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Effects of Long-Term Corticosterone Implants on Growth and Immune Function in Juvenile Alligators, *Alligator mississippiensis*

LISA A. MORICI,[1] RUTH M. ELSEY,[2] AND VALENTINE A. LANCE[1]*
[1]*Center for Reproduction of Endangered Species, Zoological Society of San Diego, San Diego, California 92112*
[2]*Louisiana Department of Wildlife and Fisheries, Rockefeller Wildlife Refuge, Grand Chenier, Louisiana 70643*

ABSTRACT Sixty juvenile alligators were implanted subcutaneously with slow release pellets of corticosterone or placebo. Alligators were divided into five different groups such that each group received a different dose. A blood sample was taken prior to and 4 days after the implants were in place to measure hormone levels. Additional blood samples were collected at 1 month and 3 months. At 4 days corticosterone levels ranged from 3,400 ng/ml in the group treated with the high dose to 40 ng/ml in the group implanted with the low dose. The extremely high dose caused 40% mortality within 4 weeks. It was evident that the pellets did not release the hormone for the expected 90 days. Circulating levels of corticosterone were back to baseline levels by 3 months. Hormone levels achieved at 4 days were a reliable predictor of subsequent growth. Rate of growth was negatively correlated with plasma corticosterone at 4 days ($r^2 = 0.711$) and at 1 month ($r^2 = 0.544$) posttreatment. Differential white blood cell counts performed after 1 month of treatment showed a clear effect of the implant. Alligators treated with corticosterone had decreased percentages of lymphocytes, eosinophils, and basophils and had a higher heterophil/lymphocyte (H/L) ratio than the placebo group. Furthermore, histological examination of the spleen revealed a significant depletion of lymphoid cells in alligators treated with the highest dose of hormone. The results from this study demonstrate that exogenous corticosterone can mimic the effects of prolonged stress in juvenile alligators. *J. Exp. Zool. 279:156–162, 1997.*

Studies of chronic stress in reptiles have demonstrated that elevated plasma corticosterone levels are associated with reproductive failure, immune suppression, and a reduction in or lack of growth (Lance, '94). It is well known that excessive levels of glucocorticoids suppress the immune system in mammals and can cause muscle breakdown and inhibit new bone formation and linear skeletal growth (Orth et al., '92). However, corticosterone's role in inhibiting growth in reptiles has not been substantiated thus far. Several studies have correlated elevated corticosterone levels with decreased growth in reptiles. A study by Elsey et al. ('90) showed that elevated plasma corticosterone levels were correlated with a reduction in growth in juvenile alligators stressed by crowding. In male green iguanas, plasma testosterone was positively correlated and plasma corticosterone level was negatively correlated with body mass and aggressive display frequency (Pratt et al., '92). Osmotically stressed juvenile alligators experienced a dramatic increase in corticosterone over a 5 week period which was correlated with lack of growth (Morici, '96). In addition to growth inhibition, immune suppression and endocrine alterations were also noted in these studies. Stress-induced immune suppression is well documented in fish (Pickering, '84; Ellsaesser and Clem, '87), birds (Siegel, '80), and mammals (Orth et al., '92), but there is little information available for reptiles, particularly the crocodilians. Therefore, this study was initiated to evaluate the long-term effects of corticosterone implants on growth, the immune response, and the endocrine system in the alligator. In this study we demonstrate that corticosterone alone, in the absence of an external stressor, suppresses growth and the immune system in juvenile alligators.

*Correspondence to: V.A. Lance, P.O. Box 551, San Diego, CA 92112.
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MATERIALS AND METHODS

Sixty alligators hatched from two clutches of artificially incubated eggs and reared in controlled environmental chambers (Joanen and McNease, '77) were used in this study. These alligators were 6 months old and weighed 464 ± 124 g at the beginning of the experiment. The animals were randomly assigned to one of five treatment groups (12 alligators per group). Alligators received either eight, four, two, or one corticosterone implants or a placebo tablet (Innovative Research of America, Sarasota, FL). Each tablet contained 100 mg of corticosterone designed to be released over 90 days. The placebo tablets contained cholesterol only.

