Corticosterone Plasma Levels of Embryo and Hatchling Broad-Snouted Caimans (*Caiman latirostris*) Incubated at Different Temperatures

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Abstract. The temperature-sensitive period is the time during development during which sex determination occurs in vertebrates that undergo temperature-dependent sex determination, such as in caimans. The interplay among temperature and steroid hormones is also known, and it has been suggested that stress hormones (corticosterone) might influence sex ratios in some reptiles. To explore this, we measured the levels of corticosterone in *Caiman latirostris* to determine if incubation temperature (31°C, 33°C, and 34°C) affects plasma corticosterone levels. Differences among nests were observed in plasma corticosterone. However, hormone levels showed no significant differences between sexes or incubation temperatures in *Caiman latirostris* embryos or hatchlings. Corticosterone levels were 0.01–2.2 ng/mL in embryos incubated at 31°C (100% of females), 0.01–4.65 ng/mL in those incubated at 33°C (100% of males), and 0.01–6.31 ng/mL in embryos incubated at 34°C (100% of males). Corticosterone levels were higher in hatchlings, being 1.11–39.18 in those produced at 31°C, 2.85–22.36 at 33°C, and 2.72–39.05 ng/mL at 34°C.

Keywords. Crocodilians; Hormones; Temperature-dependent sex determination; Temperature-sensitive period.

Resumen. El periodo termosensible es el momento durante el desarrollo embrionario en el cual ocurre la determinación sexual en vertebrados con DST, como sucede en los caimanes. Se conoce que la temperatura y las hormonas esteroideas pueden interactuar, y ha sido sugerido que la hormona de estrés (corticosterona) podría influenciar la proporción de sexos en algunos reptiles. Para explorar esto, medimos los niveles de corticosterona en *Caiman latirostris,* para poder determinar si la temperatura de incubación (31, 33 y 34°C) afecta los niveles de corticosterona plasmática. Se observaron diferencias en los niveles de corticosterona plasmática entre los nidos. Sin embargo, no se observaron diferencias significativas en los valores de corticosterona entre sexos o entre temperaturas de incubación en embriones y crías de *Caiman latirostris.* Los valores de corticosterona en embriones incubados a 31°C (100% hembras) variaron entre 0.01 hasta 2.2 ng/mL, en aquellos incubados a 33°C (100% machos) los niveles variaron desde 0.01 hasta 4.65 ng/mL, y finalmente los embriones incubados a temperatura más alta (100% machos) osciló desde 0.01 hasta 6.31 ng/mL. Las crías presentaron mayores niveles de corticosterona: aquellas producidas a 31°C variaron desde 1.11 hasta 39.18 ng/mL, a 33°C desde 2.85 hasta 22.36 ng/mL y a 34°C desde 2.72 hasta 39.05 ng/mL.

INTRODUCTION

Temperature-dependent sex determination (TSD) has been the subject of numerous studies across the diversity of reptiles. The most detailed investigations of the physiology of TSD have focused on several species of turtles (Desvages and Pieau, 1992; Bergeron et al., 1998; Wibbels et al., 1998; Warner and Shine, 2008), various lizards (Girling and Cree, 1995; Warner et al., 2009), and some crocodilians, especially in *Alligator mississippiensis* (Daudin, 1802) (Ferguson and Joanen, 1982; Lance and Bogart, 1994; Pieau, 1996). These studies demonstrated different relationships between sex ratio and constant incubation temperatures. In TSD reptiles, the differentiation of the gonads into ovaries or testes depends on the incubation temperature during a critical period of embryonic development known as the thermosensitive period (TSP; Mrosovsky and Pieau, 1991). Further, an interplay among temperature and steroid hormones during TSP in TSD systems has been described (Nakamura, 2010; Navara, 2013).

