

Plasma Catecholamines and Plasma Corticosterone Following Restraint Stress in Juvenile Alligators

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ABSTRACT Ten juvenile alligators, mean body mass 793 g, hatched from artificially incubated eggs and raised under controlled conditions, were held out of water with their jaws held closed for 48 hr. An initial blood sample was taken and further samples collected at 1, 2, 4, 8, 24, and 48 hr. Epinephrine, norepinephrine, and dopamine were measured in plasma aliquots of 1.5 ml using high pressure liquid chromatography with electrochemical detection. Corticosterone was measured by radioimmunoassay. Plasma glucose was measured using the Trinder method and plasma calcium, cholesterol, and triglycerides were measured in an autoanalyzer. Epinephrine was about 4 ng/ml at the initial bleed, but declined steadily to <0.4 ng/ml by 24 hr. Norepinephrine was also about 4 ng/ml at the initial bleed, but rose to over 8 ng/ml at 1 hr, and then declined to <0.2 ng/ml at 24 hr. A second, but smaller increase in plasma norepinephrine was seen at 48 hr. Plasma dopamine was low at the initial bleed (<0.7 ng/ml), rose to over 8 ng/ml at 1 hr, then declined to <0.2 ng/ml. Plasma corticosterone rose progressively for the first 4 hr, declined at 8 hr and 24 hr, then rose again at 48 hr. Plasma glucose rose significantly by 24 hr and remained elevated for 48 hr. Plasma calcium increased at 1, 2, and 4 hr then returned to levels not significantly different from the initial sample at 24 and 48 hr. The white blood cells showed changes indicating immune system suppression. By the end of the treatment the heterophil/lymphocyte ratio increased to 4.7. These results suggest that handling alligators, taking multiple blood samples, and keeping them restrained for more than 8 hr is a severe stress to the animals. *J. Exp. Zool.* 283:559-565, 1999. © 1999 Wiley-Liss, Inc.

Plasma testosterone in adult male alligators (Lance and Elsey, '86) and plasma estradiol in adult female alligators (Elsey et al., '91) decline rapidly following simple restraint stress. This decline in male and female reproductive hormones is mirrored by a rapid rise in plasma corticosterone. Stress-induced increase in corticosteroids is believed to directly suppress gonadal steroid secretion in mammals and other vertebrates (Gao et al., '96). In alligators plasma glucose also rises rapidly following restraint stress (Lance, '94). This rise in glucose is believed to be the result of acute catecholamine secretion (Coulson and Hernandez, '83), but there are no published data on plasma catecholamine levels in alligators. In this study we looked at changes in plasma corticosterone, plasma glucose and plasma catecholamines in juvenile alligators in response to simple restraint stress.

MATERIALS AND METHODS

Ten juvenile alligators, (mean body mass 792.8 ± 19.7 g, mean total length 63.8 ± 0.7 cm) hatched

from artificially incubated eggs and raised under controlled conditions (Joanen and McNease, '87) were used in this study. The animals were fasted for 48 hr prior to the start of the experiment. An initial blood sample was taken by heart puncture and a monel identification tag (National Band and Tag Co., Newport, KY) placed on the web between the toes. The alligators were held in zinc garbage bins without water for the duration of the experiment and the mouth was held closed by a rubber band. Additional blood samples were collected at 1, 2, 4, 8, 24, and 48 hr. Following the last blood sample the alligators were returned to the rearing pens. The plasma was frozen at -20°C until assayed. A small aliquot of whole blood and a blood smear were taken for differential white cell counts.

Norepinephrine, epinephrine, and dopamine

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were measured in the laboratory of Dr. K.S. Matt, Tempe, Arizona, using high-pressure liquid chromatography (HPLC) as described in Steger et al. ('85) and Matt et al. ('97). Briefly 1.5 ml of plasma was pipetted into a 5-ml conical extraction tube, and dihydroxybenzylamine (DHBA) added as an internal standard. Alumina (10 mg) was added and the tube shaken for 15 min. The plasma layer was discarded and the alumina washed three times with 0.2% Tris/EDTA. The catecholamines were then extracted from the alumina with 100 μ l of a mixture of acetic acid/sodium disulfite/EDTA. Catecholamine concentrations were determined following separation on a C8 (5 μ m) column using electrochemical detection with a glassy carbon electrode.

