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Rapid suppression of testosterone secretion after capture in male American alligators (*Alligator mississippiensis*)

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Abstract

All reptiles studied to date show an increase in circulating corticosterone following capture. This rise in corticosterone has also been shown in a number of instances to result in a decline in reproductive steroids within hours after capture. As a result of these observations it has been considered imperative to collect blood samples as soon as possible after capture to get reliable measures of reproductive hormones. It has been claimed, however, that there is no effect of capture stress on reproductive steroids in juvenile alligators held for 2 h following capture. As we generally reject blood samples that are not collected within 15 min of capture we decided to reinvestigate the effect of short-term capture (2 h) on corticosterone and testosterone in male alligators. Four groups of alligators, ranging in size from 74 to 212 cm total length were captured in a 2-week period in May, the time of year when testosterone levels are highest. Two groups were captured during the day (eight bled at capture and again at 2 h, eight bled at 2 h only) and two at night (10 bled at capture and again at 2 h, 10 bled at 2 h only). Testosterone and corticosterone in alligators bled immediately on capture and at 2 h were not significantly different in the AM and PM samples so the results were combined (Initial bleed: corticosterone, 0.95 ± 0.09 ng/ml, $n = 18$; testosterone, 6.06 ± 2.09 ng/ml, $n = 18$. Two-hour bleed: corticosterone 15.68 ± 1.91, $n = 18$; testosterone, 2.75 ± 0.79, $n = 18$). Both the increase in corticosterone and the decline in testosterone at 2 h were significant ($p < 0.05$). Corticosterone and testosterone in the alligators sampled only once at 2 h were not significantly different from the 2-h values in alligators sampled twice (corticosterone 15.04 ± 1.29, $n = 18$; testosterone, 1.85 ± 0.62, $n = 18$). These results clearly demonstrate that short-term capture stress results in a significant decline in testosterone in male alligators.

1. Introduction

In recent years there has been considerable interest in the effect of capture stress on reptiles. Marked increases in circulating corticosterone levels in response to capture have been well documented in lizards (Cree et al., 2000; Dunlap and Wingfield, 1995; Grassman and Hess, 1992; Moore et al., 1991), snakes (Mathies et al., 2001; Moore et al., 2000, 2001), tuatara (Cree et al., 1990a,b; Tyrrell and Cree, 1998), turtles (Aguirre et al., 1995; Cash et al., 1997; Jessop, 2001; Licht et al., 1985; Mahmoud et al., 1989; Valverde et al., 1999), and crocodilians (Elsey et al., 1990, 1991; Franklin et al., 2003; Guillette et al., 1997; Jessop et al., 2003; Lance and Elsey, 1986, 1999a,b; Lauren, 1985; Mahmoud et al., 1996; Morici et al., 1997; Turton et al., 1997). A number of investigators have also reported declines in plasma levels of reproductive steroids in response to capture stress (Lance and Elsey, 1986; Elsey et al., 1991; Licht et al., 1985; Mahmoud et al., 1989; Moore et al., 1991, 2000, 2001; Tyrrell and Cree, 1998). As a result of such studies it is now considered critical to get blood samples from reptiles within minutes after capture (when studying reproduction) in order to avoid the inhibitory effects of stress on the secretion of reproductive hormones (Lance, 1990, 1994; Lance et al., 2000).

Other investigators have shown that in some instances this capture stress response is modified by reproductive condition; animals in breeding condition have a blunted or less vigorous rise in corticosterone than animals in non-breeding condition, and the decline

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in reproductive steroids is less severe (Cree et al., 2000; Dunlap and Wingfield, 1995; Jessop, 2001; Valverde et al., 1999, see also review by Moore and Jessop, 2003).

A number of years ago we showed that when adult male alligators were held under restraint for up to 48 h, plasma corticosterone increased rapidly and plasma testosterone decreased to non-detectable levels by 24 h. The decrease in testosterone was significant by 4 h (Lance and Elsey, 1986). Adult female alligators held under restraint showed a similar profile of corticosterone secretion and a significant decline in estradiol by 24 h (Elsey et al., 1991).

