

ABSTRACT

Title of Thesis: PHYSIOLOGICAL AND BEHAVIORAL EFFECTS OF
HANDLING AND RESTRAINT ON THE BALL
PYTHON (*Python regius*) AND THE BLUE-
TONGUED SKINK (*Tiliqua scincoides*)

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Reptiles are handled during transport, veterinary care, education programs, and as companion animals. This study investigated corticosterone levels (CS), heterophil/lymphocyte ratio (H/L), and behavioral responses to routine handling in the ball python (*Python regius*) (n=4) and the blue-tongued skink (*Tiliqua scincoides*) (n=3). We validated the RSL I₁₂₅ Corticosterone Kit for rodents for parallelism and recovery, and found this to be a precise and highly specific means of CS measurement in reptiles. To determine the optimal blood sampling time, animals were sampled throughout a 24-hour cycle under two different light regimens, one a 12L:12D and one in which a red light was on during the dark period. The diurnal pattern of CS did not differ as a result of light regimen. Python peak CS levels occurred at 2400 hours during the peak locomotor activity

period and at 1200 hours when body temperature was highest, while skinks showed no significant CS rhythm. Heterophil/lymphocyte ratio values indicated that cardiac puncture did not cause chronic stress although it may be an acute stressor. Animals were then handled gently, manually, or were container restrained (CR) for 10 minutes. Although skink CS was unaffected by treatment, CR caused an elevated level of CS in pythons which may indicate short-term stress. There were no differences in H/L ratio or changes in activity level in either species. Brief periods of routine handling of the study species in captivity thus did not cause chronic stress as measured by CS, H/L ratio, and activity parameters.

THE PHYSIOLOGICAL AND BEHAVIORAL EFFECTS OF HANDLING AND
RESTRAINT ON THE BALL PYTHON (*Python regius*) AND
THE BLUE-TONGUED SKINK (*Tiliqua scincoides*)

by

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DEDICATION

This project is dedicated to Eve - the ball python who watches me from the tank next to my desk. Her company was the inspiration that resulted in this idea for a thesis topic.

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CHAPTER I LITERATURE REVIEW

Introduction

Reptiles are handled during routine maintenance of zoo exhibits, educational demonstrations at zoos and nature centers, for venom extraction (milking), forcefeeding, or as companion animals. With a growing population of apartment dwellers, reptiles have become attractive, inexpensive, unusual, and quiet pets. Many zoos and herpetoculturists however, try to minimize the handling of reptiles because they are concerned that the more disturbed the animal is, the less likely it is to breed.

Current concern about animal welfare within the private and public sectors has prompted those who handle animals to evaluate the effects of their methods on the general well-being of their charges. There are no systematic studies of the behavioral and physiological effects of handling and restraint on reptiles. This investigation evaluated four methods of handling commonly used in reptile collections and their effects on corticosterone, immunosuppression, and behavioral changes.

The Stress Response

Stress can be defined as an organism's evolved response to cope with any physical or psychological threat to its homeostasis (Selye, 1936; Moberg, 1985; Danzter and Mormede, 1985). It is a part of normal everyday life, as

physiological systems are constantly challenged by variable stimuli ranging from environmental temperature fluctuations to anticipatory fear of predation or sudden elation.

Adaptive stress or eustress is beneficial to an organism. Gross and Siegel (1982) showed that domestic chickens housed in a medium social stress environment have better feed efficiency, weight gain, and disease resistance than birds housed in either high or low social stress environments. Increases in cholinergic hormones such as norepinephrine and adrenergic hormones such as corticosterone during the stress response have been implicated in male sexual behavior in primates (Sapolsky, 1990).

Negative stress or distress is maladaptive. If the organism cannot behaviorally or physiologically rise to the challenge of the stimulus, pathologies and death will ensue. American alligators maintained at high stocking densities show chronic corticosterone secretion, growth inhibition, reproductive inhibition, and immunosuppression (Else et al., 1990).

