SHORT COMMUNICATION

Serum concentrations of corticosterone and sex hormones and their relationship in farmed Morelet's crocodile (*Crocodylus moreletii*)

Concentraciones séricas de corticosterona y de hormonas sexuales y su relación en el cocodrilo de Morelet (Crocodylus moreletii) criado en granja

Concentrações de soro de corticosterona e de hormônios sexuais e sua relação em crocodilo de Morelet (Crocodylus moreletii) criado em fazenda

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Abstract

Background: Crocodile farming aims to produce high-quality skins from captive crocodilians. Captivity usually exposes the animals to stressful conditions, resulting in increased serum corticosterone (CORT) levels that correlate negatively with those of sex hormones and reproductive success. **Objective:** To evaluate serum concentrations of CORT and sex hormones and their relationship in farmed Morelet's crocodiles (*Crocodylus moreletii*) during the non-breeding (NBS) and breeding (BS) seasons. **Methods:** The study included 59 adult crocodiles (29 females and 30 males). One blood sample was collected in NBS (n=31) and BS (n=28) from each crocodile to determine serum concentrations of CORT, estradiol (E_2), progesterone (P_4), and testosterone (T). Crocodiles were kept in mixed-sex groups and were fed once or twice a week throughout the study. **Results:** In females, CORT was higher in NBS ($p<0.05$) but had no correlation with E_2 or P_4 in any season ($p>0.05$). In males, CORT was similar in NBS and BS ($p>0.05$) and had no correlation with T ($p>0.5$). **Conclusion:** The CORT levels had no effect on sex hormones. This could be explained by low CORT levels resulting from farming conditions where the animals were not exposed to severe or chronic stress.

Keywords: *corticosterone; crocodile farming; crocodilians; estrogen; progesterone; sex hormones; stress; testosterone.*

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Resumen

Antecedentes: La cría de cocodrilos en granja busca producir pieles de alta calidad de cocodrilianos en cautiverio. El cautiverio usualmente expone a los animales a condiciones estresantes, resultando en altas concentraciones séricas de corticosterona (CORT) que se correlacionan negativamente con los niveles de hormonas sexuales y el éxito reproductivo. **Objetivo:** Evaluar las concentraciones séricas de CORT y de hormonas sexuales y su relación en cocodrilos Moreletii (*Crocodylus moreletii*) criados en granja, tanto durante la época no reproductiva (NBS) como en la reproductiva (BS). **Métodos:** El estudio incluyó 59 cocodrilos adultos (29 hembras y 30 machos). Se recolectó una muestra de sangre de cada cocodrilo en NBS (n=31) y BS (n=28) para determinar las concentraciones séricas de CORT, estradiol (E_2), progesterona (P_4) y testosterona (T). Los cocodrilos permanecieron en grupos mixtos de machos y hembras y fueron alimentados una o dos veces por semana durante el estudio. **Resultados:** En hembras, CORT fue más alta en NBS ($p<0,05$), pero no se correlacionó con E_2 o P_4 en ninguna temporada ($p>0,05$). En machos, CORT fue similar en NBS y BS ($p>0,05$) y no tuvo correlación con T ($p>0,05$). **Conclusión:** Las concentraciones de CORT no tuvieron efecto sobre las hormonas sexuales, tal vez debido a que la CORT tuvo niveles bajos como resultado de las condiciones de manejo de la granja en las que los animales no se expusieron a estrés severo o crónico.

Palabras clave: *cocodrilianos; corticosterona; estrés; estrógenos; granja de cocodrilos; hormonas sexuales; progesterona; testosterona.*

