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Are Stress-Related Hormones Involved in the Temperature-Dependent Sex Determination of the Broad-Snouted Caiman?

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Abstract. In some reptiles, gonadal outcome is regulated by temperature during a critical period of the embryonic development. Gonadal steroid hormones are seen as effectors of the gonadal differentiation process. Recently, stress and glucocorticoids (GCs), stress-related hormones in vertebrates, have been considered as potential modulators of the sex determination process in some vertebrates that present temperature-dependent sex determination (TSD). In reptiles, corticosterone is the main GC produced, and its administration to eggs causes a bias in sex ratio in some lizards. In this context, we aim at assessing whether dexamethasone (Dex), a potent synthetic glucocorticoid, can modify the sex ratio in *Caiman latirostris,* a species with strong TSD. As a first step, we incubated embryos at masculinizing temperatures (33°C; 100% males). Different doses of Dex were topically applied to the eggshell at stage 20, prior to gonadal differentiation. We assessed embryonic development at stages 22 and 25 and evaluated some physiological and morphological hatchling traits. Embryonic mortality was not affected by dexamethasone manipulation. No effects of Dex on sex ratio were found and all animals analyzed histologically possessed testes. However, older embryos and hatchlings from Dex treated eggs were heavier, larger, and hatched earlier than control individuals. Our results do not account for Dex involvement in the process of ovarian differentiation, at least under a strong masculinizing temperature. Nevertheless, they suggest that Dex might accelerate embryo development by enhancing intermediate metabolism and/or by stimulating growth hormone secretion.

Keywords. *Caiman latirostris;* Dexamethasone; Embryo; Glucocorticoid; Gonad development.

INTRODUCTION

A variety of sex-determining systems exist across vertebrates (Grossen et al., 2010; Gamble and Zarkower, 2012; Schwanz et al., 2013). In many fishes (Heule et al., 2013; Yamamoto et al., 2014), amphibians (Nakamura, 2009; Sarre et al., 2011), and reptiles (Kohno et al., 2014) sex determination owes to a combination of genetic and environmental cues (environmental sex determination; ESD). The most extensively studied form of ESD is temperature-dependent sex determination (TSD), in which embryo sex is determined by incubation temperature. Our experimental model is the broad-snouted caiman, *Caiman latirostris* (Daudin, 1802), which exhibits a TSD system with a female–male–female pattern, i.e. females are produced at low and high temperatures and males are produced at intermediate temperatures. Only males are produced at 33°C and only females at low incubation temperatures (< 31°C; Piña et al., 2003). It has been established that the gonad is sensitive to temperature during a discrete period of development, called the thermosensitive period (TSP). In our species, the TSP encompasses stages 19–24 (Piña et al., 2007; Iungman and Piña, 2013) and is aligned with the morphological differentiation process of the gonad (Iungman, 2012).

The manipulation of either temperature or background hormones during this window can redirect the putative sex of the embryo (Nakamura, 2010). For example, it is well known that embryonic or larval treatments with natural and/or synthetic estrogens may feminize genetic male individuals, even resulting in sex inversion in fish (Guiguen et al., 1999), amphibians (Isomura et al., 2011) and avian (Clinton and Haines, 1999) and non-avian reptiles (Navara, 2013). The exogenous administration of estrogens to *Caiman latirostris* eggs overrides temperature and induces ovarian differentiation even at masculinizing temperatures (Durando et al., 2013). In recent years, stress and glucocorticoids (GCs), stress-related hormones in vertebrates, have been postulated as endogenous mediators involved in the sex determination/differentiation process. Recent studies have demonstrated that the manipulation of a natural (cortisol) or synthetic (dexamethasone, Dex) GC altered sex ratios in fishes (Hattori et al., 2009; Yamaguchi et al., 2010). It has also been shown that high levels of corticosterone in female breeding birds can bias offspring sex ratio towards daughters

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(Pike and Petrie, 2006) or sons (Gam et al., 2011; Pryke et al., 2011) in different avian species. An experimental study has shown that the corticosterone administration on eggs causes sex ratio shifts in two lizard species with TSD. However, such shifts may lead in opposite directions in different species (Warner et al., 2009).