MODELS OF ADAPTIVE STRESS RESPONSE:

In 1936, Hans Selye found that rats injected with chemicals and rats injected with ordinary saline developed peptic ulcers, atrophy of the tissues of the immune system, and enlarged adrenals. Since the same effects were found in both groups, he considered this a general response and

developed the concept of the General Adaptation Syndrome (GAS) (Fig. 1). In an evolutionarily advantageous effort to restore homeostasis and protect the organism from any real or perceived damage, the body's energy resources are immediately reallocated away from non-critical processes like growth and reproduction to more essential needs such as increased circulation to skeletal muscle. The GAS, which is a coordinated response involving the sympathetic nervous system and the hypothalamic-pituitary-adrenal axis, (Moberg, 1985) proceeds in three stages once the stimulus is perceived as a stressor and the central nervous system is activated: alarm, resistance, and exhaustion.

Alarm Response: The autonomic response to a stress stimulus (stressor) was first identified by Cannon (1929). This became better known as the "fight or flight" response, during which the animal either directly challenges the stressor or attempts to escape from it. When the central nervous system first perceives a threat, nerve impulses mediated by the hypothalamus have immediate effects on the sympathetic nervous system and the adrenal medulla, which releases epinephrine and norepinephrine to supplement and maintain the "fight or flight" responses. Physiological responses include increased circulation to critical organs (i.e. heart, brain, skeletal muscle) and decreased circulation to non-critical organs (i.e. gastrointestinal

Central Nervous System Perceives Stressor



ALARM

First exposure to stressor

- Fight or flight
- Adrenal release of catecholamines
- Increase circulation to critical organs
- Increase clotting ability and erythrocyte formation
- Increase respiration
- Liver glycogenolysis
- Decrease circulation to non-critical organs



RESISTANCE

Continued exposure to stressor

- Glucocorticoids released from adrenal
- Reduce inflammation
- Fat, protein, and carbohydrate catabolism
- Gluconeogenesis
- Immunosuppression



EXHAUSTION

Animal cannot behaviorally or physiologically escape stressor

- Adrenal and thyroid atrophy
- Fatigue
- Tissue atrophy
- Cardiac hypertension



DEATH

FIG. 1. Summary of the major characteristics of the General Adaptation Syndrome (GAS) based on descriptions by Selye (1936) and Moberg (1985).

tract, kidney), as well as increased clotting ability and erythrocyte formation, increased catabolism via bronchiodilation, increased respiration rate, greater sweat production, and liver glycogenolysis.

Stage of Resistance: If the stressor does not kill the organism during the alarm stage, the body continues to develop a resistance through hypothalamic release of corticotropin releasing factor and vasopressin which stimulate the anterior pituitary to produce adrenocorticotrophic hormone (ACTH). High levels of epinephrine and norepinephrine also stimulate ACTH release. ACTH stimulates the adrenal cortex to produce glucocorticoids and mineralocorticoid. The adrenal then hypertrophies from excess ACTH stimulation. Mineralocorticoid cause the retention of sodium to maintain the body's water and pH balance. Glucocorticoids reduce inflammation, and increase catabolism of proteins and fat stores for gluconeogenesis, catalytic enzymes, and substances for cellular reproduction. Increased levels of corticosteroids can inhibit ACTH release from the anterior pituitary in a negative feedback loop. Growth hormone releasing factor and thyrotropin releasing factor (TRF) are also released by the hypothalamus to the anterior pituitary which produces growth hormone (GH) which, in the liver, increases gluconeogenesis and increased fat catabolism and

thyroid stimulating hormone (TSH) which, via the thyroid, produces thyroxine which increases carbohydrate catabolism. Behaviorally, the animal is constantly alert and cautious and continually trying to evade the stressor (Moore *et al.*, 1991; Sapolsky, 1990). Corticosteroids can pass through lymphoid tissue and bind to the lymphocyte cells, thus decreasing their production and weakening the acquired immune system via lowered antibody-antigen binding. The blood neutrophil (mammals) or heterophil (birds and reptiles) to lymphocyte ratio tends to increase (Siegel 1980; Lance 1990).