Resumo

Antecedentes: A criação de crocodilos em fazenda procura produzir couros de alta qualidade de crocodilos em cativeiro. O cativeiro geralmente expõe os animais a condições estressantes, resultando em altas concentrações de soro de corticosterona (CORT) que têm correlação negativa com os níveis de hormônios sexuais e o sucesso reprodutivo. **Objetivo:** Avaliar as concentrações de soro de CORT e hormônios sexuais e sua relação em crocodilos Moreletii (*Crocodylus moreletii*) criado em fazenda, nas estações de não reprodução (NBS) e reprodução (BS). **Métodos:** O estudo incluiu 59 crocodilos (29 fêmeas e 30 machos). Uma amostra de sangue foi coletada de cada crocodilo em NBS (n=31) e BS (n=28) para determinar as concentrações de soro de CORT, estradiol (E_2), progesterona (P_4) e testosterona (T). Ao longo do estudo, os crocodilos permaneceram em grupos mistos de machos e fêmeas e foram alimentados uma ou duas vezes por semana. **Resultados:** Em fêmeas, CORT foi maior em NBS ($p<0,05$), mas não teve correlação com E_2 ou P_4 em qualquer estação ($p>0,05$). Em machos, CORT foi parecido em NBS e BS ($p>0,05$) e não teve correlação com T ($p>0,05$). **Conclusão:** As concentrações de CORT não tiveram efeito sobre os hormônios sexuais talvez porque o CORT foi baixo como resultado das condições de tratamento em fazenda que não expuseram os animais a estresse severo ou crônico.

Palavras-chave: *corticosterona; crocodilianos; estresse; estrogênios; fazenda de crocodilos; hormônios sexuais; progesterona; testosterona.*

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Introduction

Crocodile farming is commonly conducted under improper management practices, causing stress in the animals and a concurrent increase in corticosterone (CORT) plasma levels (Isberg and Shilton, 2013; Finger *et al.*, 2015), which is the main glucocorticoid (GCs) in reptiles (Cockrem, 2013). While acute increases in GCs after short-term stress are beneficial for the survival of the individual (Dantzer *et al.*, 2014), long-term increases in GCs after chronic stress have deleterious effects on reproduction, immunity, and growth (Sapolsky *et al.*, 2000). In farmed individuals, including crocodilians, chronic stress affects their health, reproduction, and productivity (Morici *et al.*, 1997). High CORT correlates negatively with concentrations of sex hormones and reproductive success (Lance and Elsey, 1986; Elsey *et al.*, 1991; 1994; Guillette *et al.*, 1995).

Data on the relationship between plasma CORT levels and sex hormones are available on the American alligator (*Alligator mississippiensis*; Lance and Elsey, 1986; Elsey *et al.*, 1990; 1991; Guillette *et al.*, 1997) and the Cuban crocodile (*Crocodylus rhombifer*; Augustine *et al.*, 2020). Nevertheless, there is no information in this regard in Morelet's crocodile (*Crocodylus moreletii*). Morelet's crocodile is native to Mexico and is included in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2022). Hence, its legal use for conservation, research, or commercial purposes occurs in farms (Sánchez-Herrera *et al.*, 2011; Cedeño-Vázquez *et al.*, 2012) where crocodiles are usually housed in groups according to their size. However, confinement of crocodiles increases their aggressive behavior, particularly in the breeding season, and leads to high levels of GCs (Elsey *et al.*, 1990; 1994). Evaluation of CORT and sex hormones patterns in crocodilians can help monitor animal welfare and reproductive status (Augustine *et al.*, 2020).

The present study hypothesized that CORT correlates negatively with sex hormones levels in intensively managed Morelet's crocodiles. Therefore, we assessed serum concentrations of CORT and sex hormones and their relationship in farmed *C. moreletii* female and male adults during non-breeding (NBS) and breeding (BS) seasons.

Materials and Methods

Ethical considerations

The experiment complied with guidelines by the Bioethics and Animal Welfare Commission of the School of Veterinary Medicine and Zootechnics of Universidad Veracruzana (Act 07/22, November 24, 2022).

Study characteristics

The study was conducted in Veracruz, Mexico (Lat. 19°22' N, Long. 96°22' W) at El Colibrí de la Antigua crocodile farm during two consecutive years (2017-2019) to evaluate hormone concentrations in NBS (November) and BS (March). The breeding season in *C. moreletii* starts with courtships in February or March and ends with egg laying in July (Pérez-Higareda, 1980; Lazcano, 1982). During the study, mean annual temperature and precipitation were 23.5°C and 34 mm during NBS, and 26.7°C and 15 mm in BS.

Experimental animals

The study included 59 apparently healthy adult Morelet's crocodiles (≥1.51 m length, 29.2 ± 9.1 kg live weight; 29 females and 30 males). Crocodiles were randomly selected from the general population of adult individuals that had reached commercial size. In NBS, blood samples were collected from 31 individuals (15 females, 16 males), and in BS from 28 (14 females, 14 males). Different crocodiles were included in each evaluation to determine hormone concentrations that could be representative of the population and not concentrations in specific individuals. In addition, since the crocodiles had reached

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commercial size, they were slaughtered a few days after sampling.