GCs are steroidal hormones that play an important role in both development and growth due to their involvement in the metabolic homeostasis (Vakili and Cattini, 2012). Corticosterone is the major GC produced by adrenal steroidogenic cells in reptiles, and an increase in its plasma concentration is a frequent indicator of stress (Gunderson et al., 2003; Anderson et al., 2014). In nature, GCs in the developing embryos seem to be largely of maternal origin (McCormick, 1999; Lovern and Adams, 2008; Uller et al., 2009) and are probably transferred to the growing oocytes through vitellogenesis (Elf et al., 2002, 2003). As such, stress in breeding females causes high levels of circulating GCs that are laid into the egg yolk (Lovern and Wade, 2003; Hayward and Wingfield, 2004). Yolk corticosterone has been associated with offspring phenotypes of some reptiles (Uller et al., 2009), including sex ratio in some lizard species (Warner et al., 2007, 2009). The GCs seem to interfere with the biosynthesis of sexual steroids, since the enzymes involved in their synthesis are also involved in the glucocorticoid synthesis. It has been observed that GCs increase the production of major androgens in fishes (Hattori et al., 2009), whereas in rats they inhibits testosterone production in progenitor Leydig cells (Xiao et al., 2010). Thus, we ask if Dex administration at male-producing temperatures might bias sex ratio in *Caiman latirostris* embryos during TSP.

MATERIALS AND METHODS

Egg collection and incubation

Three broad-snouted caiman wild clutches (n = 113 eggs) were collected soon after laying and transported in