Stage of Exhaustion: A persistent stressor will exhaust the ability of the animal to adapt. The adrenal and thyroid atrophy. The stomach is ulcerated and healthy tissue atrophies since glucose is not being stored. Cardiac hypertension increases and kidneys are damaged. The body becomes fatigued and cannot behaviorally control the stimulus that has affected it physiologically. Often, the animal dies at this stage.

The Selye Model suggests that the stress response is non-specific. Different stressors should trigger the same physiological response among animals. However, this model has been shown to be too general and has been widely refuted (Moberg, 1985; Dantzer and Mormede, 1985; Seigel, 1980; Sapolsky, 1990).

Recent investigations demonstrate that the degree to which animals show the GAS depends on many factors, including genetics, previous experience, social situation, perception, and characteristics unique to the individual (Berlyne, 1960; Dantzer and Mormede, 1983b; Greenberg and Crews, 1990; Mason, 1968a; Moberg, 1985; Sapolsky, 1990 and 1989; Suomi, 1990).

Mason (1968b) showed that typical stressors do not always activate the expected adrenal response and that psychological factors determine how the neuroendocrine system will respond as a whole to the stressor. He found that novelty and uncertainty are common activators of the classic stress response. Fasting rhesus monkeys, for example, do not show elevated cortisol levels when given non-nutritive pellets when no physical discomfort is involved. Avoidance conditioning raises cortisol levels, but high environmental temperatures do not. All three of these factors could be perceived as stressors, but the response to each was variable. Veal calves raised outdoors and introduced to an indoor housing facility show a greater cortisol increase than do those transferred from one outdoor enclosure to another, which also indicates the effect of novelty (Dantzer et al., 1983a). Lambs that can avoid electric shock by passing through an unfamiliar door go from a high to low cortisol response in five days in their familiar environment even though shock frequency and

intensity has not decreased (Siegel and Moberg, 1980). This indicates that habituation can lower adrenocortical response. Experience is important in how animals deal with stress. A young monkey reared with a dog as a surrogate mother is more able to psychologically and behaviorally deal with a novel situation than a monkey reared with an inanimate object as its surrogate mother (Mason, 1978).

The Selye model has been refined by researchers such as Moberg, Mason, Dantzer and others to be more inclusive. Results from this research provided the framework for a more comprehensive stress response model developed by Moberg (1985) (Fig. 2). Every individual does not respond equally to the same challenge because of differences in perception of the threat. The magnitude and duration of the response depends on perception. The stimulus must be perceived as a stressor by the central nervous system which organizes the body's biological defense. Perception and biological response are affected by experience, genetics, sex, and physiological state. Even if there is no genuine threat, an animal perceiving the stimulus for the first time can perceive it as a stressor. The biological defense will depend on the stressor and the individual.

The biological response followed by the change in biological function encompasses Selye's alarm and resistance stages. The biological response can be behavioral such as lowering the head in a submissive posture, autonomic as in