Numerous experiments administering exogenous steroid hormones at male- and female-producing

temperatures have demonstrated their role in sex differentiation (Imhof and Piña, 2005; Parachú Marcó et al., 2010). The feminizing effects of exposure to estradiol and the masculinizing effects of aromatase inhibition during development are well established in non-mammalian vertebrates (Lance and Bogart, 1992; Bowden et al., 2000; Amateau and McCarthy, 2004; Warner et al., 2014). Another steroid hormone, corticosterone, also has an effect on sex ratio in birds (Pike and Petrie, 2003). For example, experimental manipulation of circulating corticosterone levels in reproductive quails, *Coturnix japonica* Temminck and Schlegel, 1849, suggests that high levels of this hormone can bias offspring sex ratios toward females (Pike and Petrie, 2006). However, when corticosterone values were measured in embryos of different species, ambiguous results in relation to sex were found. While there were no apparent sex differences on corticosterone plasma levels in chick embryos, *Gallus domesticus* Linnaeus, 1758 (Tanabe et al., 1986), in the turkey, *Meleagris gallopavo* Linnaeus, 1758, embryo plasma corticosterone levels in females were approximately twice those of males at a similar developmental stage, but decreased shortly after hatching, when sex differences disappeared (Wentworth and Hussein, 1985). In turtle embryos, circulating corticosterone levels were found to be four times higher in females than in males (White and Thomas, 1992), but this is not consistent among reptiles. For instance, elevated steroid levels affected embryos of two lizard species with TSD in very different ways. High corticosterone levels during embryonic development produced more females in *Amphibolurus muricatus* (White, 1790) and more males in *Bassiana duperreyi* (Gray, 1838) (Warner et al., 2009). However, dexamethasone (a glucocorticoid synthetic) administration at male producing temperatures resulted in no bias sex ratio towards females in *Caiman latirostris* (Daudin, 1802) (Iungman et al., 2015). In *Alligator mississippiensis,* plasma corticosterone values were higher in female than in male embryos during the last weeks of incubation (Medler and Lance, 1998). However, three weeks after hatching, plasma corticosterone concentration decreases in females to equal that of males, suggesting a possible role in the sex differentiation pathway in alligators.

Table 1. Distribution of the four *Caiman latirostris* clutches among the three incubation temperatures. Numbers in each cell refer to quantity of embryos sacrificed at stage 24, hatchlings used to measure corticosterone, and animals maintained under farming conditions, respectively.

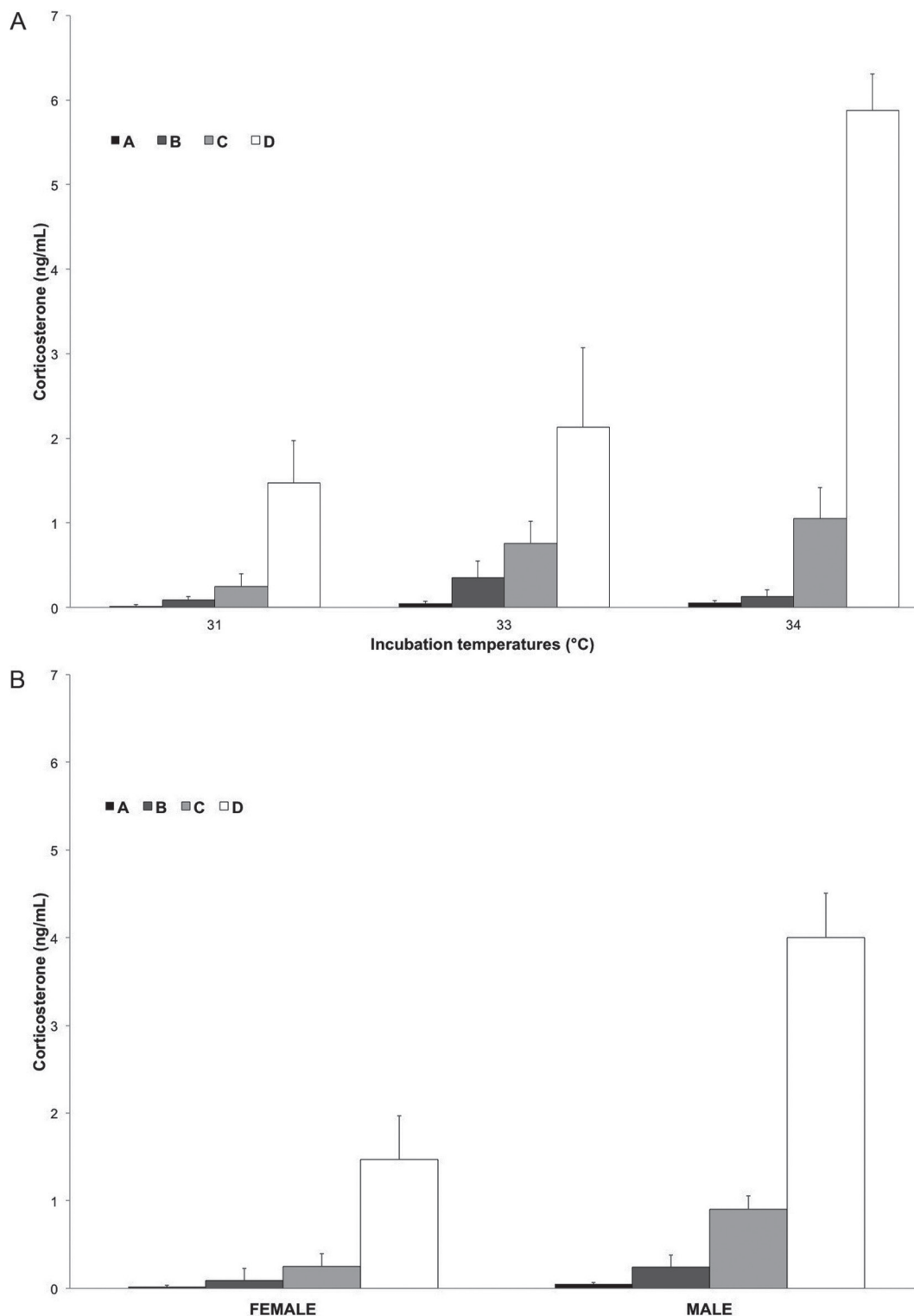| Nest	Incubation Temperature		
	31°C	33°C	34°C
A	4, 4, 4	4, 3, 4	4, 3, 4
B	4, 4, 2	4, 4, 2	4, 4, 2
C	4, 4, 6	4, 4, 6	4, 4, 6
D	4, 4, 2	4, 3, 3	2, 3, 3
Total	16, 16, 14	16, 14, 15	14, 14, 15