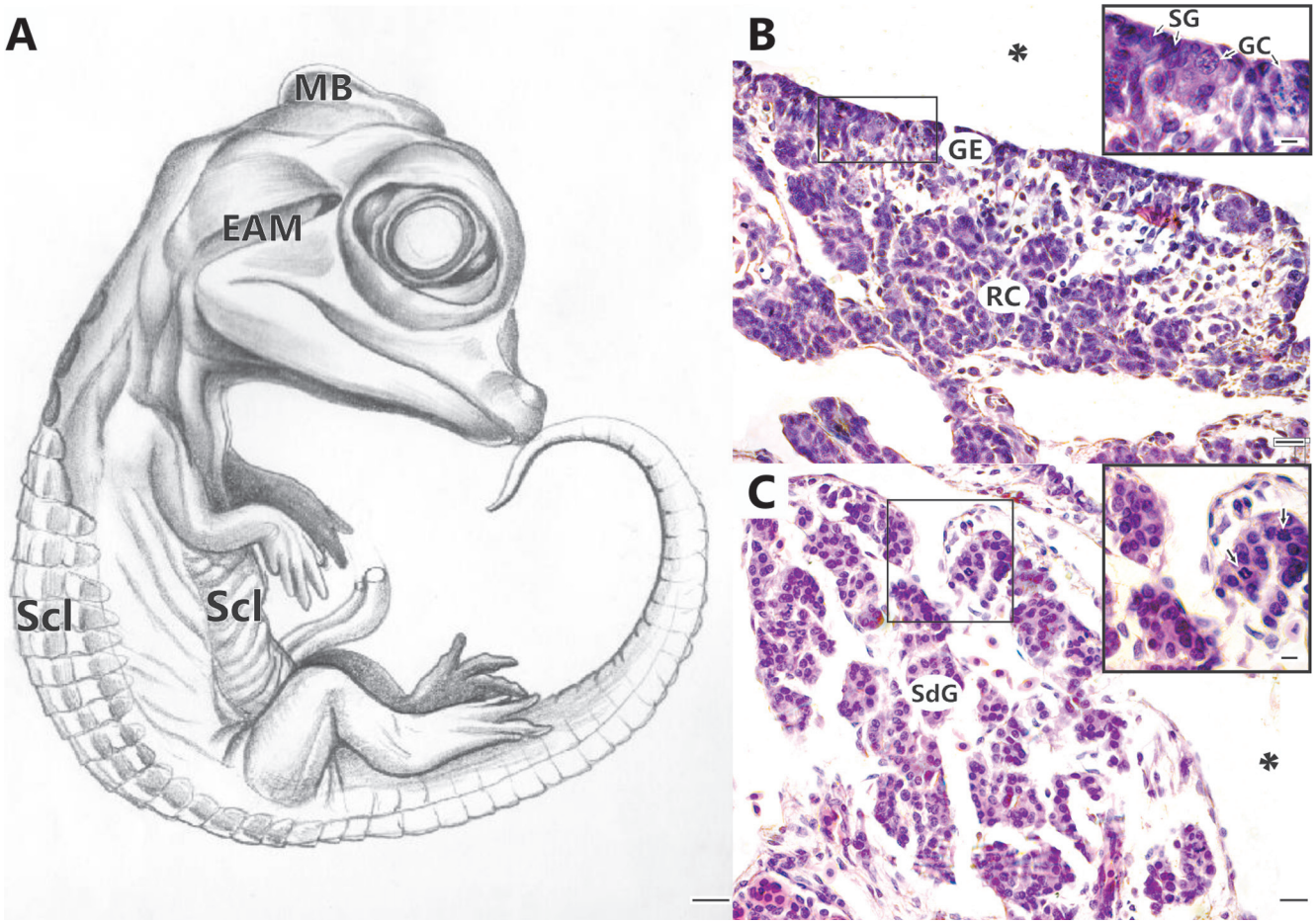

Figure 1. Topical application of dexamethasone at stage 20. **(A)** A 27-day *Caiman latirostris* embryo; scales (Scl) are visible dorsally (bar = 1 mm). **(B)** Transverse section of an undifferentiated gonad stained with hematoxylin-eosin (bar = 10 μm). Note germinal epithelium (GE) on the coelomic edge and medullary rete cords (RC). **(C)** Transverse section of adrenal gland exhibiting steroidogenic cells (SdG) with mitotic activity (arrow) stained with hematoxylin-eosin (bar = 10 μm). Rectangles define areas shown in greater magnification (bar = 2 μm). EAM, external auditory meatus; GC, germ cell; MB, midbrain; SC, somatic cell; *, coelomic cavity.

coolers to the laboratory where they were artificially incubated. Upon arrival, embryo viability was determined by the presence of opaque eggshell banding; eight eggs were excluded from the experiment because no opaque band was evident (0 from nest A; 4 from nest B; and 4 from nest C). One egg per clutch was dissected to stage embryos; no nests were more advanced than stage 12 (Iungman et al., 2008). After being numbered and measured, the eggs were placed in one of two incubators (SEMEDIC, I-290D) that were set at a constant 33 ± 0.2°C, a temperature producing 100% males in this species (Piña et al., 2003). In order to avoid any incubator-specific effects we included a replicate for each treatment, although no significant variation among incubators was recorded in any of the ANOVA models employed. Within each incubator, eggs from each clutch were distributed equally among four treatments and placed into an individual plastic chamber. All treatments were applied topically to the eggshell at stage 20 of embryonic development (Fig. 1A), prior to the first morphological signs of gonadal differentiation (Iungman, 2012). Eggs were treated as follows: (a) Dex (Sigma Chemical, St. Louis, MO) in low dose (0.014 ppm) or (b) high dose (1.4 ppm), both suspended in 50 μL 95% ethanol, (c) vehicle control in which only 50 μL 95% ethanol was applied, and (d) control with no solutions applied in any way. Before treatment administration, two eggs from each clutch were opened from control groups in order to check the adrenal- gonad development (Fig. 1B, C). The average egg weight was 69.1 ± 3.7 g. Dex doses were chosen according to the exogenous estradiol that induces gonadal feminization at a male-producing temperature in *Caiman latirostris* (Stoker et al., 2003). Temperature was monitored daily by HOBO temperature loggers (Onset Computer, Pocasset, MA). All eggs were laid on a mixture of vermiculite and water (1:1) and maintained at approximately 90% humidity.

Experimental protocol

One clutch was used to assess the effect of dexamethasone (Dex) on embryonic development at stages 22 and 25 of incubation. We used the Iungman et al. (2008) descriptions to define *C. latirostris* developmental stages. Stage 22 was chosen because at this time the morphological differentiation of male sex occurs (Iungman, 2012), while the latter corresponds to a stage in which the thermosensitive period has already ended (Piña et al., 2007). Whole embryos were removed from eggs and they were staged, weighed (yolk-free; ± 0.1 g), and measured for total and dorsal-cranial length (± 0.01 mm). Later, their adrenal-kidney-gonads were dissected for histology.

The remaining two clutches were allowed to reach their full term development (stage 28) and hatchling data were recorded (hatching success, incubation period and phenotypes). Hatchling caimans were weighed (including the internalized yolk-sac; ± 0.1 g), measured for total length (± 1 mm) and dorsal-cranial length (measured from the anterior tip of the snout to the median posterior edge of the supraoccipital; ± 0.01 mm), and sexed histologically. Caimans were euthanized using a lethal dose of sodium pentobarbital.

Histological sex determination

Embryo and hatchling (total = 64 individuals) sex was determined according to the histological criteria established by Iungman (2012). The adrenal-kidney-gonad complex was fixed in 10% phosphate-buffered formalin and processed for routine histology, and 5-μm-thick semiserial sections stained with hematoxylin-eosin or periodic acid-Schiff were used to evaluate the presence of sex defining structures. Sections were examined and photographed using a Leica DM 5000 B light microscope.

