Short Communication

Unexpected lower testosterone in faster growing farmed saltwater crocodile (*Crocodylus porosus*) hatchlings

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ABSTRACT

Agricultural production of the saltwater crocodile (*Crocodylus porosus*) is an emergent industry in northern Australia with many of the factors affecting production remaining unknown. In this study, we sought to expand upon our previous findings of reference corticosterone and immune function by reporting baseline sex hormone levels [testosterone (TEST) and estradiol (ESTR)] and their association with growth. This was achieved by sampling 253 hatchling crocodiles repeatedly at 3, 6, and 9 months of age. Sampling age had a significant effect on both TEST ($p < 0.001$) and ESTR ($p < 0.001$) suggesting climatic/abiotic factors have an influence even in prepubescent crocodiles. Stress, as measured by plasma corticosterone, had no detectable effect on plasma ESTR or TEST levels. Unexpectedly however, TEST was higher in slower-growing crocodiles, which is contrary to what has been reported for the American alligator. ESTR was not associated with growth.

1. Introduction

Production of saltwater crocodile (*Crocodylus porosus*) skins for the international leather trade is a necessary component of crocodile conservation in Australia by providing economic incentives for sustainable use. To achieve this objective, crocodile farming must remain economically viable. As such, previous studies have been conducted to understand the significant biotic and abiotic factors affecting various production traits including age at harvest (Isberg et al., 2005), juvenile survival (Isberg et al., 2006a) and number of scale rows (Isberg et al., 2006b; Miles et al., 2010). However, many of the factors that could underlie these production traits remain unknown. For example, the effect of stress (corticosterone; CORT) has been described is some detail including its effect on growth and/or suppressed immune function (Elsey et al., 1990; Turton et al., 1997; Finger et al., 2015). However, the effect of sex steroids on these traits have not been explored in juvenile *C. porosus*. In fact, the possibility that producers may manipulate these hormone levels, akin to previous work in cattle (e.g. Perry et al., 1991), is of interest in a commercial setting. However, modifying levels of sex steroids may have other unintended effects. For example, increasing androgen levels has been shown to increase aggression in lizards (Marler and Moore, 1989) although this has not been tested in crocodilians. Due to previous work illustrating that testosterone (TEST) levels were higher in faster growing Nile crocodiles (*Crocodylus niloticus*; Morpurgo et al., 1992), we hypothesized that faster growing *C. porosus* would similarly exhibit higher levels of TEST and estradiol (ESTR).

2. Methods and materials

Details of the experimental design have been previously described by Finger et al. (2015). Briefly, 253 male hatchling *C. porosus* were sampled three times at an average age of 113 (Sampling 1), 189 (Sampling 2), and 275 (Sampling 3) days post-hatch. Sampling 1, 2, and 3 corresponded to the months of June/July (cool, dry season), September/October (hot, dry season), and November/December (hot, wet season) respectively (Finger et al., 2015). As described in Finger et al. (2015), crocodiles were fed five times weekly and housed in enclosed sheds. Water temperature was maintained at 32 °C, but air temperature fluctuated with ambient, seasonal conditions. All experimental protocols were approved by the University of Sydney Animal Ethics Committee (approval number: N00/5-2012/3/5729).

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At each Sampling, blood samples, from the occipital sinus, were taken from the same subset of crocodiles ($n = 40$) although head length (HL) and total length (TL; Isberg et al., 2005) measurements were obtained from all 253 animals (Finger et al., 2015). Raw mean (± standard error; SE) TL for Sampling 1, 2, and 3, was 453.80 ± 4.27 mm, 518.40 ± 5.17 mm, and 624 ± 7.46 mm, respectively. To obtain baseline CORT, TEST, and ESTR levels, each Sampling was divided into four weeks with only 10 crocodiles blood sampled per week. A timer was started when we entered the pen to calculate the cumulative time (CumTime) between entering the pen, capture, and obtaining a blood sample for each individual (Finger et al., 2015). Sampling was standardized at 10:00 h, an hour after cleaning finished.

Plasma TEST (DES6622, Demeditec Diagnostics GmbH, Kiel, Germany) and ESTR (DESLV4188, Demeditec Diagnostics GmbH, Kiel, Germany) levels were determined by enzyme-linked immunosorbent assays (ELISAs). All kits were assayed following manufacturer-specific protocols at Berrimah Veterinary Laboratories (Berrimah, NT, Australia). Prior to the assay, ESTR was extracted from plasma following the protocol of Lance et al. (2003). Briefly, 150 µL of plasma was mixed with 1350 µL of ethyl acetate:hexane mix (3:2 dilution) and vortexed for 10 s. Subsequently, this volume was snap-frozen in a dry ice-acetone slush and dried under an air stream on a 55 °C block heater. After drying, extracted ESTR was reconstituted in 150 µL of PBS.

As described by Finger et al. (2015), all analyses were performed using ASReml (Version 3) and GenStat (Version 16.1). The phenotypic traits plasma ESTR and TEST were natural log-transformed prior to analysis to meet model assumptions.