Previous results on alligator embryos showed that low incubation temperatures (female producing temperatures) produce higher levels of corticosterone (Medler and Lance, 1998). In *Caiman latirostris,* incubation at 34.5°C is considered a stressful temperature, since it is associated with a high mortality rate (Piña et al., 2003). In a recent study, we also observed that incubation at 34°C produces 100% males and does not reduce hatching rates (Parachú Marcó et al., unpublished data). So, within this framework, we incubated *C. latirostris* eggs at female (31°C) and at two male incubation temperatures (33°C and 34°C) to determine if there are differences in corticosterone levels based on incubation temperature or sex in *C. latirostris* embryos after TSP and at hatching time.

MATERIALS AND METHODS

Caiman eggs from four different nests (Nest A: 35 eggs; Nest B: 36 eggs; Nest C: 45 eggs; and Nest D: 38 eggs; $n = 154$ eggs) were collected shortly after oviposition in nature during the 2013 breeding season and transported to the *Proyecto Yacare* facilities in Santa Fe province, Argentina. Once in the laboratory, egg viability was determined by checking the presence of the opaque banding on the eggshell. Only those eggs considered to be viable were included in the experiment ($n = 134$). Nests A–D had 35, 31, 43, and 29 viable eggs, respectively. One egg from each clutch was opened to determine developmental stage based on Iungman et al. (2008). All clutches had structural age < stage 12 at the beginning of the experiment. Each clutch was randomly distributed among the three incubation temperature treatments with two replicates (six incubators in total; Table 1). Eggs were placed in plastic trays with moist vermiculite as substrate (Larriera et al., 2008) in constant-temperature incubators at either 31 ± 0.5°C, 33 ± 0.5°C, or 34 ± 0.5°C. Eggs were maintained at high humidity, but it was not measured (for a detailed methodology see Iungman et al., 2008). Temperature was monitored by a HOBO™ Data Logger (Onset Computer Corporation, Pocasset, MA, USA) placed among the plastic trays, which was checked daily throughout the incubation period. Embryos from each nest at each incubation temperature were sacrificed at stage 24 (one stage after the end of the TSP), corresponding to 42 days at 31°C, 39 days at 33°C, and 38 days at 34°C (Iungman et al., 2008), and a blood sample was taken from each ($n = 46$). All samples were collected from 9:00–12:00 h in order to avoid any bias in the results due to possible circadian variations in hormone levels. The remaining eggs were maintained in the incubators until hatching. Immediately after hatching, another blood sample was taken from the dorsal postcranial sinus of a group of animals ($n = 44$), and all hatched caimans ($n = 88$) were maintained under farming conditions. All embryos of the study and 14 (of 29) hatchlings