Statistics

Analyses were performed using SAS software, version 9.1 (SAS Institute, 1997). One-way ANOVA followed by the Tukey test was used to assess the effects of treatments (independent variable) on phenotypes and incubation period (i.e., days between oviposition and hatching). All variables met the assumptions of parametric analysis. We used egg mass as a covariate in analysis of offspring body mass, and nest of origin was included as a blocking factor. Differences were considered significant at $P \leq 0.05$.

RESULTS

Gonadogenesis and sex outcome

The expected temperature-dependent sex outcome was not inverted by Dex treatment and all caimans developed their gonads to testes. In contrast to our previous studies in which gonads exhibited signs of masculinization at stage 22, in the present study the gonads were still undifferentiated in that stage, i.e. they contained a few large germ cells scattered about somatic cells on the coelomic edge (see Fig. 1B). At stage 25, germ cells migrated into the medullar region where they were enclosed within the developing seminiferous tubules (Fig. 2 shows the typical morphology of testis-determination). At hatching (stage 28), testes were characterized by well-differentiated seminiferous tubules filled with developing spermatogonia and Sertoli cells and outlined by a basement membrane and peritubular myoid cells (Fig. 3). The histological structure for both undifferentiated gonads

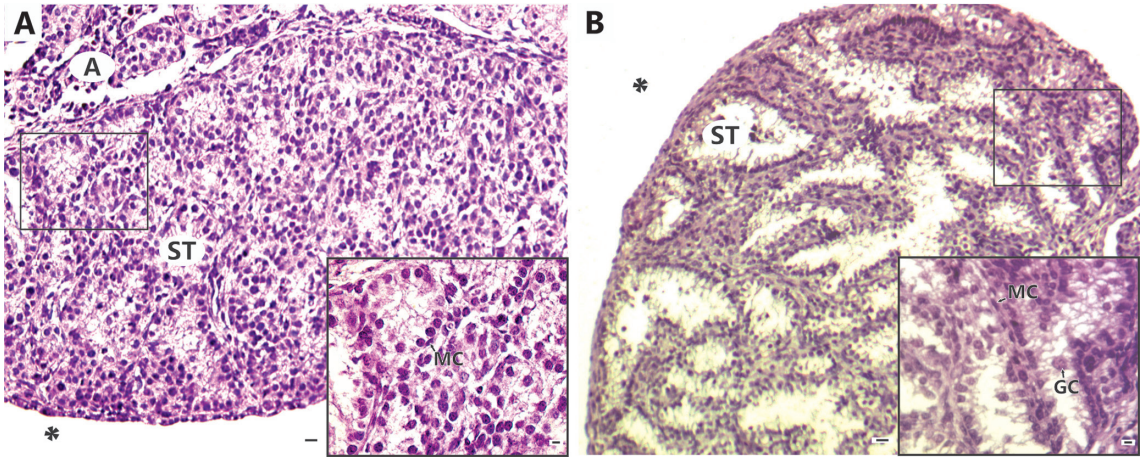

Figure 2. Transverse testis sections of *Caiman latirostris* at stage 25 stained with hematoxylin-eosin. **(A)** Testis from control male (bar = 9 μm). **(B)** Testis from male with low dose of dexamethasone (bar = 9 μm). Rectangles define areas shown in greater magnification (bar = 3 μm). Observe that in both views the gonad exhibits seminiferous tubules (ST). GC, germ cell; MC, peritubular myoid cell; *, coelomic cavity.