Another indicator of acute stress in crocodilians is a rise in plasma glucose within minutes after restraint (Lance, 1994; Lance et al., 2000), though this response appears to be induced by autonomic responses and independent of the adreno-hypothalamic axis (deRoos et al., 1989; Lance et al., 2000).

Guillette et al. (1997) reported that juvenile alligators held in cloth sacks for 2 h showed a marked rise in plasma corticosterone from approximately 1 ng/ml to levels in excess of 20 ng/ml, but that "capture and holding of animals in cloth bags for 2 h does not induce significant changes in plasma sex steroid concentration" (Guillette et al., 1999). In our initial studies on capture stress in adult male alligators we did not take a second blood sample until 4 h after capture. As we are currently studying sexual maturation and seasonal variation in sub-adult alligators (and generally reject blood samples that are not collected within 15 min of capture) we decided to reinvestigate the effect of short-term stress in sub-adult and adult male alligators.

2. Materials and methods

We collected sub-adult and adult male alligators on the Rockefeller Wildlife Refuge in South West Louisiana during the month of May when testosterone levels are highest (Lance, 1989). Immature alligators also show elevated testosterone in May but circulating levels are some tenfold lower than in adults (Lance, 2003). Alligators were captured from airboats at night or snared from the bank during the day. Sixteen alligators were collected during the day and 20 at night. The alligators were divided into four groups: two groups during the day and two at night, (1) bled immediately at capture, the mouth held shut with rubber bands, held in a sack for 2 h then bled again before release, (2) caught, held in a sack without an initial bleed then bled after 2 h. The groups consisted of eight alligators bled only once (after 2 h in a bag) during the day, 10 alligators bled only once (after 2 h in a bag) during the night, 10 alligators bled initially and again at 2 h during the night, and eight alligators bled twice (initially and 2 h later) during the day (Table 1). We attempted to collect only sub-adult male alligators (i.e., >180 cm total length), but of the 36 collected, eight males had total body lengths between 195 and 212 cm. These potentially sexually mature alligators, however, were distributed among three of the four groups. Total body lengths ranged from 74 to 212 cm with a mean of 157.6 ± 6 cm. Mean body length of alligators collected during the day (166 ± 26 cm, $n = 16$) was not significantly different from that of alligators collected at night (149 ± 41 cm, $n = 20$).

Table 1
Treatment groups

Group	Treatment	n	Total length, cm (range)
1	AM single sample	8	159.4 ± 8.7 (124.5–198.0)
2	PM single sample	10	127.80 ± 11.8 (74.0–169.0)
3	AM double sample	8	173.84 ± 9.4 (132.0–205.7)
4	PM double sample	10	170.8 ± 10.4 (134.6–212.1)

Blood samples were collected from the dorsal venous sinus using a 10 ml heparinized syringe fitted with an 18-gauge needle. The syringes filled with blood were placed on ice until returned to the laboratory where the blood was transferred to plastic tubes and then centrifuged, and the plasma separated and placed in a −20 °C freezer until assayed for steroids, glucose, and protein.

2.1. Radioimmunoassay

2.1.1. Testosterone

Duplicate 100 μl plasma samples (samples with concentrations greater than 4 ng/ml were diluted and reassayed) were extracted in 2 ml of an ethyl acetate:hexane mixture (3:2) by shaking for 30 s on a vortex mixer (Lance and Elsey, 1986). The tubes were then snap-frozen in a dry-ice–methanol mixture, the organic phase decanted into 12×75 mm disposal glass tubes, and evaporated to dryness under a stream of filtered air in a 37 °C water bath. To the dry tubes 500 μl of PBS (phosphate-buffered saline w/0.1% gel; pH 7.0), was added, and the tubes shaken for 30 s. Tritiated testosterone (10,000 cpm) in 100 μl PBS and 100 μl antibody in PBS (ICN, Costa Mesa, CA) added, and the mixture allowed to equilibrate overnight at 4 °C. Unbound steroids were removed by addition of 250 μl of a dextran–charcoal mixture (6.25 g charcoal, 0.625 g dextran in 100 ml PBS) and allowed to sit for 30 min at 4 °C. Following centrifugation at 8 °C for 15 min and 3000 rpm the supernatant containing the bound fraction was decanted into glass scintillation vials, 5 ml scintillation fluid added, and the radioactivity measured in a Beckman liquid scintillation counter, LS 6500.