Corticosterone was measured in duplicate 100 μ l aliquots of plasma by radioimmunoassay as previously described (Lance and Lauren, '84). Samples were extracted with 20 vols of ethyl acetate:n-hexane (3:2), the solvent evaporated under a stream of nitrogen gas and the dried extract reconstituted in 500 μ l of PBS buffer, pH 7.0. Antibody and tritiated corticosterone were added and the tubes held at 4°C overnight. Unbound steroids were separated from bound with dextran charcoal. Tritiated corticosterone was obtained from NEN (Boston, MA). Corticosterone antibody was obtained from ICN (Costa Mesa, CA). Plasma glucose was measured in a spectrophotometer at 505 nm using the Trinder method (Sigma). There was insufficient plasma to do a chemistry panel for the 8-hr sample, but at least five samples from all the other times were analyzed using a Hitachi 911 autoanalyzer. Differential white cell counts were obtained from blood smears stained with Wright-Giemsa and examined using oil immersion at 1000 \times magnification. Data were analyzed using a repeated measure single factor ANOVA followed by Scheffe's multiple range test.

RESULTS

A typical result of the HPLC analysis is shown in Figure 1. Good separation and accurate quantification of each of the catecholamines was achieved. Plasma epinephrine was very high at the initial bleed, declined by about 30% at 1 hr and by about 60% at 2 hr ($P < 0.05$), and by 24 hr was barely detectable (Fig. 2). Norepinephrine was as high as epinephrine at the initial bleed, but doubled by 1 hr ($P < 0.05$). By 2 hr, norepinephrine had declined by 50%, continued to decline until 24 hr, but increased again at 48 hr (Fig. 3). Plasma dopamine was low at the initial bleed, rose dramatically at

one hr ($P < 0.05$) then declined to close to baseline by 4 hr, rose again at 8 hr then declined to baseline at 24 and 48 hr (Fig. 3).

Plasma corticosterone rose initially for 4 hr ($P < 0.05$) then fell back to baseline at 24 hr and rose again dramatically at 48 hr (Fig. 4).

Plasma glucose rose significantly by 24 hr and remained elevated for 48 hr (Table 3). Hematocrit (Fig. 5) declined from about 18 to less than ten by 48 hr ($P < 0.05$). Total white cell numbers remained unchanged, but the numbers of the different cell types changed markedly. Heterophils increased throughout the 48 hr and all others cell types, especially lymphocytes decreased (Table 1). At 48 hr the percentage of heterophils increased to 60% of the total and the lymphocytes decreased to 13% such that the H/L ratio increased to 4.7 (Table 2). Plasma sodium, potassium, and chloride remained unchanged. Similarly, there were no significant changes in cholesterol or triglyceride, but plasma calcium increased significantly at 1, 2, and 4 hr (Table 3).

DISCUSSION

Basal levels of catecholamines in alligators are not known, but based on what has been published on a number of vertebrate species (Hart et al., '89) and one or two reptiles, we speculate that plasma epinephrine in the juvenile alligators was already elevated at the initial bleed and declined thereafter, despite the repeated stress of handling. Norepinephrine at a similarly high level at the initial bleed doubled to over 8 ng/ml at 1 hr post-treatment before declining to baseline by 24 hr. The initial epinephrine levels in the alligator (4 ng/ml) are approximately twice those reported in an aquatic turtle subjected to forced submergence in anoxic water (Keiver et al., '92) and twice those reported for the lizard, *Dipsosaurus dorsalis* (Gleeson et al., '93). Baseline epinephrine values in another lizard, *Urosaurus ornatus*, were, however, more than twice those of the alligator (Matt et al., '97). These large differences in "baseline" values for epinephrine reflect the difficulty of obtaining a blood sample without disturbing the animal (Hart et al., '89). The sustained stress in the alligators did not result in any further secretion of epinephrine but did result in increased secretion of the other two catecholamines. Dopamine also spiked at 1 hr and declined to baseline at 24 hr. The origin of the high levels of circulating dopamine in the alligator is not known. In most mammals, plasma dopamine is generally very low, but shows a significant increase in response to