Crocodiles were housed in outdoor concrete enclosures with both females and males. Each enclosure was 18 m length×8 m breadth×1.4 m height and had a pool of 17×6×1.4 m filled with water at a depth of 80 cm, a basking area at each side, and a shaded area of 12×1.5 m. The stocking density was similar in both seasons of the year, and it was 0.1 to 0.2 individuals/m^2.

Crocodiles were fed a mixture of minced chicken and fish prepared at the farm (40% crude protein) at amounts of 10% of their live weight once or twice a week throughout the study. The food was spread throughout the basking area so each crocodile could get some. They received mebendazole, as a dewormer, mixed with food every four months.

Blood sample collection and processing

Blood samples were collected to determine serum concentrations of CORT and sex hormones (estradiol [E_2], progesterone [P_4], and testosterone [T]). For blood sample collection, the crocodiles were captured from their enclosure one at a time. Once captured, they were physically restrained, and their snout taped shut to assure their own and the handlers' safety. Sex was determined and animals were examined visually to exclude sick, wounded, or emaciated individuals.

From each animal, a blood sample (3 to 5 mL) was aseptically collected from the post-occipital venous sinus within 3 min of capture (Romero and Reed, 2005), transferred into 6 mL plastic tubes without anticoagulant, and kept in a cooler for 1 to 2 h until the sampling of all the individuals was completed. Then, blood samples were centrifuged at 810 x g for 10 min to obtain the serum, which was stored at -20ºC until hormone analysis. Blood samples were collected once during the NBS and once in BS in each year of the study, totaling four blood samplings (November 2017/2018 and March 2018/2019). After sampling, each crocodile was measured and weighed to determine its total length and weight.

Determination of serum hormone concentrations

Serum concentrations of CORT were measured in both females and males; E_2 and P_4 in females, and T in males, all by solid phase enzyme-linked immunosorbent assay (ELISA) using commercial kits (DRG International, Inc., Springfield, NJ, USA). The hormone assays were validated for crocodiles using one mammalian known sample as positive control in each run of each hormone to confirm that the assay detected the hormone and measured its levels in serum. Validation values of the standard curves constructed to calculate the results for each hormone were: Corticosterone: R=0.9891, Rsqr=0.9881, Adj Rsqr: 9852, SE of estimate: 0.0279; Estradiol: R=0.9987, Rsqr=0.9974, Adj Rsqr=0.9949, SE of estimate=0.0123; Progesterone: R=0.9984, Rsqr=0.9968, Adj Rsqr=0.9936, SE of estimate=0.0085; Testosterone: R=0.9990, Rsqr=0.9980, Adj Rsqr=0.9961, SE of estimate=0.0512.

Corticosterone. The DRG® Corticosterone ELISA kit was used (DRG International, Inc., Springfield, NJ, USA). The assay indicated cross-reactivity with CORT (100%), P_4 (7.4%), deoxycorticosterone (3.4%), 11-dehydrocorticosterone (1.6%) and cortisol (0.3%). The assay range was 0 to 240 nmol/L, with sensitivity <1.6 nmol/L. The intra- and inter-assay coefficients of variation (CV) were 3.1 and 6.0%. The resultant concentrations were transformed from nmol/L into ng/mL by dividing them by 2.89.

Estradiol. The kit used was DRG® Estradiol ELISA (DRG International, Inc., Springfield, NJ, USA). The assay indicated cross-reactivity with estradiol-17β (100%), estrone (0.2%) and estriol (0.05%). The assay range was 9.7 to 2,000 pg/mL, and 9.7 pg/mL sensitivity. Intra- and inter-assay CVs were 4.5 and 7.8%.

Progesterone. The DRG® Progesterone ELISA kit was used (DRG International, Inc., Springfield, NJ, USA). The assay indicated cross-reactivity with P4 (100%), 11-desoxycorticosterone (1.1%), pregnenolone (0.35%), 17α-hydroxyprogesterone (0.3%) and CORT (0.2%). The assay range was 0 to 40 ng/mL, and 0.04 ng/mL sensitivity. Intra- and inter-assay CVs were 6.4 and 6.6%.