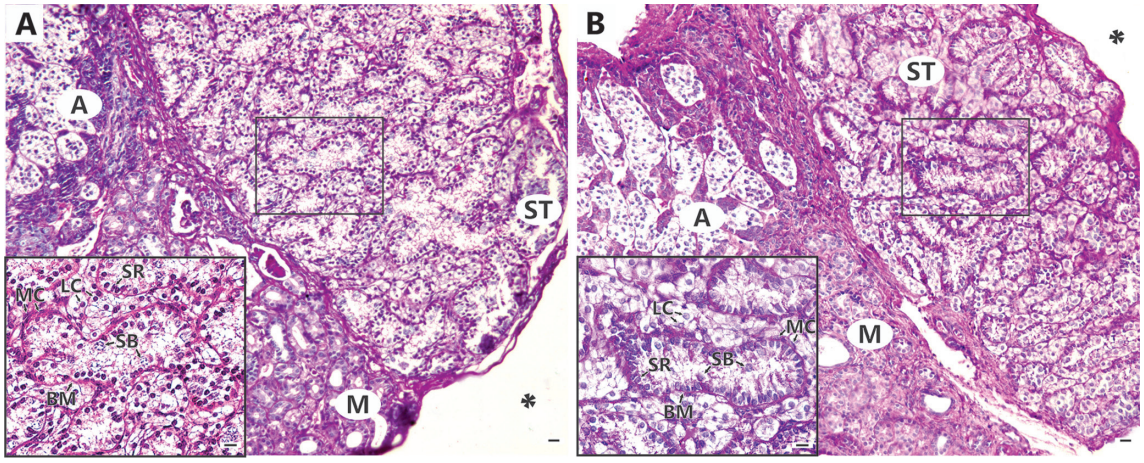

Figure 3. Transverse sections of *Caiman latirostris* testis at hatching stained with periodic acid-Schiff. **(A)** Testis from control male (bar = 6 μm). **(B)** Testis from male with low dose of dexamethasone (bar= 6 μm). Rectangles define areas shown in greater magnification (bar = 3 μm). Note that the sex-specific extracellular matrix of basement membrane (BM) and peritubular myoid cell (MC) is present in both control and treated embryos. A, adrenal gland; LC, Leydig cell; M, mesonephros; SB, type B spermatogonia; SR, Sertoli cell; ST, seminiferous tubules; *, coelomic cavity.

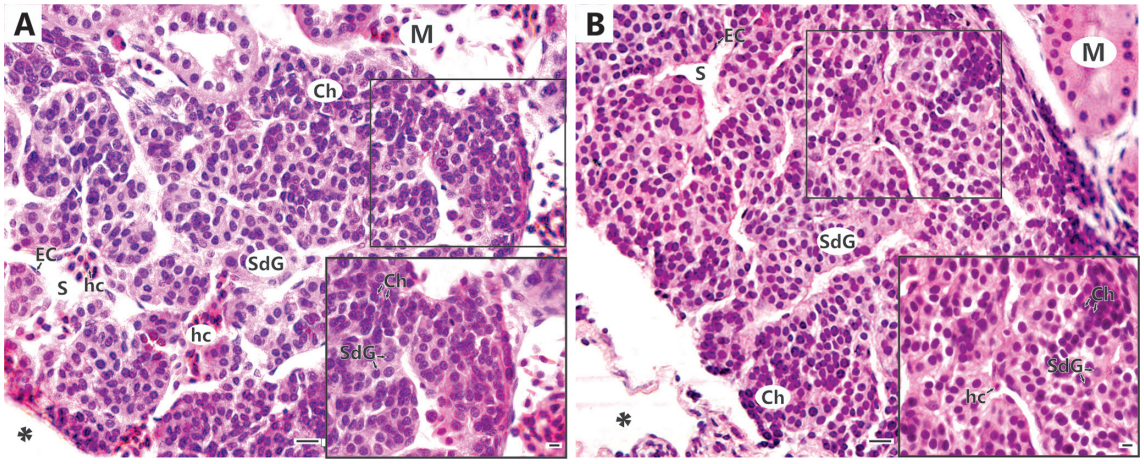

Figure 4. Transverse adrenal gland sections of *Caiman latirostris* at stage 25. **(A)** Male embryos from control groups (bar = 10 μm). **(B)** Male embryos from high dexamethasone treated groups in B (bar = 10 μm). Rectangles define areas shown in greater magnification (bar = 4 μm). Two types of cells could be detected: steroidogenic cell (SdG) and chromaffin cell (Ch). Observe that in high dexamethasone treated embryos there seems to be no sign of adrenal reduction. EC, endothelial cell; hc, hematopoetic cell; S, sinusoid;*, coelomic cavity.

and testes was consistently comparable in Dex-treated and control animals. Figures 2B and 3B show the gonad histology of embryos and hatchlings from treated testes.

The histological aspect of the adrenal gland at stage 22 involved just steroidogenic cells forming loop-shaped cords (see Fig. 1C), although at stage 25 it was sparsely populated by groups of chromaffin cells confined to the cortex (Fig. 4). This adrenal gland structure was maintained until hatching. No obvious differences were observed between control and Dex-treated adrenal tissues (Figs. 3, 4).

Survival and incubation period length

Mortality during incubation was less than 13% in all groups after topical Dex application and, therefore, did not cause sex ratio deviations. Embryos treated with low doses of Dex developed faster than those belonging to other groups and were the earliest to hatch ($P < 0.0001$; Fig. 5). However, faster development did not correlate with stage of development, and all embryos reached the same stage regardless of the Dex treatment.

Phenotypes

Dex application increased body size in older embryos and hatchlings. Embryo size did not differ among treatments at stage 22 ($P > 0.6$; Fig. 6A and B), although at stage 25 they were heavier ($P = 0.002$) and longer in both total and dorsal-cranial length ($P < 0.0001$), under Dex treatments (Fig. 6A and B). Hatchlings from dexamethasone-treated eggs weighed more than those from control treatments ($P < 0.0001$; Fig. 6C) and were also longer in total and dorsal-cranial length ($P < 0.0001$; Fig. 6D).

