temperatures, as occurs in *Alligator mississippiensis* (Lang and Andrews 1994). This MF pivotal temperature has always been very close to the upper limit of thermal tolerance in other previously studied crocodilians: 34° C in *Alligator mississippiensis*; 33.5° C in *Caiman crocodilus*; 33° C in *Crocodylus palustris*, *Crocodylus porosus*, and *Crocodylus johnstoni* (Lang and Andrews 1994), and 34.5° C in *Caiman latirostris* (Piña et al. 2003). Incubation at the MF transition temperature leads to allow hatching success (only 16% when incubation was at 34.5° C; Piña et al. 2003) and a decrease in hatchling survival (Piña et al. 2003). This suggests that hatchlings produced at the

MF pivotal temperature transition may have a minimal effect in wild populations.

Incubation temperature did not significantly influence hatching success, though this may be an artifact of small sample sizes and large variations (standard deviation) among nests. Under incubation at 34° C, we were expecting that embryos would be subjected to thermal stress due to the proximity of the higher tolerance limit of embryo development (34.5° C) reported for *C. latirostris* (see Piña et al. 2003). However, in our experiment, caiman embryos incubated at 34° C did not show a decline in hatching success compared to individuals incubated at lower incubation temperatures. While 34° C may not be a lethal temperature for *C. latirostris* incubation, we would not recommend it as an optimal incubation regime for commercial purposes. Incubation at 34° C has resulted in lethal temperature for other species (e.g., *Caiman crocodilus*, *Crocodylus palustris*, *Crocodylus moreletti*, *Crocodylus siamensis*, *Crocodylus porosus*, and *Gavialis gangeticus*, reported by Webb et al. 1987; Lang and Andrews 1994), and it could result in increased hatchling mortality, a factor not assessed as part of this study.

Our work indicates that an incubation temperature of 32° C produces approximately 70% females and incubation at 34° C produces 100% males in *C. latirostris*. The TRT covers > 31° C to < 33° C (female to male) and > 34° C to > 34.5° C (male to female). For the first time we are able to suggest possible limits of lower and upper T_{piv} for *C. latirostris* as 32° C < T_{piv} < 33° C, and 34° C < T_{piv} < 34.5° C. Because the MF transition occurs at higher incubation temperature than 34° C, and 34.5° C reduces hatching success and survivorship (Piña et al. 2003), we suggest that high temperature females constitute an overall low (possibly nonexistent) proportion of the total population.

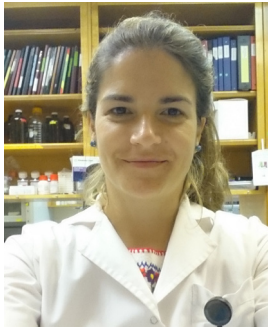
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