Testosterone. The DRG® Testosterone ELISA kit was used (DRG International, Inc., Springfield, NJ, USA). The assay indicated cross-reactivity with T (100%), 11β-hydroxytestosterone (3.3%), 19-nortestosterone (3.3%), androstenedione (0.9%) and 5α-dihydrotestosterone (0.8%). The assay range was 0.08 to 16 ng/mL, with 0.08 ng/mL sensitivity. Intra- and inter-assay CVs were 3.5 and 7.1%.

Data analysis

Differences in hormone concentrations between NBS and BS in females and males were analyzed with the Student's t-test for independent variables. A difference of $p<0.05$ was considered significant. The Spearman's correlation coefficient was used to determine the relationship between concentrations of CORT and E_2, P_4 and T. The statistical program used was Statistica 10® (StatSoft®, Inc., OK, USA).

Results

NIn females, CORT and P_4 concentrations were higher ($p<0.05$) in NBS, while E_2 levels were similar ($p>0.05$) in NBS and BS (Table 1). No correlation ($p>0.05$) was observed between CORT and E_2 or P_4 concentrations in any season (Table 2).

In males, CORT concentrations were similar ($p>0.05$) in NBS and BS, while T levels were higher ($p<0.05$) in BS (Table 1). There was no correlation ($p>0.05$) between CORT and T concentrations in any season (Table 2).

Table 1. Serum concentrations (mean ± SEM) of corticosterone and sex hormones in adult females and males of Morelet's crocodile (*Crocodylus moreletii*) during the non-breeding (NBS) and breeding (BS) seasons.

	Non-breeding season	Breeding season
Females		
Corticosterone (ng/mL)	47.8 ± 24.8^{a}	15.5 ± 9.1^{b}
Estradiol (pg/mL)	203.0 ± 110.2^{a}	251.6 ± 191.4^{a}
Progesterone (ng/mL)	2.0 ± 1.7^{a}	0.4 ± 0.5^{b}
Males		
Corticosterone (ng/mL)	27.1 ± 17.5^{a}	23.4 ± 14.1^{a}
Testosterone (ng/mL)	2.3 ± 1.8^{a}	8.9 ± 5.1^{b}

Different superscript letters ($^{a, b}$) within rows indicate statistical difference by season ($p<0.05$).

Table 2. Correlation between serum concentrations of corticosterone and sex hormones in adult females and males of Morelet's crocodile (*Crocodylus moreletii*) during the non-breeding (NBS) and breeding (BS) seasons.

	Non-breeding season	Breeding season
Females		
Corticosterone and estradiol	r=0.02 p=0.91	r=-0.47 p=0.08
Corticosterone and progesterone	r=0.35 p=0.23	r=0.65 p=0.058
Males		
Corticosterone and testosterone	r=0.24 p=0.36	r=0.16 p=0.57

Discussion

Captive crocodilians will commonly experience some level of stress (Elsey *et al.*, 1994); in consequence, they will have increased plasma CORT (Guillette *et al.*, 1995; Cockrem, 2013). In reptiles, CORT levels are usually higher in BS when they might be necessary for reproduction (Tokarz and Summers, 2011). This was assumed in the present study due to farming conditions, as crocodiles would be exposed to several stressors especially in BS because of increased energy demands in such period (Tokarz

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and Summers 2011), and high CORT would decrease sex hormones levels. However, none of this occurred. This could be explained as CORT was not high because the animals did not require a CORT release to mobilize energy during BS, like a report in male rattlesnake (*Crotalus atrox*; Taylor *et al.*, 2004), or that animal management was adequate and thus they had low stress levels, particularly males, which showed similar CORT in NBS and BS. It is known that *C. moreletii* does well in captivity and shows high tolerance towards conspecifics (Lang, 1987; Ojeda *et al.*, 1998), which could contribute to low stress and CORT levels. However, since there are no reference values for CORT in *C. moreletii*, it is not possible to accurately know if the levels found in the present study could be considered as normal or if they were elevated, considering that these are captive individuals.

Females had higher CORT in NBS, which could be a female response. Differences in CORT levels are common in reptiles and result from variations, at individual or population level, of the adrenocortical response to the same stressor caused by age, reproductive status, and season of the year (Dunlap and Wingfield, 1995; Moore *et al.*, 2001; Moore and Jessop, 2003). However, as mentioned before, there are no reference values for CORT in *C. moreletii* that allow to know if lower levels found in the present study could be considered normal or if in both seasons CORT was, indeed, elevated. Therefore, it is necessary to conduct more studies on captive *C. moreletii* to establish normal values of CORT in females and males to help monitor the stress status throughout the year.