DISCUSSION

The results of this experiment account for a Dex implication in the embryonic development of *Caiman latirostris,* since it generated larger embryos with shortened incubation periods; however, no Dex effect was found on offspring sex ratio towards females, at least under a strong masculinizing temperature (33°C, Piña et al., 2003; Iungman, 2012). Our discussion should be interpreted taking into account that embryonic responses vary according to Dex exposure. We could suppose that a single Dex application would have an analogous response to acute stress (high GC values for a short period), which possibly differs from continuous applications leading to chronic stress. Stage 20 was chosen because at this time the gonad is undifferentiated according to Iungman (2012), which was confirmed histologically in our series. However, at stage

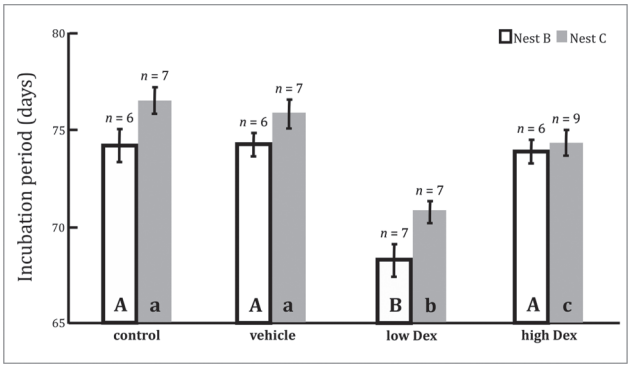

Figure 5. Effect of dexamethasone on *Caiman latirostris* incubation period. Analyses comparing control (control), vehicle control (vehicle), and dexamethasone in low (low Dex) and high (high Dex) doses. Note that the effect of the latter depended on the nest of origin, which was taken as a blocking factor in the one-way ANOVA. Different letters inside the bars indicate statistical differences between treatments: capital letters for nest B and lower-case letters for nest C. *n* = sample size.

22 (two stages after Dex application) the control and Dex treated gonads did not show typical signs of differentiation, as we expected, suggesting that gonadal differentiation took place later than stage 22 in our series. Medler and Lance (1998) reported that the corticosterone endogenous levels can be detected in *Alligator mississippiensis* (Daudin, 1802) embryos before the sex-determining period. In contrast, the timing of estrogen or androgen detection in plasma occurs during the thermosensitive period or shortly afterward, i.e. during gonadal differentiation (Valenzuela and Shikano, 2007). Therefore, we believe that a temporal gap between our topical application stage (stage 20) and a steroidogenically active gonad might explain the absence of a Dex effect on sex outcome. Another possibility would be that our dosages were not potent enough to override the temperature effect and bias sex ratio. We believe that the doses could be even higher, given that our highest dose (1.4 ppm Dex) did not affect the adrenal gland morphology (see Fig. 4) as should be expected under pharmacological doses or chronic stress situations (Kawashima et. al., 2010; Aslam et al., 2014).

Although no published information about reptiles is available, it has been registered that during avian development the corticotrophs (pituitary cells that synthetize and secrete adrenocorticotropic hormone, ACTH) are the first functional cell types to appear in the anterior pituitary (Carlton and Porter, 1999). This avian ontogenetic differentiation profile correlates with the embryonic serum capacity to induce *in vitro* glucocorticoid release (Porter et al., 1995). In studies based on the development of avian and rodent species, the expression of corticotrophin-releasing hormone (CRH) is first detected in the hypothalamus early in development (Keegan et al., 1994). The CRH peptide is the major secretagogue of ACTH (Sheng et al., 1996), whose increased circulation levels stimulate subsequent GC biosynthesis and release (matching the increased mitotic activity in the interrenal

tissue; Medler and Lance, 1998; Schulte et al., 2007). Altogether, a functional hypothalamus-pituitary-adrenal axis is established early in development and possibly prior to any other hypothalamus-pituitary-axis (Schulte et al., 2007).