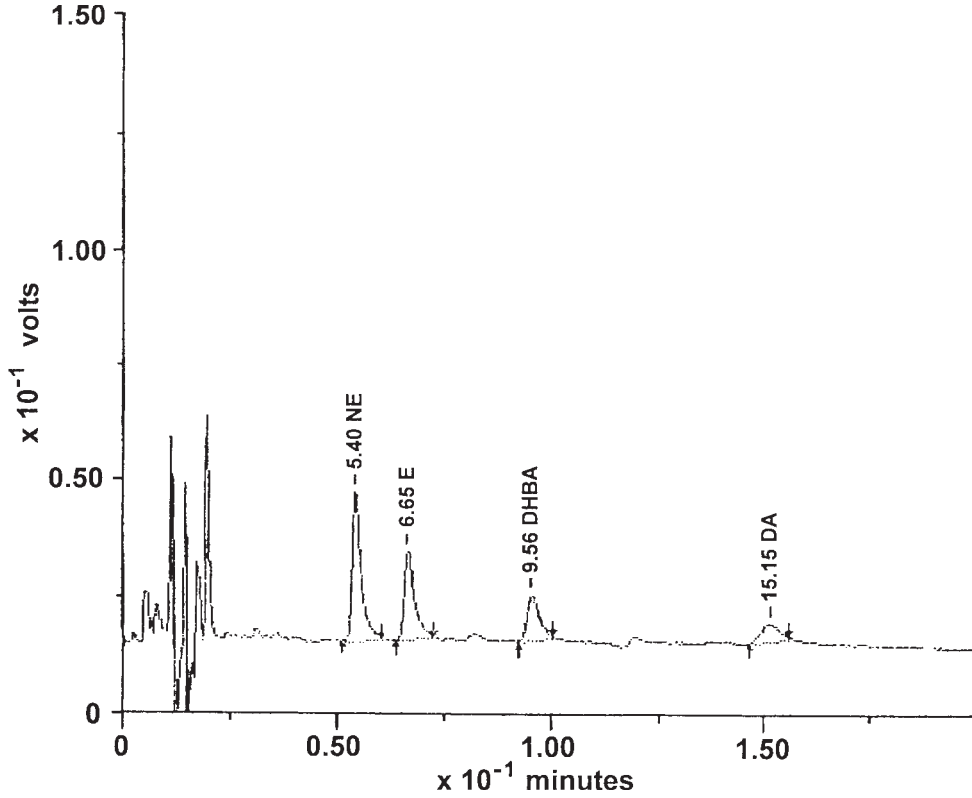


Fig. 1. HPLC profile of plasma catecholamine analysis of alligator plasma at the initial sample. The numbers above the peaks represent the retention time in minutes and the letters above the peaks represent the analyte: E, epinephrine; NE, norepinephrine; D, dopamine; and DHBA, dihydroxybenzamine, the internal standard.