Explanatory variables included the factor Sampling ($k = 1, 2, 3$). As described by Finger et al. (2015), a CumTime-adjusted CORT (CORTadj) predictor variable was created. Similarly, a HLres variable was also created using residuals from the significant terms for a model for head length (Finger et al., 2015). Since the data are repeated measures, AnimalID was included as a random effect to link the observations of an individual. Clutch was also included as a random effect to account for any clutch effects. The assumption of residual variance homogeneity between sampling periods was evaluated and accounted for where necessary. All results are reported as back-transformed model-based means ± SEs. The significance of all terms was determined at the 5% significance level (Finger et al., 2015).

3. Results

3.1. Testosterone

The overall mean TEST level was 254.4 ± 23.2 pg/mL. TEST was significantly affected by Sampling ($p < 0.001$) and HLres ($p = 0.004$; Fig. 1). TEST levels at both Sampling 1 (343.8 ± 41.9 pg/mL) and 3 (280.6 ± 33.7 pg/mL) were significantly higher than Sampling 2 (170.9 ± 16.8 pg/mL), respectively (5% LSD; Fig. 2). Interestingly, larger animals (HL) had lower TEST levels ($-1.02 ± 0.01$) but CORTadj was not significant ($p = 0.75$). There was a significant effect of Clutch on TEST levels.

3.2. Estradiol

The overall mean ESTR concentration was 2.86 ± 0.24 pg/mL. As observed with TEST, ESTR was significantly affected by Sampling ($p < 0.001$). However, contrary to TEST, Sampling 2 ESTR (4.41 ± 0.47 pg/mL) was significantly higher than ESTR at Samplings 1 (2.21 ± 0.19 pg/mL) and 3 (2.40 ± 0.23 pg/mL; 5% LSD; Fig. 2). Neither CORTadj ($p = 0.62$), HLres ($p = 0.71$; Fig. 1) nor Clutch ($p = 0.13$) were significant.

4. Discussion

The aim of this study was to determine if plasma sex steroids had an underlying association with crocodile growth and/or stress as measured by CORT. In this commercial setting, as reported by Finger et al. (2015), there was no significant effect of CORT on HL as justified by the low baseline levels of CORT experienced. Similar in this study, CORT was not a significant covariate for either ESTR or TEST. Interestingly though, the lowest TEST levels were observed at Sampling 2 when both CORT and ESTR were at their peak (Fig. 2).

For crocodile producers, understanding how sex steroids impact on crocodile growth is important. Similar to previous work conducted in wild American alligators (*Alligator mississippiensis*; Rooney et al., 2004), we observed no effect of animal size on ESTR concentrations. Unexpectedly though, we observed a negative relationship between HL and plasma TEST (Fig. 1). This is in contrast to previous observations in captive Nile crocodiles and wild alligators (Morpurgo et al., 1992; Lance et al., 2015; Rooney et al., 2004). There are a few possible explanations for this different observation. Firstly, the experimental design employed by Rooney et al. (2004) considered a larger size range of animals (52–172 cm total length) and excluded smaller individuals (<38 cm snout-vent length) because they had TEST values <50 pg/mL. Secondly, not all alligators were bled immediately after capture, instead some were held in cloth bags for almost 2 h until blood sampling (Rooney et al., 2004), which may have suppressed alligator TEST levels (Lance et al., 2004). Thirdly, the husbandry and housing of the *C. niloticus* described in Morpurgo et al. (1992) is substantially different to this study (Finger et al., 2015), in particular, the stocking densities. In fact, after reducing the stocking density and size variation at 12-months of age, although non-significant, the TEST levels of the smaller 18-month old *C. niloticus* were higher than the medium size animals. Most interestingly though is the decrease in plasma TEST of the medium-sized crocodiles between the 12- and 18-month samplings. In contrast, the crocodiles in this study were kept at consistent stocking densities with minimal size variation due to constant grading between pens. Grading may have affected CORT levels and subsequently, TEST or ESTR (Lance et al., 2004). However, the CORT levels were the lowest reported for *C. porosus* (Finger et al., 2015) and we observed no effect of CORTadj on either TEST or ESTR. Moreover, grading was not conducted on sampling days to minimize disturbance.

In this study, the highest TEST levels were during Sampling 1 (June/July), which coincided with the cooler, dry season of the Australian tropics (Webb et al., 1991; Fig. 2). The next highest levels were at Sampling 3 (November/December) during the initial stages of the wet season and corresponding to the start of the *C. porosus* breeding season (November to March; Webb et al., 1977). The lowest TEST levels were at Sampling 2 (September/October) coinciding with the "build-up", a hot and humid period with little to no rain prior to the commencement of the wet season. This temporal variation of TEST in hatchling *C. porosus* has also been observed in the American alligator (Lance et al., 2015), however the effects were antithetical. In juvenile and adult alligators, TEST levels were highest immediately prior to the start of the breeding season, whereas the lowest levels were found in colder months (Lance, 1989; Lance et al., 2015; Rooney et al., 2004). Concurrent with this increase in TEST, adult males also exhibit increased testicular mass and spermatogenesis in preparation for breeding (Lance, 1989).

As with TEST, seasonal variation in ESTR levels were also observed (Fig. 2). The highest ESTR levels in hatchling *C. porosus* were at Sampling 2, a few months before the start of the wet season and reproductive activities in sexually mature individuals. This was the converse to the trend of TEST (Fig. 2). Similar to our results, Lance et al. (2003) also observed temporal variation in juvenile