2.1.2. Corticosterone

Corticosterone was measured by radioimmunoassay as previously described (Lance and Elsey, 1999a,b; Morici et al., 1997). Duplicate 150 μl aliquots of plasma (for samples with concentrations >6 ng/ml a smaller

volume of plasma was taken for subsequent assay) were extracted with 2 ml of ethyl acetate:hexane (3:2), the solvent evaporated under a stream of filtered air in a water bath at 37 °C, and the dried extract reconstituted in 500 µl PBS buffer, pH 7.0. Antibody (ICN, Costa Mesa, CA), 100 µl, and tritiated corticosterone,100 µl (Perkin–Elmer, Boston, MA), were added and the tubes held at 4 °C overnight. Unbound steroids were separated from bound with dextran–charcoal, the mixture centrifuged and the supernatant decanted directly into scintillation vials. A pool of alligator plasma and commercial human serum pools (for testosterone only) from Diagnostic Products, Los Angeles, CA were run in each assay for quality control. Interassay coefficient of variation for testosterone and corticosterone were 11.5 and 10.2%, respectively, and intraassay coefficient of variation for testosterone and corticosterone were 5.4 and 7.2%, respectively. The sensitivity of the corticosterone assay was 20 pg/tube and that of testosterone 5 pg/tube.

2.2. Glucose and protein

Glucose was measured in 25 µl plasma samples spectrophotometrically at 450 nm using the glucose oxidase–peroxidase enzymatic method (Sigma).

Protein was measured in 20 µl plasma samples spectrophotometrically at 540 nm using the biuret method (Sigma).

2.3. Statistics

Data were subjected to unpaired t test, paired t test, or ANOVA followed by Scheffé's multiple range test.

3. Results

3.1. Testosterone

Plasma testosterone in alligators bled immediately upon capture ranged from 0.085 to 33.93 ng/ml. The difference in mean testosterone levels between the initial bleed and the 2-h bleed was significant (paired t test, $t = 2.352$, $p = 0.031$). Plasma levels of testosterone at 2 h in the groups bled twice or only once at 2 h were not significantly different from one another ($t = -0.881$, $p = 0.385$).

The mean testosterone values for alligators caught during the day and bled immediately and those caught at night and bled immediately were not significantly different from one another, 6.92 ± 4.13 ng/ml ($n = 8$) vs 5.38 ± 2.02 ng/ml ($n = 10$) ($t = -0.357$, $p = 0.759$), therefore the results from the two groups were combined. Similarly the mean testosterone at 2 h in all four groups: group one, 2.66 ± 1.04 ng/ml ($n = 8$); group two, 1.21 ± 0.75 ng/ml ($n = 10$); group three, 2.84 ± 1.27 ng/ml ($n = 8$); and group four, 2.67 ± 1.08 ng/ml ($n = 10$) were not significantly different from one another. Group two had more small alligators than the other groups, three of which were less than 85 cm, thus the mean total length of this group was marginally, but significantly smaller ($p < 0.04$) than the other groups (Table 1). Testosterone and corticosterone levels in this group, however, were not significantly different from the other three groups. Plasma testosterone during the breeding season (March–May) in male alligators shows a significant relationship to total body length, but numbers of very large individuals that are clearly sexually mature sometimes have very low testosterone (Lance, Elsey, and Trosclair, in preparation). The eight potentially sexually mature alligators with total body lengths >1.8 m all had measurable testosterone, but in three of them the concentration was <1.0 ng/ml (e.g., Table 2).

Table 2
Alligators with very low testosterone

Alligator I.D.	Total length (cm)	Initial testosterone (ng/ml)	2-h Testosterone (ng/ml)
3343	132.0	0.106	0.075
3345	167.6	0.095	0.079
3354	150.0	0.085	0.080
3356	195.6	0.147	0.190

The results of the pooled data for testosterone are shown in Fig. 1.