Alligators were placed in an ice bath until a surgical plane of anesthesia was obtained. Small bilateral incisions were then made approximately 3 cm anterior to the cloaca, and small pockets for the implants were formed with the use of hemostats. After insertion of the tablets, the incision was closed with sutures. Animals were weighed to the nearest gram and total length measured to the nearest 0.5 cm. The alligators were then assigned individual web tags for identification (#3 monel web tags; National Band and Tag Co., Newport, KY) and were placed together in one large environmental chamber maintained at 31°C (for a description see Joanen and McNease, '87). Similar low stocking densities are associated with very low plasma corticosterone levels and optimum growth in juvenile alligators (Joanen and McNease, '77; Elsey et al., '90). Alligators were fed a dry pelletized alligator ration (Burris Mill Feed Inc., Franklinton, LA) five times a week.

Blood samples were taken prior to implantation and then 4 days, 1 month, and 3 months postimplantation. A 1 ml blood sample was obtained by cardiac puncture with heparinized syringes. All samples were collected between 0800 and 1100 h to avoid the known circadian variation in corticosterone and aldosterone (Lance and Lauren, '84; Morici, '96). Time for blood collection was kept under 3 min for each animal to avoid a rise in corticosterone due to handling stress. Blood samples were kept on ice until the plasma was separated on a clinical desktop centrifuge. The plasma was then immediately frozen and maintained at –20°C until assayed. Corticosterone levels were measured in duplicate 100 μl aliquots of plasma by radioimmunoassay as described in Lance and Lauren ('84). Aldosterone levels were measured in duplicate 200 μl aliquots of plasma by radioimmunoassay using the RSL ^{125}I Aldosterone kit (ICN Biomedicals, Inc., Costa Mesa, CA). Plasma glucose levels were determined using the Sigma (St. Louis, MO) Diagnostics kit for quantitative, enzymatic determination at 505 nm.

From the blood samples drawn at 1 month postimplant, blood smears were obtained for analysis of white blood cells. White blood cell smears were stained with Wright-giemsa, and differential white cell counts were performed using oil immersion at ×1,000. At the end of the experiment, five animals were sacrificed from each group, and the spleen and adrenal gland were removed and fixed in 10% buffered formalin. The tissues were then embedded in paraplast, sectioned at 4 μm, and stained with hematoxylin and eosin. Cell diameter and cytoplasm-to-nuclear ratios were measured in the adrenal tissues. Splenic tissue was histologically examined at ×40 for lymphoid cell proliferation or depletion.

The data were subjected to a multifactorial repeated measures analysis of variance. Intergroup comparisons were made with Scheffe's F test.

RESULTS

Alligators that received corticosterone implants experienced a reduction in growth, immune suppression, and adrenal steroid inhibition. In addition, severity of suppression was dose-dependent. In the group that received eight tablets, 40% died after 1 month, and 60% died by 3 months. This group was therefore not included in the statistical analyses.

Growth

After 3 months, alligators that received two or more tablets weighed significantly less than the placebo group ($P < 0.05$) (Fig. 1). Whereas the placebo group increased in mass by 148%, the groups with two and four tablets increased by only 77% and 46%, respectively. Alligators treated with only one tablet of corticosterone increased in mass by 102%, as compared to an increase of 148% in the placebo group. However, this difference fell short of statistical significance. The surviving alligators that received eight tablets increased in mass by 68%. Body mass at 3 months showed a strong negative correlation with plasma corticosterone at 4 days postimplant ($r^2 = 0.711$) (Fig. 2).