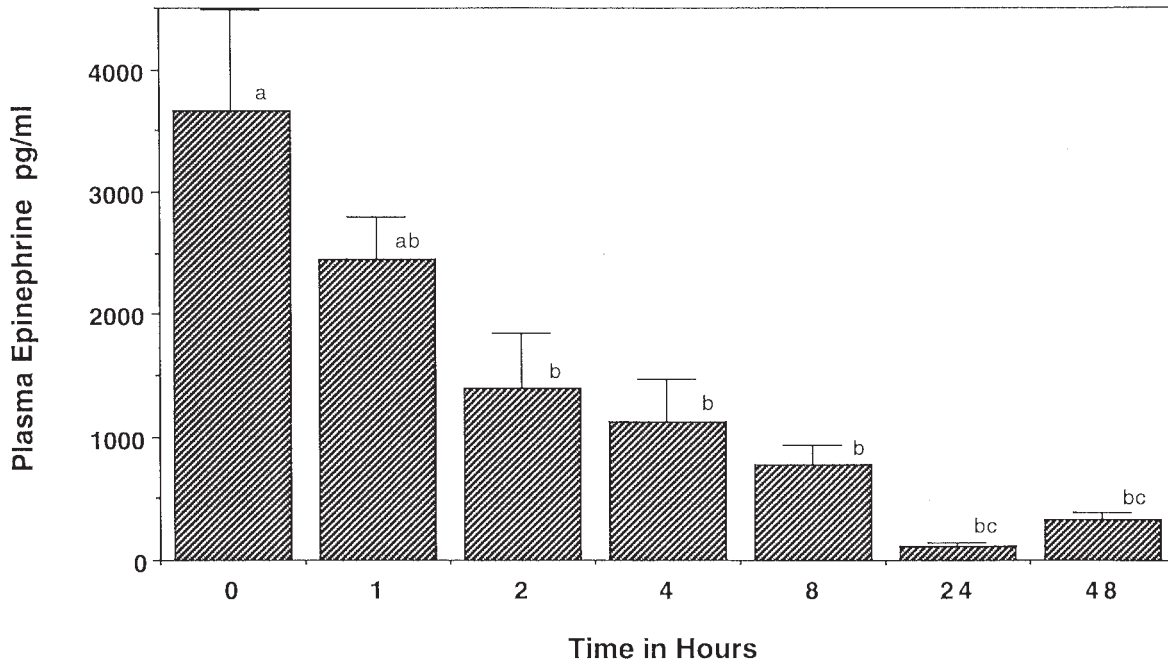


Fig. 2. Plasma epinephrine in juvenile alligators during 48 hr of restraint. Values are presented as mean \pm standard error of the mean (SEM). Significant differences between sampling times are indicated by letters. Columns that share a letter are not significantly different from one another.

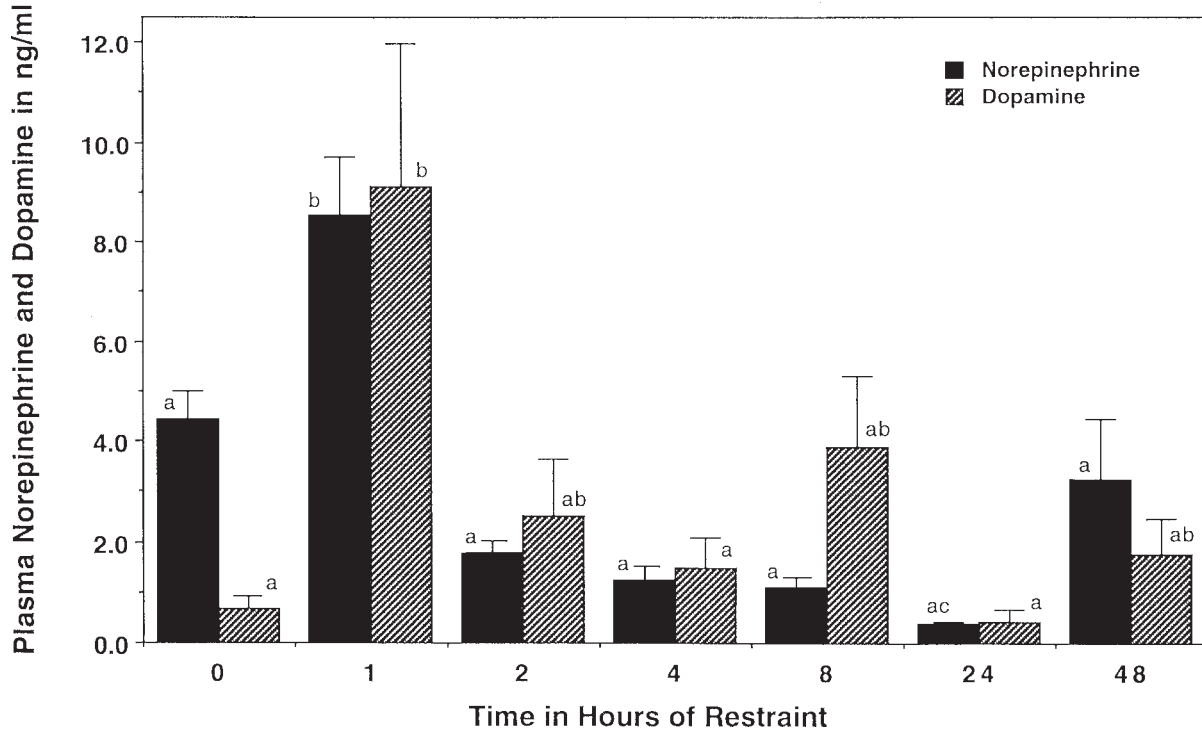


Fig. 3. Plasma norepinephrine and dopamine in juvenile alligators during 48 hr of restraint. Values are presented as mean \pm SEM. Hatched bars, dopamine; solid bars, norepinephrine. Significant differences are noted as in Fig. 2.