3.2. Corticosterone

Plasma corticosterone in alligators bled immediately at capture ranged from 0.52 to 1.72 ng/ml. Mean corticosterone of alligators sampled during the day was 0.95 ± 0.15 ng/ml ($n = 8$), and was not significantly different from that of alligators bled immediately at capture at night, 0.94 ± 0.11 ng/ml ($n = 10$). The difference from initial sample and 2-h samples was highly significant ($t = 5.606$, $p < 0.001$). Likewise the mean corticosterone at 2 h in the group bled once, 15.04 ± 1.29 ng/ml ($n = 18$), was not significantly different from the 2-h sample in alligators bled twice, 15.68 ± 1.91 ng/ml ($n = 18$) ($t = 0.280$, $p = 0.7814$) (Fig. 1).

3.3. Glucose

Glucose concentration in the plasma in alligators sampled twice was 4.72 ± 0.2 mM/L at the initial bleed and 7.10 ± 0.3 nM/L at 2 h. The difference was highly significant, $p < 0.001$. Plasma glucose in alligators bled once at 2 h was $6.47 + 0.2$ mM/L and was not significantly different from the 2-h mean in the doubly sampled group (Fig. 2).

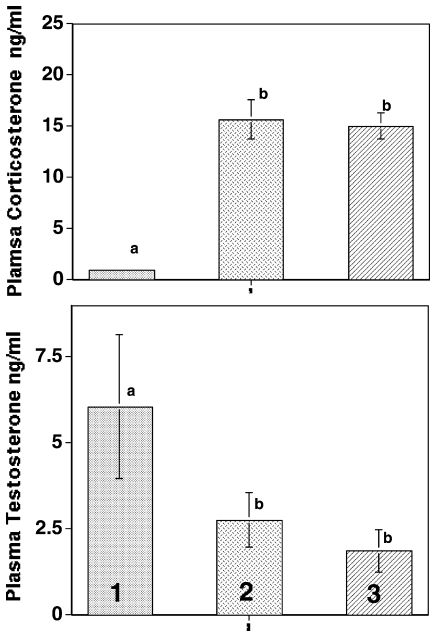

Fig. 1. Mean plasma corticosterone in ng/ml (upper panel) and mean plasma testosterone in ng/ml (lower panel) in male alligators immediately after capture and at 2-h post-capture. Group 1 ($n = 18$) represents samples immediately at capture, group 2 ($n = 18$) represents samples collected at 2 h following the immediate sample, and group 3 ($n = 18$) represents samples collected at 2 h only. The standard errors of the means (SEM) are indicated by the lines at the top of the bars. Lower case letters when different indicate differences are highly significant.

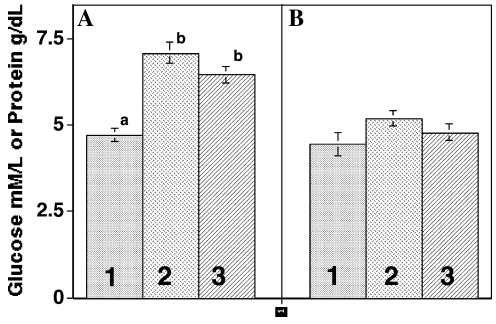

Fig. 2. Mean plasma glucose in mM/L (panel A) and mean plasma protein in g/dL (panel B) in male alligators. Groups and statistics as in Fig. 1.

3.4. Protein

Mean protein concentrations were not significantly different among all three groups (Fig. 2).

4. Discussion

Male alligators held in bags for 2 h show an increase in corticosterone, an increase in glucose, and a decline in testosterone. The stress of simply being held in a bag for 2 h is sufficient to cause a significant decline in testicular secretion of androgen. This decline of approximately 50% from initial plasma hormone concentrations could result in serious errors when estimating the reproductive condition of alligators. Thus, if studying reproductive steroids in this species it is imperative to collect blood samples as soon as possible after capture. There was no difference in corticosterone levels between animals that were bled once at 2 h, and those bled at capture and again at 2 h. Taking two blood samples does not appear to be any more stressful than taking a single blood sample at 2 h. In our previous study on restraint stress in adult male alligators (total body length >2.2 m), we kept the animals tied to a board and took multiple blood samples over a period of 48 h, a perhaps far more stressful experience than simply being held in a bag, yet mean corticosterone at 4 h in that experiment was less than 10 ng/ml (Lance and Elsey, 1986) as compared to mean levels of more than 15 ng/ml at 2 h in this study.