Corticosterone

After 4 days, plasma corticosterone levels in all groups were significantly elevated over baseline ($P < 0.001$). However, all groups receiving corticosterone tablets were significantly higher than the placebo group. The significant increase in cor-

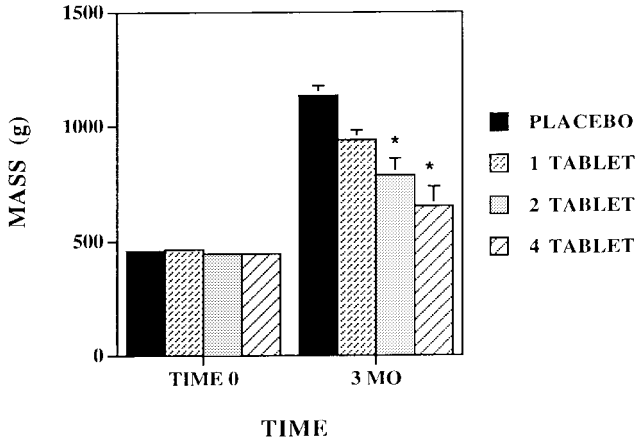

Fig. 1. Effects of corticosterone on body mass over a 3 month period. Columns and bars indicate means and standard errors of the means (SEM), respectively. *Indicates significant difference between treatment group and control group ($P < 0.05$).

ticosterone (3.8 to 42.8 ng/ml) in the placebo group after 4 days most likely resulted from the stress associated with handling, surgery, and bleeding. After 1 month of treatment, corticosterone levels in all groups dropped significantly ($P < 0.05$). However, alligators which received two or four tablets had significantly higher levels of corticosterone than all other groups. By 3 months, corticosterone levels in all groups had returned to baseline levels of 1–3 ng/ml (Fig. 3).

Aldosterone

Plasma aldosterone levels measured 22.1 ± 1.5 pg/ml at the time of the initial bleed. At 4 days postimplant, all alligators treated with corticosterone had undetectable levels of plasma aldosterone, while levels in the placebo group remained unchanged. After 1 month, plasma aldosterone had returned to baseline levels in all groups and remained relatively stable throughout the remaining 2 months.

Glucose

Plasma glucose levels measured 7.0 ± 0.7 mmol/liter prior to treatment. Four days after implantation, glucose levels were significantly elevated (10.6 ± 0.7 mmol/liter) in all treated groups ($P < 0.05$). However, a repeated measures ANOVA determined that treatment was not a factor, whereas