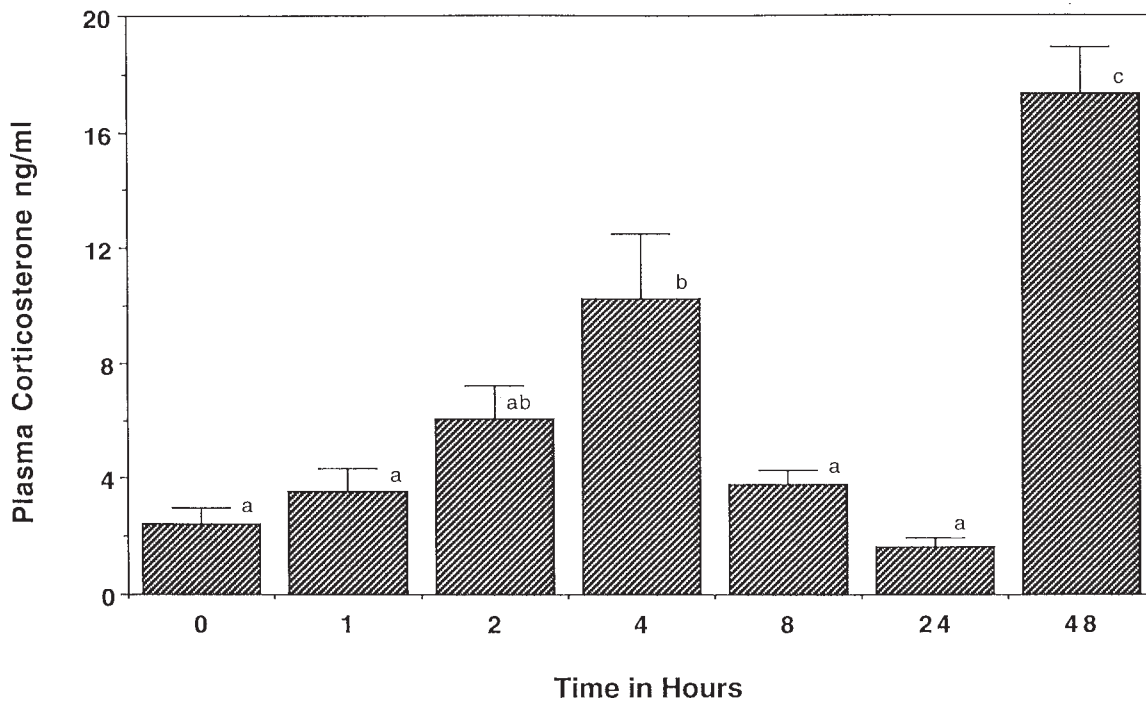


Fig. 4. Plasma corticosterone in juvenile alligators during 48 hr of restraint. Values are presented as mean \pm SEM. Significant differences are noted as in Fig. 2.

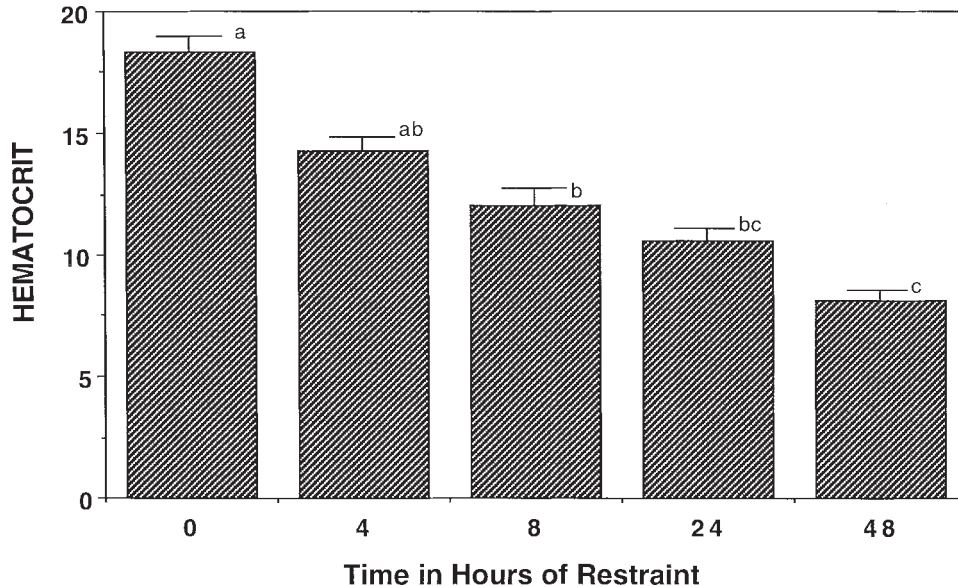


Fig. 5. Hematocrit values of juvenile alligators during 48 hr of restraint. Values are presented as mean \pm SEM. Significant differences are noted as in Fig. 2.