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of 34°C were sexed by histological techniques. Animals of other temperatures (31°C and 33°C) were not sacrificed because we have enough information to support sex ratio of *C. latirostris* at incubation temperatures of 31°C and 33°C (Piña et al., 2003; Piña et al., 2007; Stocker et al., 2008; Iungman, 2012). The sex of all hatched animals was determined when they were 4 months old by visual examination of the cliteropenis. All the animals were treated

Figure 1. Plasma corticosterone in *Caiman latirostris* at stage 24 of development at different incubation temperatures. **(A)** Comparison between nests and incubation temperature, **(B)** comparison between nests and sexes (31°C female against 33 and 34°C male temperature). Average of nest D at 34°C was calculated based in only two measurements. Values are presented as $X \pm$ SE. Letters A–D within the figure represent different nests (see text for details).

following established ethical principles for research with laboratory, farm and wild animals (National Scientific and Technical Research Council, 2005), minimizing stress and suffering by suitable management methods.

Blood samples from embryos (n = 46) and hatchlings (n = 44) were centrifuged at 3,000 rpm for 15 min, and the plasma was separated and stored at –20°C for hormone determination. Corticosterone was measured by radioimmunoassay after extraction of 100 μL aliquots of plasma with diethyl ether (Jahn et al., 1995). Samples were run in duplicate. The intra-assay coefficient of variation was less than 5%, and inter-assay was less than 10%. The lower detection limit of the assay was 0.1 ng/tube.

To compare hatching success among incubation temperatures, a χ^2 test was used. Because the data did not conform to the assumptions of a parametric ANOVA, a non-parametric Kruskal-Wallis test was used to determine statistical differences in plasma corticosterone concentrations among incubation temperature, sex, and nests in embryos and hatchlings of *Caiman latirostris.*

RESULTS

No differences in hatching success were found among temperature treatments at 31°C (88%), 33°C (85%), and 34°C (78%) (χ^2 = 5.07, df = 2, P = 0.074). All females were obtained at 31°C (n = 46; 16 embryos, and 30 hatchlings), and all males at 33°C (n = 45; 16 embryos, and 29 hatchlings) and 34°C (n = 43; 14 embryos, 29 hatchlings) incubation temperatures. The different nests had different plasma corticosterone concentrations in caiman embryos at stage 24 (H = 25.37, P < 0.007). Although Nest D seems to present differences in corticosterone plasma levels among incubation temperature or sex, the statistical analysis did not support it (H = 2.92, P = 0.2304; H = 4.58, P = 0.094; Fig. 1A–B). Incubation at 33°C and 34°C (100% males) had similar levels of corticosterone in embryos (H = 0.01, P = 0.9126). Corticosterone levels were 0.01–2.2 ng/mL in embryos incubated at 31°C, 0.01–4.65 ng/mL in those incubated at 33°C, and 0.01–6.31 ng/mL 34°C (at this temperature we only determined corticosterone level of two embryos at Nest D, and they showed the highest level of all embryos).

Hatchlings presented higher levels of corticosterone than embryos (H = 60.84, P < 0.001). At hatching, plasma corticosterone concentrations among nests presented similar values (H = 17.22, P = 0.1016), and neither sex (H = 11.78, P = 0.1079) nor incubation temperature (H = 17.22, P = 0.1016) influenced corticosterone plasma levels (Fig. 2A–B). Similarly, as we found in embryos, hatchlings incubated at 33°C and 34°C (100% males) had similar levels of corticosterone (H = 2.59, P = 0.1073). Corticosterone range were from 1.11–39.18 ng/mL at 31°C, 2.85–22.36 ng/mL at 33°C, and 2.72–39.05 ng/mL at 34°C.