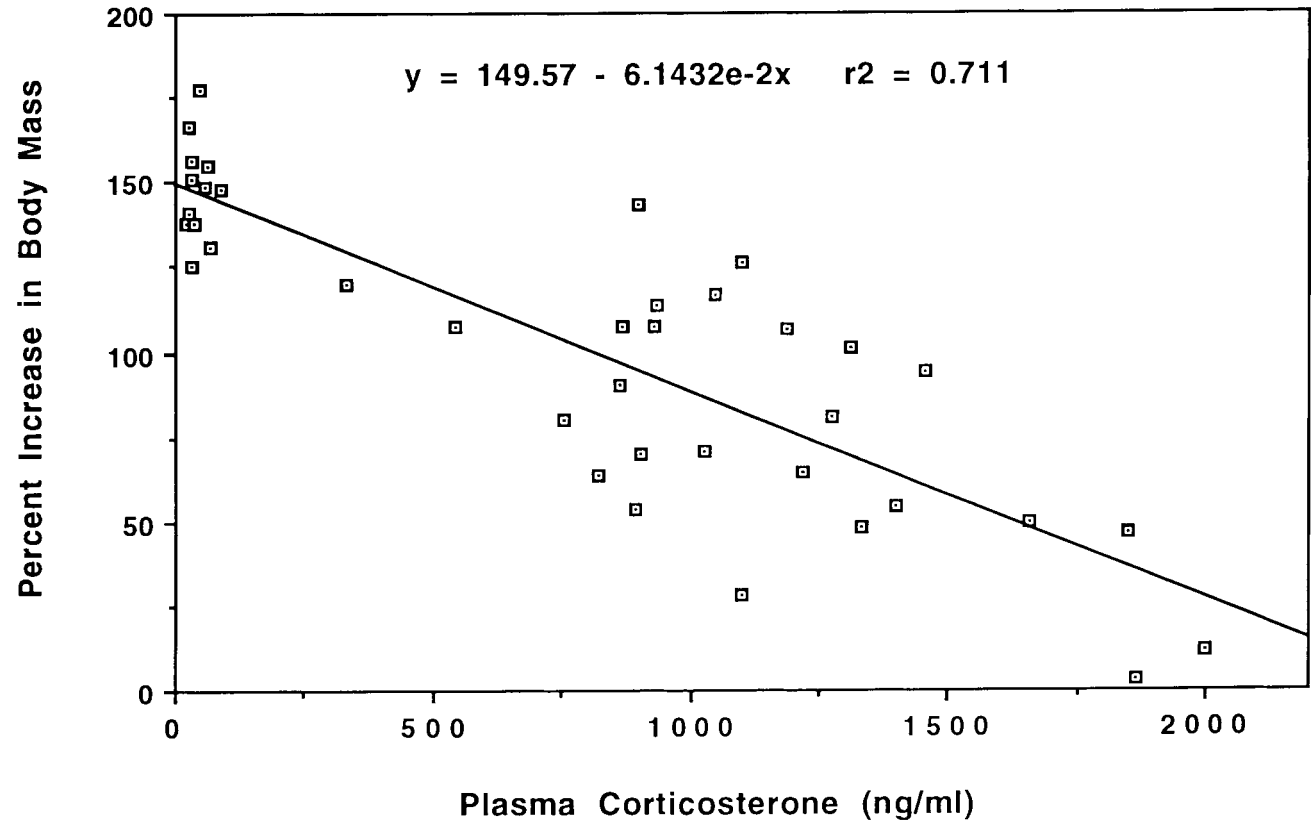

Fig. 2. Scatterplot of day 4 plasma corticosterone levels vs. percent increase in body mass at 3 months. $R^2 = 0.711$. N = 38.

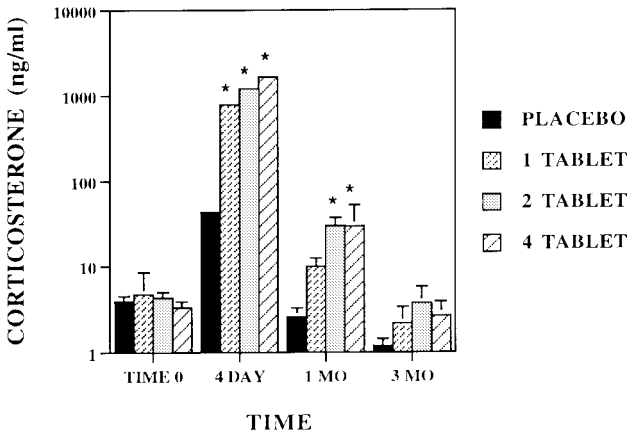

Fig. 3. Plasma corticosterone levels in juvenile alligators over a 3 month period. Columns and bars indicate means and SEMs, respectively. An asterisk on top of a column indicates a significant difference between the treatment group and control group ($P < 0.05$). Note the log scale on the y axis.

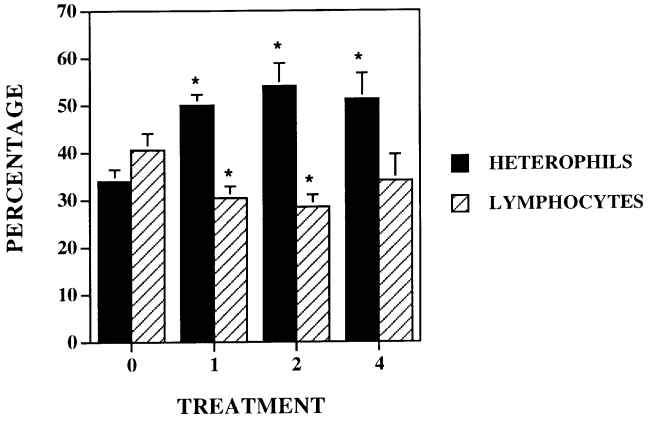

Fig. 4. Changes in the percentage of heterophils and lymphocytes in the plasma of juvenile alligators after 1 month of corticosterone treatment. Columns and bars indicate means and SEMs, respectively. An asterisk on top of a column indicates a significant difference between the treatment group and control group ($P < 0.05$). The numbers at the base of the columns indicate the number of corticosterone tablets implanted.

time was significant ($P < 0.0001$). One month later, glucose levels in the treated groups remained slightly elevated from baseline values but were not significantly higher than the placebo group. By the end of the 3 month study period, plasma glucose levels had returned to pretreatment levels in all groups.