In females, the E_2 similarity observed in NBS and BS was contrary to the expected (higher E_2 in BS). In tropical crocodilians, circulating E_2 increases in breeding females four to five months before oviposition, as it stimulates vitellogenin production and growth of preovulatory follicles (Uribe and Guillette, 2000; Calderón *et al.*, 2004; Milnes, 2011) and because the oviduct grows during such time (Guillette *et al.*, 1997; Milnes, 2011). Thus, plasma E_2 is elevated when vitellogenic follicles are present, namely, right before ovulation (Coutinho *et al.*, 2000). The reason for not obtaining higher E_2 in BS could be that the timing and number of samplings did not allow to detect the expected differences that should occur in BS. The samples collected in March corresponded to the start of BS; hence, maybe March was too early into BS to show elevated E_2. Thus, it is necessary to evaluate E_2 in adult females several times throughout the year to establish normal values for different moments within each season to determine the stage of the breeding cycle in which females are.

Serum P_4 levels were higher in NBS, contrary to what was expected and to findings in American alligator females indicating that plasma P_4 was barely detectable throughout the year and only increased in the periovulatory period (Lance, 1989) and then declined after oviposition (Guillette *et al.*, 1997). In egg-laying Cuban crocodile (*Crocodylus rhombifer*) fecal P_4 metabolites were higher in the nesting season than in BS and NBS (Augustine *et al.*, 2020), likely because P_4 promotes the formation and development of eggs in reptiles (Custodia-Lora and Collard, 2002). In this study, P_4 was not evaluated in the nesting season but at the start of BS, when females were not close yet to ovulating. That might be the reason for not observing differences in P_4 between NBS and BS. Hence, it is necessary to evaluate P_4 at different moments within BS in adult females to establish normal values for its different stages, including oviposition and nesting, to improve the management of farmed females.

In the present study, CORT did not correlate with E_2 and P_4. The lack of correlation between CORT and E_2 was contrary to the negative correlation observed in female American alligators suggesting that acute stress decreases E_2 levels (Elsey *et al.*, 1991). Exposure of females to stressors can decrease E_2 levels in some reptiles (Elsey *et al.*, 1991; Ganesh and Yajurvedi, 2002) but the relationship between plasma CORT and E_2 or P_4 varies greatly with species and reproductive stage (Tokarz and Summers, 2011).

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In males, higher levels of T in BS were expected and agreed with the results obtained in American alligators (Lance, 1989; Guillette *et al.*, 1997) and Cuban crocodiles (Augustine *et al.*, 2020), and with reports indicating that plasma T increases concurrently with courtship and copulation in male crocodilians, corresponding with the restart of gonadal activity that begins four months before nesting, and that T declines abruptly at the end of BS (Milnes, 2011). On the other hand, CORT had no correlation with T in NBS and BS, which is contrary to a report where T levels in male reptiles correlate positively with baseline CORT (Eikenaar *et al.*, 2012). In male American alligators, CORT and T were negatively correlated, indicating that CORT inhibits T secretion in males (Lance and Elsey, 1986).

In general, one explanation for the lack of correlation between CORT and E_2, P_4, or T in NBS and BS could be that CORT was not high enough to influence sex hormones levels, suggesting that crocodiles were not experiencing significant or chronic stress in either season in the present study.

In conclusion, CORT levels did not correlate to those of sex hormones in any sex or season suggesting that crocodile management was adequate for their well-being and optimal reproduction. Only two samplings were conducted throughout each year, which might be insufficient to determine the effect of husbandry on reproduction and welfare. Therefore, it is necessary to conduct more studies on farmed *C. moreletii* individuals with multiple samplings at different times during NBS and BS to determine basal concentrations of CORT and sex hormones. This would help to better understand the effects of management on reproduction and welfare and to determine optimal husbandry conditions for captive Morelet's crocodiles.

Declarations

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Conflict of interest

The authors declare that they have no conflicts of interest regarding the work presented in this report.

Author contributions

AGC: study design, field and laboratory work and data analysis. CAA: study design and supervision, project administration, data analysis, manuscript writing. LLB: study design and supervision, manuscript writing. SVP: study design and supervision. JEMM: data analysis and manuscript revision. FMP: study supervision and manuscript revision.

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