DISCUSSION

Caiman embryos at the end of TSP (stage 24) and hatchlings showed high variability in corticosterone levels. In *Alligator mississippiensis* embryos at stage 24, pooled samples of corticosterone concentrations ranged between 2–4.5 ng/mL (males and females; Medler and Lance, 1998). In the present work, individual stage 24 *Caiman latirostris* embryos from four different clutches exhibited corticosterone concentrations ranging from 0.01 ng/mL to nearly 7 ng/mL. However, there appears to be an effect of clutch on these values. This amplitude of corticosterone levels suggests that other factors might have an influence on corticosterone levels. These differences might be due to varying concentrations of steroid hormones from maternal origin deposited in the yolk, as has been reported in different avian species (Schwabl, 1996; Schwabl et al., 1997). It has been shown that corticosterone in avian egg yolk correlates positively with corticosterone in maternal circulation at the time of laying, and that high corticosterone yolk levels can influence offspring development and adult phenotype (Hayward and Wingfield, 2004; Love and Williams, 2008). Some studies in reptiles have reported on the role of maternal yolk sex steroid hormones (estradiol or testosterone) in offspring sex determination, and the correlation among steroid hormone levels and sex ratio sometimes differs among species (Janes et al., 2007; Warner et al., 2009). However, corticosterone levels in reptile embryos have not been studied in depth. Warner et al. (2007) reported the first data showing that maternally derived corticosterone yolk levels were associated with offspring sex in *Amphibolurus muricatus,* a lizard with TSD, but a more recent study showed that the effects of corticosterone differ among species (Warner et al., 2009). In this framework, more experimental work on corticosterone yolk and embryo plasma levels is needed to clarify their effects on sex determination in reptiles.

Piña et al. (2003) reported that when *Caiman latirostris* eggs were incubated at 34.5°C hatching success was drastically reduced (reaching 16.2%). On the other hand, previous reports on different crocodile species showed that 34°C is a lethal incubation temperature in *Caiman crocodilus* (Linnaeus, 1758), *Crocodylus moreletii* (Duméril and Bibron, 1851), *Crocodylus palustris* Lesson, 1831, *Crocodylus porosus* Schneider, 1801, *Crocodylus siamensis* Schneider, 1801, and *Gavialis gangeticus* (Gmelin, 1789) (Webb et al., 1987; Lang and Andrews, 1994; Deming, 2004). In this context, by incubating at 34°C we were expecting to find higher corticosterone levels than other incubation temperatures; however, corticosterone levels and hatching success were similar among incubation temperatures. This would indicate that temperatures as high as 34°C would not be a factor of stress for *Caiman latirostris* during incubation, or that embryos do not increase corticosterone levels under stressful incubation conditions.