White blood cells

White blood cell differentials were performed 1 month posttreatment. All of the treated groups experienced a significant increase ($P < 0.01$) in the percentage of heterophils (Fig. 4). Alligators which received four tablets had a significantly lower percentage of eosinophils and basophils ($P < 0.05$) than the placebo group (Fig. 5). Alligators treated with two tablets experienced a significant decrease in percent lymphocytes and basophils, while alligators treated with only one tablet experienced a significant decrease in percent lymphocytes and eosinophils ($P < 0.05$). No significant change in the percentage of azurophils occurred for any of the treatment groups. Furthermore, the heterophil/lymphocyte ratio (H/L ratio) was significantly higher (1.8–2.0) than placebo (0.94) in all treated groups ($P < 0.05$).

Histology

No differences were found in cell diameters or cytoplasm to nuclear ratios of the adrenal tissue for any of the groups. Histological examination of the spleen revealed a significant difference in lymphoid cell number between the eight tablet group and the placebo group. Splenic tissue from the placebo group contained large numbers of lymphocytes (greater than 200) surrounding arterioles with a layer width of 13 μm or greater (Fig. 6A). However, spleens from the eight tablet group were severely depleted of lymphoid cells (less than 100) with a layer no thicker than 6 μm (Fig. 6B). The spleens from alligators receiving one, two, and four tablets showed more variability and were not consistently different from the placebo group.

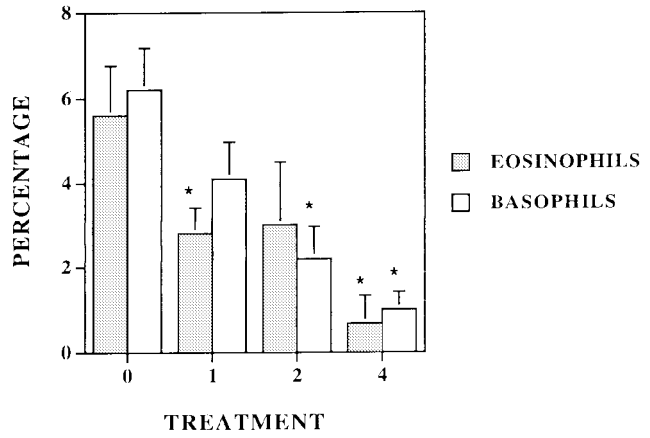

Fig. 5. Changes in the percentage of eosinophils and basophils in the plasma of juvenile alligators after 1 month of corticosterone treatment. Columns and bars indicate means and SEMs, respectively. An asterisk indicates a significant difference between the treatment group and control group ($P < 0.05$). The numbers at the base of the columns indicate the number of corticosterone tablets implanted.