hemorrhage (Hart et al., '89). Dopamine is found in a number of sympathetic systems, including the adrenal medulla, and is a precursor of norepinephrine. Although the plasma levels of norepinephrine and dopamine do not show a clear concordance, it appears that in the alligator stimuli that result in norepinephrine release can also result in dopamine release. In the tree lizard, *Urosaurus ornatus*, restraint resulted in increases in all three catecholamines, but norepinephrine increased to levels several orders of magnitude higher than those seen in the alligator (Matt et al., '97). The source of the plasma catecholamines in the alligator is not known. In ducks subjected to a forced dive, 80% of the circulating norepinephrine came from the adrenal medulla and not the peripheral sympathetic nerve endings (Lacombe and Jones, '90). It is probable that the increased catecholamines following restraint stress come from a similar source in the alligator.

The pattern of corticosterone secretion in the

juvenile alligators during the 48 hr of restraint is almost identical that seen in adult male (Lance and Elsey, '86) and female (Elsey et al., '91) alligators held under restraint for a similar period. In all three experiments corticosterone rose initially and then declined to baseline levels at 24 hr, but rose again to even higher levels at 48 hr. The cause of this biphasic pattern is unknown, but the fact that almost identical results are seen in three separate experiments with both adult and juvenile alligators suggests a similar mechanism is operating. The lowest levels of all three catecholamines were also seen at 24 hr, but at no other time following restraint did the values of corticosterone and the catecholamines show a similar pattern. The juvenile alligators used in this experiment lost a great deal more blood, as a percentage of body mass, than the adult alligators subjected to a similar restraint stress (Lance and Elsey, '86; Elsey et al., '91), but showed an identical pattern of corticosterone secretion. This

TABLE 1. Differential white cell counts following restraint stress in juvenile alligators

	4 hr	8 hr	24 hr	48 hr
Total WBC	6420 \pm 986	6646 \pm 729	7000 \pm 552	5746 \pm 485
Total heterophils	3001 \pm 303	3592 \pm 439	4390 \pm 296*	3757 \pm 316
Total azurophils	1464 \pm 445	1136 \pm 149	1298 \pm 252	1061 \pm 163
Total lymphocytes	1452 \pm 288	1480 \pm 441	1176 \pm 206	792 \pm 167*
Total basophils	505 \pm 76	272 \pm 49	220 \pm 59	101 \pm 27*
Total eosinophils	599 \pm 68	502 \pm 116	318 \pm 58	226 \pm 44*

*Indicates significantly different from 4 hr sample $P < 0.05$.

TABLE 2. White cell percentages following restraint stress in juvenile alligators

	4 hr	8 hr	24 hr	48 hr
% Heterophils	39.0 ± 2.9	48.9 ± 4.2	59.3 ± 2.7*	62.2 ± 2.8*
% Azurophils	20.3 ± 2.4	17.6 ± 1.9	17.9 ± 2.1	18.6 ± 2.2
% Lymphocytes	21.8 ± 2.3	20.9 ± 3.7	15.4 ± 2.8	13.2 ± 2.1*
% Basophils	8.6 ± 1.2	4.3 ± 0.4*	2.9 ± 0.6*	1.6 ± 0.4*
% Eosinophils	10.3 ± 1.4	8.3 ± 1.4	4.4 ± 0.5*	4.4 ± 0.8*

*Indicates significantly different from initial sample $P < 0.05$.

unusual pattern could be the result of negative feedback at the initial rise in corticosterone as seen at 24 hr, but the chronic and perhaps more potent stress (hypovolemia, dehydration) following the 24-hr bleed may have overridden the suppression of ACTH secretion and thus resulted in a greater stimulation of corticosterone production. There are no similar data for other species.