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Temperature plays an important role in various aspects of the life history and physiology of ectotherms (Angilletta et al., 2004; Clarke and Pörtner, 2010; Iungman and Piña, 2013; Martins et al., 2014). In wild geckos, basal corticosterone was positively correlated with body temperature (Cree et al., 2003). In that context, we expected a positive relationship between incubation temperature and corticosterone concentrations during the induction of a
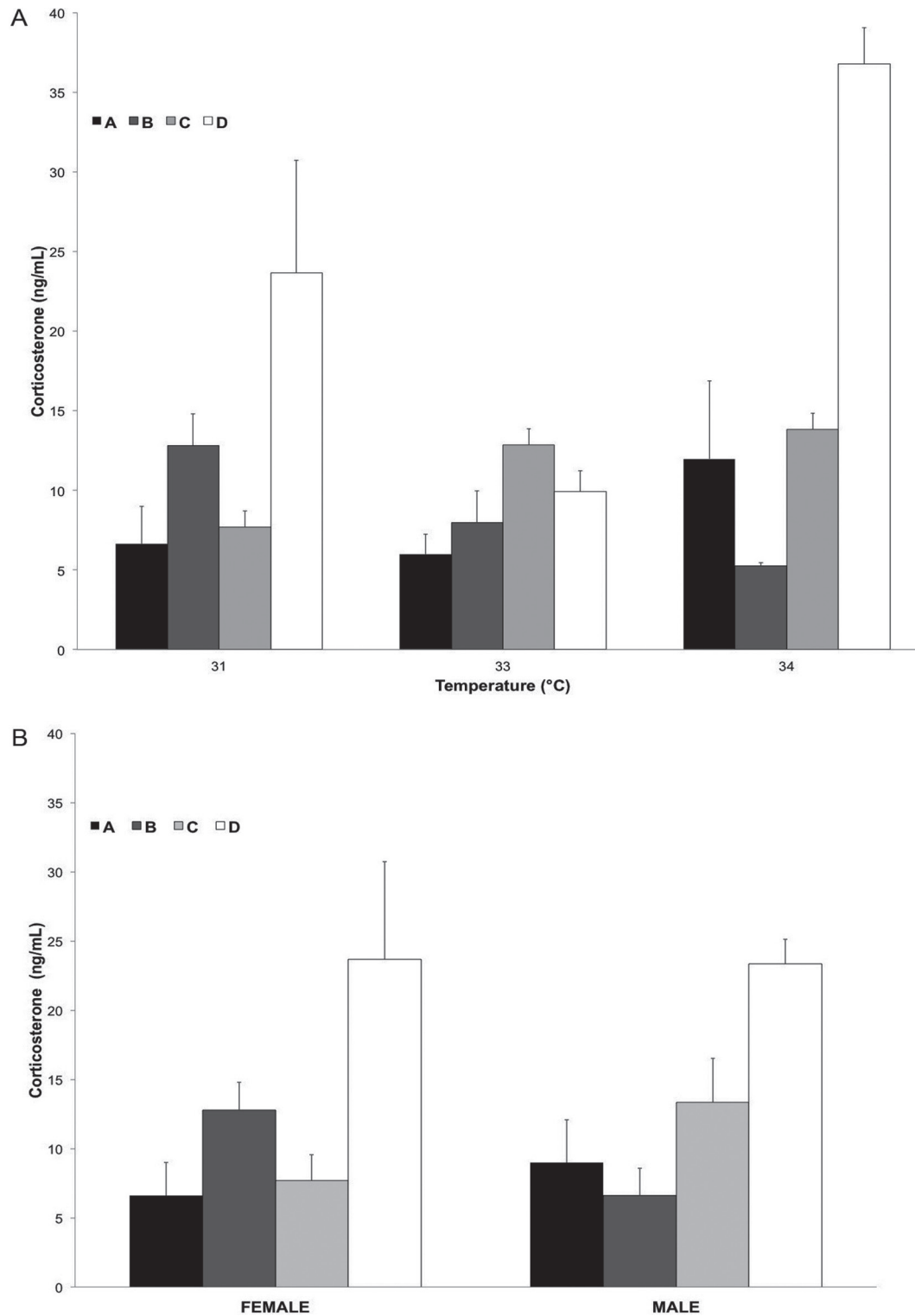
Figure 2. Plasma corticosterone in *Caiman latirostris* hatchlings incubated at different incubation temperatures. **(A)** Comparison between nests and incubation temperature. **(B)** Comparison between nests and sexes (31°C female against 33 and 34°C male temperature). Values are presented as $\bar{X} \pm$ SE. Letters A–D within the figure reprepesent different nests (see text for details).

thermal stress response (Tyrrell and Cree, 1998; Cree et al., 2003). However, in the present study we did not detect differences in corticosterone levels among incubation temperatures or sexes in *Caiman latirostris* embryos at stage 24, although we observed differences among clutches.

We are aware of only one study that has examined the effects of incubation temperatures on corticosterone levels in crocodilian embryos (Medler and Lance, 1998). In that study, the authors found that female *Alligator mississippiensis* embryos (obtained at low incubation temperature) exhibited slightly higher corticosterone values than observed in males (obtained at high incubation temperature) from stage 25 onwards and continued to rise until hatching. Three weeks after hatching, plasma corticosterone levels declined dramatically in both sexes and sex-specific difference disappeared. Our results did not show differences in corticosterone levels at the time of hatching, and the nest effect observed in embryos was not observed at hatching. While there was no significant effect of corticosterone levels in embryos or hatchlings in this study, we believe that larger sample sizes might uncover differences. Thus, the difference between 33°C and 34°C that seems to be evident in the graph in the case of Nest D could be transformed into statistical evidence.

In this paper we have partially dissociated the effect of temperature and sex determination because we were able to compare two treatments that produce only males (33°C and 34°C) and found that there were no differences in corticosterone levels, knowing that 34°C is very close to lethal. Also, this study showed that incubation temperature does not appear to alter corticosterone in *Caiman latirostris*. Due to the controversial role of corticosterone on sex differentiation, additional research should be performed, and future studies must try to integrate incubation temperature and sex ratio in the same treatment. It would be interesting to measure corticosterone levels at 32°C, an incubation temperature that can generate both sexual phenotypes in *C. latirostris,* to determine if any interplay between temperature and this steroid hormone exists.

ACKNOWLEDGMENTS

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