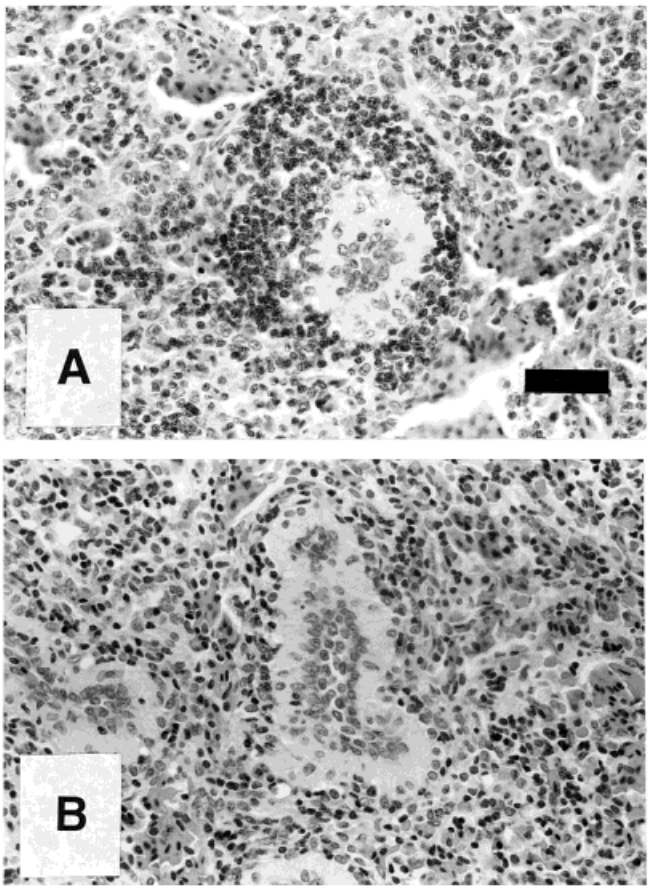

Fig. 6. **A:** Splenic tissue from an alligator receiving a placebo tablet. There is a large number of lymphocytes surrounding the arteriole. Bar = 40 μm. **B:** Splenic tissue from an alligator implanted with eight tablets of corticosterone. Magnification as in A. The region surrounding the arteriole is depleted of lymphocytes.

DISCUSSION

Corticosterone levels approached 3,400 ng/ml in alligators implanted with eight tablets of corticosterone. These supraphysiological levels were fatal for most of the juvenile alligators. The five surviving alligators from the eight tablet group after 3 months had mean levels of corticosterone of only 1 ng/ml, which was the same as the placebo group. It is interesting to note that these five alligators which received very large doses of corticosterone continued to grow, albeit at a reduced rate. Alligators given a placebo tablet increased in body mass by 148%. While all treated groups increased in mass over the 3 month period, the increase was significantly less than the placebo group despite the apparent absence of elevated corticosterone after 1 month. These results suggest that the excessive corticosterone levels for only 3–5 weeks were sufficient to suppress growth in these groups over the remaining study period. It is not clear, however, whether this was an acute response or whether the growth rate was chronically inhibited. Increase in body mass showed a strong negative correlation with plasma corticosterone levels. At 4 days post treatment, a correlation of $r^2 = 0.711$ was noted (Fig. 2). However, by 3 months posttreatment, a weaker correlation ($r^2 = 0.544$) was observed. The lack of a strong correlation at 3 months is most likely the result of corticosterone levels returning to baseline levels in all groups by the end of the study. These data are consistent with the results observed by Elsey et al. ('90) in which alligators stressed by crowding failed to grow as fast as alligators reared in low density conditions. In that study, a similar negative correlation between plasma corticosterone and growth rate ($r^2 = 0.544$) was reported. Moreover, the placebo group from our study grew at a similar rate as the control group in the study by Elsey et al. ('90). The results of the current study strongly suggest that exogenous corticosterone alone can inhibit growth. It is therefore reasonable to conclude that any external stressor (such as crowding) which produces an increase in corticosterone may lead to a suppression of growth.

Field conditions did not permit analysis of total circulating white blood cells which require analysis within 24 h. However, it was clear that elevated corticosterone did result in a significantly altered differential white blood cell picture. Glassman et al. ('81) characterized the white blood cells in alligators. Heterophils are the equivalent of mammalian neutrophils, and azurophils are similar to monocytes. In the treated groups, a significant decrease in percentage of lymphocytes, eosinophils, and basophils was seen. There was, however, no detectable change in percent azurophils. A decrease in lymphocytes, basophils, and eosinophils and no change in azurophils was also observed in alligators stressed by sequential bleeding (V. Lance, unpublished data). The observed decrease in percent lymphocytes is also consistent with the findings of Saad and El Ridi ('88). In their study, injection of pharmacological doses of hydrocortisone in the lizard *C. ocellatus* produced a severe depletion of lymphocytes. Glucocorticoids produce a marked decrease in human peripheral lymphocyte numbers in about 4 h posttreatment. The effect is due to redistribution of lymphocytes from the intravascular compartment to the spleen, lymph nodes, thoracic duct, and bone marrow (Orth et al., '92). In mice and rabbits, corticosterone can induce lysis of the lymphocytes in these