Fasted plasma glucose in alligators varies with season, but in captive reared juveniles is between 5 and 6 mmole/liter (Coulson and Hernandez, '83). Plasma glucose was about 50% above normal fasted levels at the initial bleed, but increased significantly at 24 hr and remained elevated at 48 hr. Glucose will remain elevated for days simply as a result of handling and bleeding alligators (Lance, '92). A similar increase in plasma glucose in juvenile alligators can be induced by epinephrine injection (Lance and Elsey, unpublished). As plasma epinephrine and norepinephrine were elevated at the initial bleed, probably due to the disturbance involved in catching them, the initial glucose levels were also elevated. The data suggest that the increase in plasma glucose is a result of catecholamine secretion in response to acute stress and not due to corticosteroid secretion. Pharmacological doses of cortisone (Coulson and Hernandez, '83) caused only a slight increase in blood glucose, but in alligators implanted with pharmacological doses of corticosterone, plasma glucose remained unchanged (Morici et al., '97).

Long-term effects of corticosteroids on bone metabolism in humans have been well documented. Excessive corticosteroid treatment is known to lead to severe bone loss in humans (Reid, '97),

but an increase in calcium in response to acute stress has not, to our knowledge, been reported. Plasma lactate rises dramatically in alligators when acutely stressed (Coulson and Hernandez, '83), probably in response to somatic nerve activity (deRoos et al., '89). Under conditions that lead to anaerobic glycolysis reptiles are believed to prevent acute lactic acidosis by mobilizing calcium and magnesium from bone and forming lactate complexes with these ions (Jackson and Heisler, '82). It is likely that this is what is occurring in the alligators undergoing restraint stress.

In a study on the response to cold shock in juvenile alligators we noted a massive increase in triglycerides and a decline in cholesterol at 24 and 48 hr following the exposure to ice water (Lance and Elsey, '99). In this study no change in either lipid was noted. There is little information on the effect of stress on lipid metabolism in poikilotherms, but the relatively short-term changes in corticosterone and the modest changes in catecholamines seen in this study were unlikely to have been sufficient to result in any marked fluctuations in circulating plasma lipid.

The gradual decline in hematocrit in response to blood sampling has been noted in earlier studies on juvenile alligators (Lance, '94) and is probably due to dilution of the blood via fluid shifts from interstitial and intracellular stores. In all cases in which we have taken relatively large volumes of blood, the animals seem to recover normally in that no loss of appetite or weight was noted in the weeks following the experiments.

In this study, and in previous studies we have shown that restraint stress (Lance, '92) or im-

TABLE 3. Plasma chemistry of juvenile alligators following restraint stress¹

	0 hr	1 hr	2 hr	4 hr	24 hr	48 hr
Glucose	8.09 ± 0.65	8.50 ± 0.52	8.67 ± 0.92	10.75 ± 0.68	15.29 ± 1.56*	12.46 ± 1.00
Calcium	3.1 ± 1.1	6.9 ± 1.4	10.3 ± 0.7*	9.8 ± 0.9*	4.7 ± 0.8	4.9 ± 0.0
Triglyceride	35.5 ± 10.5	39.2 ± 7.2	34.2 ± 5.8	39.6 ± 5.3	33.6 ± 4.9	26.4 ± 5.9
Cholesterol	66.5 ± 6.5	75.7 ± 9.1	79.7 ± 13.9	73.2 ± 6.8	73.4 ± 7.9	78.7 ± 9.5

¹Glucose and calcium in mmol/liter; cholesterol and triglycerides in mg/dL.

*Indicates significantly different from 0 hr ($P < 0.05$).

plants of corticosterone (Morici et al., '97) result in a decrease in lymphocytes and an increase in heterophils. Glassman et al. ('81) noted a similar change in alligator white cells following infection with *Aeromonas hydrophila*. An increase in the H/L ratio has been used as a measure of stress in chickens (Gross et al., '83) and in sea turtles (Aguirre et al., '93). The H/L ratio of 4.7 in the alligators at 48 hr is far higher than that seen in sea turtles (Aguirre et al., '95), or that seen in alligators subjected to cold shock (Lance and Elsey, '99), or to alligators implanted with pharmacological doses of corticosterone (Morici et al., '97). These results suggest that handling alligators, taking multiple blood samples and keeping them restrained for more than 8 hr is a severe stress to the animals.

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