It is well known that manipulation of steroidal hormones during the period of sex determination may override the temperature effects. Estrogen is one of the most important ovary inducers in all vertebrates groups except for placental mammals (Nakamura, 2010), and they are synthesized after induction of aromatase expression (*cyp*19a). Exogenous estrogen treatments during development will transiently sex-inverse chicken (Clinton and Haines, 1999) and permanently sex-inverse non-avian reptiles (Navara, 2013), amphibians (Isomura et al., 2011), and fishes (Guiguen et al., 1999). In the broad-snouted caiman, male-to-female sex-inversion can be induced by estrogen treatments of eggs incubating at a male-producing temperature (Parachú Marcó et al., 2010; Durando et al., 2013). Moreover, treatment of eggs with aromatase inhibitors or antiestrogen disrupt ovary differentiation or induce testis differentiation at feminizing temperature (Lance and Bogart, 1992; Dorizzi et al., 1996) or at temperatures that otherwise yield both sexes (Pieau et al., 1999). Importantly, warm temperature and estrogen act synergistically: less estrogen is required to sex-inverse embryos from intermediated temperatures than from extreme male-producing temperatures (Merchant-Larios and Díaz-Hernádez, 2012).

The impact of prenatal GC exposure on sex ratio has led to conflicting findings among vertebrates, involving not only sex direction skews but also how they are achieved. In fish with TSD, cortisol pushes sex ratio in the male direction even when larvae are reared at "sexually neutral" temperatures (Hattori et al., 2009; Yamaguchi et al., 2010). Moreover, cortisol concentrations in fish reared at male temperatures were higher than those at female temperatures, suggesting that masculinization in these groups is due to an increase in cortisol concentration induced by high temperatures during the sexual