tissues (Dougherty and White, '45), but in humans this is rarely observed (Fauci, '78). In fish, a decrease in the number of circulating white blood cells is common in the stress response (Pickering, '84; Ellsaesser and Clem, '87). Furthermore, prolonged stress or chronic elevations of corticosteroids can cause a depletion of lymphocytes from major lymphoid tissues in fish (Chilmonczyk, '82). This loss suggests that lymphocytes are being lysed in response to corticoids rather than simply being redistributed to various tissues. In the present study, it was not possible to perform total lymphocyte counts in the alligator tissues due to field conditions. However, histological examination of the spleen of alligators treated with the high dose of corticosterone revealed a severe depletion in lymphoid cells (Fig. 6). Therefore, either 1) the supraphysiological levels of corticosterone caused lysis of the lymphocytes or 2) lymphocytes were redistributed to other tissues. It should also be noted that glucocorticoids cause the opposite effect on granulocytes, causing them to leave the bone marrow and enter the circulating blood. This mechanism may account for the increased percentage of heterophils observed for the treated alligator groups in the present study.

Glucocorticoid-related immunosuppression has been characterized by higher levels of corticosterone, increased neutrophilia, and larger H/L ratios in green sea turtles (Aguirre et al., '95). A similar increase in the H/L ratio was seen in the treated alligators in this study. After 1 month of treatment, the placebo group had a mean H/L ratio of 0.94, whereas alligators implanted with corticosterone had H/L ratios ranging from 1.8–2.0. H/L ratios have proven a reliable measure of stress in birds (Gross and Siegel, '83), and our data suggest the same may be true for the alligator.

Baker ('54) showed that cortisone and desoxycorticosterone suppressed the inflammatory response to implanted foreign bodies in rats. Desoxycorticosterone acted locally via implantation, while cortisone acted both locally and systemically via subcutaneous injection. In the present study, it was evident that the healing process was suppressed in alligators implanted with corticosterone. There was an accumulation of scar tissue at the implantation site in alligators treated with corticosterone (Fig. 7), whereas the surgical site in alligators that received placebo tablets healed with minimum scarring.

In this study, alligators which received corticosterone had undetectable levels of circulating aldosterone 4 days after treatment. It was recently demonstrated that aldosterone production in the alligator is stimulated by adrenocorticotropic hormone (ACTH) secretion from the pituitary (Morici, '96). Corticosterone levels were extremely elevated 4 days after surgery. Therefore, production of aldosterone via ACTH secretion may have been suppressed due to negative feedback inhibition of ACTH by corticosterone. After 3 months, corticosterone levels had returned to baseline levels in all groups. Similarly, plasma aldosterone in the treated groups had also returned to pretreatment levels. Negative feedback inhibition of gonadal steroids via the pituitary gonadotropins as a result of increased corticosteroids has been well documented in reptiles. Suppression of testosterone and estradiol in male and female alligators was correlated with elevated plasma corticosterone (Lance and Elsey, '86; Elsey et al., '91). Also, Tokarz ('87) showed that male lizards, *Anolis sagrei*, experienced a dramatic decline in plasma testosterone following implantation of corticosterone. This study suggests that adrenal steroidogenesis can

Fig. 7. Accumulation of scar tissue at the implantation site in alligators treated with corticosterone is apparent 4 months after surgery.

also be inhibited, at least for a short period of time. It has been demonstrated in the rat that the sensitivity of the pituitary to exogenous glucocorticoids can be impaired during chronic stress. While pellets of corticosterone cause an acute dose-dependent decrease in ACTH production, ACTH responds to a novel superimposed stress despite sustained elevation of glucocorticoid (Aguilera, '94). Therefore, future research is needed to determine if the pituitary-adrenal response in alligators to chronic administration of exogenous glucocorticoids is similar to that of mammals.

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