The range of values for corticosterone, both at initial sample and at 2-h post-capture, we report are in the same range as those reported by Guillette et al. (1997). Surprisingly, we found no differences in corticosterone levels between animals bled immediately at capture during the day and those bled immediately at night. A similar observation was noted in the study by Guillette et al. (1997) in that corticosterone levels in animals bled early in the evening were no different from those bled late in the evening. Alligators raised in captivity, however, show a distinct circadian rhythm in corticosterone levels; plasma levels at 8 PM are significantly higher than levels at noon (Lance and Lauren, 1984; Lance et al., 2000).

As was reported previously for this species (Lance, 1992, 1994) plasma glucose increased significantly following capture stress, presumably in response to catecholamine secretion (deRoos et al., 1989; Lance and Elsey, 1999a; Lance et al., 2000). Similar increases in glucose following restraint stress have been reported for *Crocodylus johnsoni* (Jessop et al., 2003) and *Crocodylus porosus* (Franklin et al., 2003). Plasma protein was included in the analyses to test for possible dilution of the blood sample with lymph or cerebrospinal fluid that sometimes occurs when collecting blood from the dorsal sinus. The results indicated that the samples were within normal range and similar to what has been published previously for the species (Coulson and Hernandez, 1964).

Several of the alligators over 2 m in length had testosterone levels less than 1 ng/ml (we have also encountered very large male alligators with surprisingly low circulating testosterone (Lance and Elsey, unpublished). Although there does appear to be a strong positive correlation between total body length and plasma testosterone in male alligators during the breeding season (Lance, Elsey, and Trosclair, in preparation) a single testosterone value is not always a good predictor of the

size or sexual maturity of the animal. Plasma corticosterone and plasma testosterone in the initial bleed showed a significant correlation ($R = 0.627$, $p = 0.0054$), but when a larger number of samples from a separate study (Lance and Elsey, in preparation) were analyzed the correlation was not significant. This observation clearly needs more research because in male desert tortoises testosterone and corticosterone levels are highly correlated (Lance et al., 2001).

Four alligators in the AM double-sample (group 3) had initial plasma testosterone of less than 0.2 ng/ml, but these animals were by no means the smallest in size (Table 2). Despite the apparent drop in testosterone levels (e.g., alligator number 3345, from 0.095 to 0.079 ng/ml) we cannot consider these values meaningful. When attempting to get an accurate measure of testosterone in plasma volumes of 100–200 μl and an assay sensitivity of about 10 pg/tube, small changes in concentrations from an initial value of less than 0.150 ng/ml in either direction are almost impossible to verify. We repeated assays on these samples with very low testosterone concentrations using volumes as high as 400 μl and were still unable to demonstrate a clear stress response. Since a number of smaller alligators had plasma levels much higher than these four examples, and all responded to the capture stress (all alligators with an initial testosterone plasma concentration of 0.5 ng/ml or greater showed a stress-induced decline at 2 h), we may conclude that the testes of alligators exhibiting very low circulating levels of testosterone are essentially "shut down" and incapable of responding to increased levels of corticosterone. A similar situation may have occurred in the studies by Guillette et al. (1997, 1999) in which only small alligators were sampled (30–130 cm total length compared to 74.0–212 cm in this study) and plasma testosterone levels appeared to be less than 0.5 ng/ml. In the 1997 paper (Guillette et al., 1997) no body lengths were given, but the highest mean testosterone reported for a group of alligators was only 0.23 ng/ml. Given such low values it is not surprising that any stress-induced changes in plasma testosterone were undetectable.

In conclusion, our results unambiguously demonstrate that holding male alligators for only 2 h results in a significant decline in circulating testosterone, and emphasize the importance of collecting blood samples as soon as possible after capture for studies in which reproductive steroids are to be measured.

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