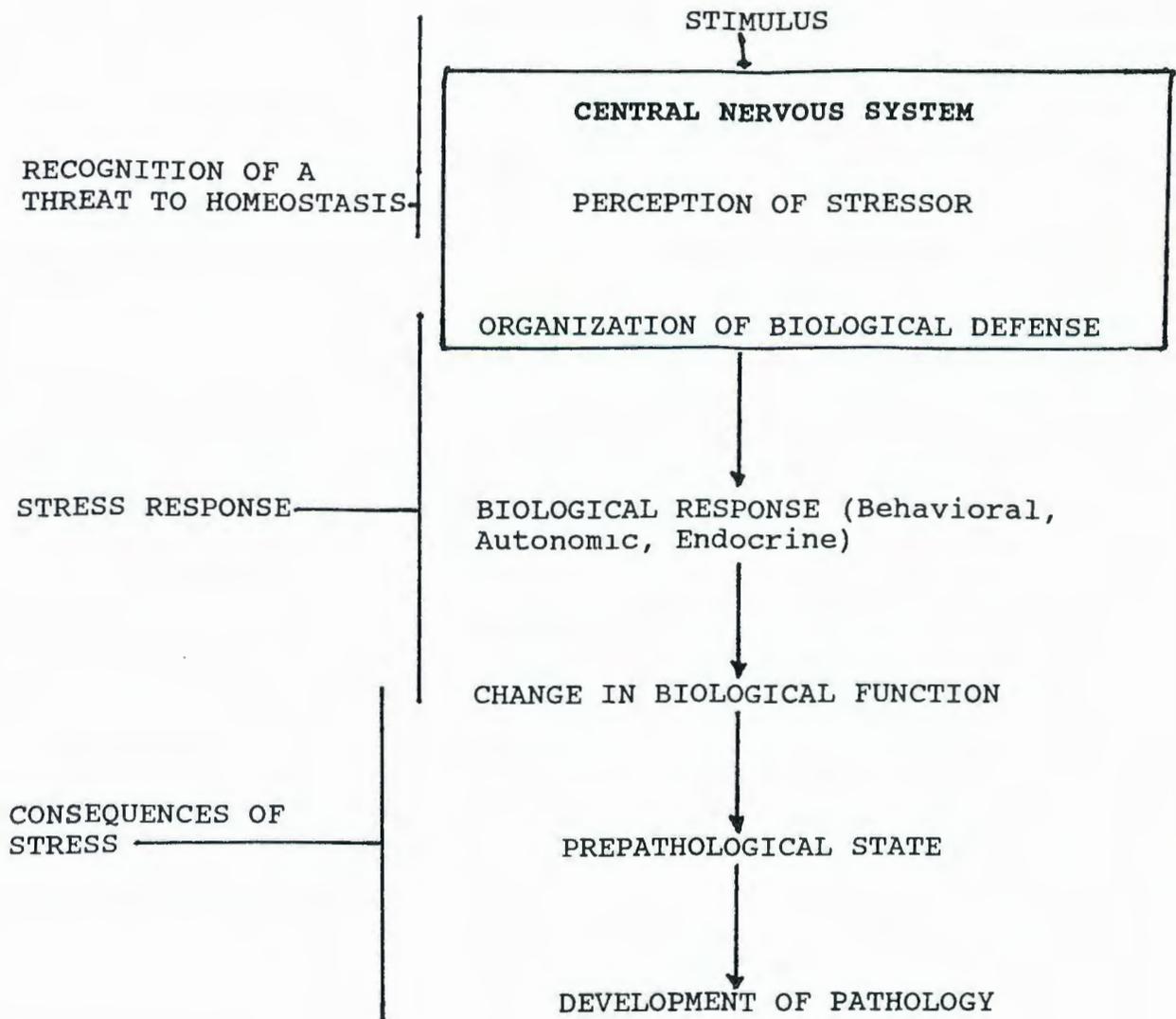


FIG. 2. Summary of refinements to the General Adaptation Syndrome (Moberg, 1985).

the immediate "fight or flight" response, or neuroendocrine which relies on glucose mobilization or any combination of these factors. The resulting change in biological function includes gluconeogenesis using amino acids and lipids that normally go towards growth and reproduction. Lambs stimulated by electric shock will not gain weight although feed intake remains constant (Siegel and Moberg, 1980). Increased corticosteroid levels also impair the immune response and reproduction. It is unlikely that a severely stressed animal will, for example, grow a thick coat of fur before aestivation if its caloric intake is going into evading the stressor and not normal biological functions.

The stress response is followed by stress consequences that encompass Selye's exhaustion stage. The consequences depend on the duration and magnitude of the stressor and include prepathological states and the development of pathologies. Prepathologies are both mental and physical risks. Immunosuppression from high glucocorticoid levels increases, for example, disease susceptibility. Increased activity leads to hypertension. Hypertension can cause cardiac failure - a pathology; and disease susceptibility can lead to a variety of pathologies. Some pathologies could actually be a summation of subthreshold stressors (Moberg, 1985).