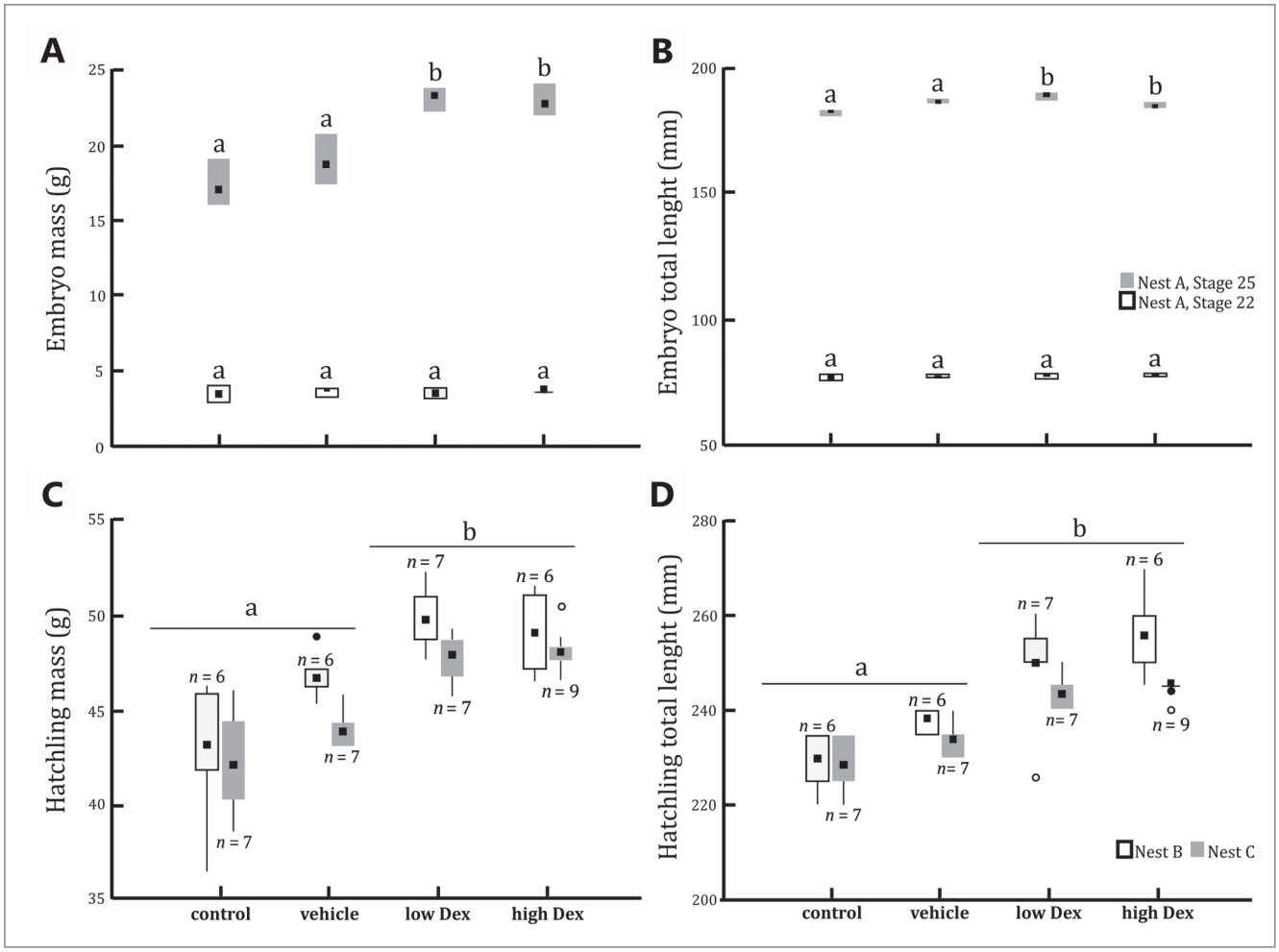

Figure 6. Dexamethasone influence on offspring size of *Caiman latirostris*. Analyses comparing control (control), vehicle control (vehicle), and dexamethasone in low (low Dex) and high (high Dex) doses. **(A)** Embryos in stage 22 (n = 4). **(B)** Embryos in stage 25 (n = 3). (C–D) Hatchlings. No nest of origin effect was found. The lower-case letters above the bars indicate statistical differences. The n = sample size.

differentiation period (Hattori et al., 2009). It has also been pointed out that high cortisol concentrations in fishes suppress androgen production (Hattori et al., 2009), as well as aromatase mRNA, which prevents estrogen production (Kitano et al., 1999; Fernandino et al., 2013). Nevertheless, the situation seems to be different in TSD reptiles. For example, in oviparous lizards the corticosterone application on eggs deviates sex ratio towards the production of females rather than males in *Amphibolurus muricatus* (White, 1790) (Warner et al., 2007), the opposite effect is recorded in *Bassiana duperreyi* (Gray, 1838) (Warner et al., 2009), and the sex outcome is unaffected in *Ctenophorus fordi* (Storr, 1965) (Uller et al., 2009). As far as we know, this is the first attempt to induce skewed sex ratio in crocodilians by exogenous administration of Dex. The levels of endogenous corticosterone in alligator embryos were measured, yielding higher levels in females than males after the thermosensitive period (Medler and Lance, 1998), although Parachú Marcó et al. (2015) did not find sex differences in the corticosterone production of embryo caiman at low and high temperatures.

The pathways underlying sex ratio adjustments have not been assessed in any reptiles. GCs also affect the sex of species with a sex chromosome system. For example, increases in maternal corticosterone during egg production have been linked to female- (Pike and Petrie 2005, 2006; Bonier et al., 2007) or male-biased (Gam et al., 2011; Pryke et al., 2011) primary sex ratios in some avian species. It has also been observed that such sex ratio adjustments are produced by a corticosterone influence on sex-chromosome segregation at the first meiotic division (Correa et al., 2005; Aslam et al., 2014). In mammals, studies *in vitro* have shown a possible interference of GC in the biosynthesis of male steroids (Xiao et al., 2010). It has been reported that GCs or Dex decrease testosterone biosynthesis by inhibiting the androsterone production in progenitor Leydig cells (Martin and Tremblay, 2008). The contrasting effects of GCs on offspring sex ratios seem to be associated with the multiple mechanisms of sex determination exhibited by taxa and related to the fitness gains from strategic offspring sex manipulation. Our results indicate that Dex is either not involved in gonadal feminization or not strong enough to counteract the masculinization process initiated by temperature.

Phenotypes effects induced by dexamethasone manipulation suggest that fitness is likely to be impacted, as hatchling size predicts subsequent survival (Warner and Shine, 2007). Our results agree with previous studies in lizards that demonstrated that prenatal GC exposure increases body size (Uller and Olsson, 2006). While the prevailing view is that excessive amounts of GCs are accompanied by impaired growth rates in juveniles or adults, our data suggest a positive effect of Dex on embryonic body size. Growth, in terms of height and weight, is a complex physiological process in which several growth factors are involved in a coordinated fashion and, certainly, the growth hormone (GH) is a major contributor (Giustina et al., 2008). In this context, some reports indicate that GCs are required for somatotroph (GH- producing cells of the anterior pituitary) differentiation and, thus, its functions during embryonic development (Kawashima et al., 2010; Vakili and Cattini, 2012). Our observations could be interpreted taking into consideration such reports. Nevertheless, GCs might have deleterious effects on some physiological parameters like the unbalanced blood sugar that might affect embryo or hatchling survival negatively, which is a topic for further studies.

In summary, we have demonstrated that the manipulation of Dex (a potent synthetic GC) at the beginning of TSP affects offspring phenotypes in ways likely to alter their fitness. The Dex effect on the somatotroph might be an important area for future research, especially in light of endocrine interactions during development.

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