COMPARISON OF STRESS BETWEEN MAMMALS, BIRDS, AND REPTILES

The physiological manifestations of stress are similar among mammals, birds, and reptiles. In all three classes, glucocorticoids are secreted by the adrenals which respond to ACTH stimulation. The two adrenals are located each near a kidney although, in reptiles, proximity to the kidney varies by order (Chester-Jones, 1987). The structure of the reptilian and avian adrenal differs only slightly from that found in mammals (Sandor, 1972). In mammals, the catecholamine-secreting tissue (chromaffin) lies in a distinct zone surrounded by glucocorticoid-secreting tissue (cortical). Birds and reptiles lack coalescence of chromaffin tissue, and the degree of cellular intermingling varies by species (Greenberg and Wingfield, 1987). The major adrenal steroid in mammals except rodents is cortisol. Birds, reptiles, and amphibians produce corticosterone. The basal level of corticosteroid secretion in reptiles is similar to that found in birds (Lance, 1990; Greenberg and Wingfield, 1987; Siegel, 1980) and less than that found in mammals.

Although the only stress-related hormone in reptiles on which there is substantial information is corticosterone (Lance, 1990), there are several non-stress factors that can also affect its basal level. For example, as observed in pelagic birds, reptilian glucocorticoids have the added function of stimulating water and sodium movement (Porter,

1972). It is suggested that sea snakes may have higher corticosterone levels than terrestrial and freshwater snakes due to high salt excretion (Duggan, 1981).

As in birds, basal corticosterone levels vary with season, daily activity, and reproductive status (Greenberg and Wingfield, 1987). Chan and Collard (1972) were the first to demonstrate circadian rhythms of corticosterone in reptiles. They found that, in the desert iguana, levels peaked around midday when the animal was most active. Levels in Indian soft-shell turtles (*Lissemys punctata punctata*) are highest at midnight and lowest at midday (Mahapatra et al., 1987). Although no correlation to behavioral activity was investigated, soft-shell turtles typically bask at midday and forage in the late evening (Capula, 1989). American alligators in the wild and in captivity show peaks at 800 and 2000 hrs which correspond to peaks in their natural biphasic activity cycle (Lance and Lauren, 1984). Whiptail lizards show peaks at periods of high activity and previtellogenesis and depressions during vitellogenesis (Grassman and Crews, 1990). Similar results were reported in the viviparous lizard (*Lacerta vivipera*) (Dauphin-Villemant and Xavier, 1987). In temperate zones, the adrenal becomes inactive during the first half of hibernation and hypertrophies in the spring, which increases glycemia and decreases chromaffin granulocytes (Cooper and Jackson, 1981). Though there is little data on seasonal

effects on tropical reptiles, it is suggested that rhythms depend on rainy seasons and prey availability (Seigel and Ford, 1987). Currently, there are no clear trends in corticosterone rhythm among closely related species that correspond to seasonal or daily activity patterns (Gibbons and Semlitsch, 1987). In birds, however, migratory restlessness can be stimulated by daily corticosterone injections which simulates a seasonal change in basal corticosterone level. Orientation during evening navigation may, in part, be due to the daily corticosterone rhythm (Martin and Meier, 1973). Mahapatra, et al. (1987) found that age does not affect diurnal corticosteroid rhythms in the soft-shell turtle. Age does not affect the diurnal rhythm in birds or mammals (Assenmacher and Jallagas, 1980; Allen and Kendall, 1967).

Normal glucocorticoid levels are also influenced by social status. In free-ranging baboons the basal cortisol level of a dominant male is lower than that of a subordinate male which may be considered chronically stressed. However, if threatened acutely, the rate of cortisol increase is greater in the dominant animal (Sapolsky, 1990). Avian studies of corticosterone and social status have yielded conflicting results (See review in Mench, 1991). In reptiles, *Anolis carolinensis* and *Iguana iguana* males show an inverse basal relationship between corticosterone levels and dominance status (Greenberg and Wingfield, 1987;

Phillips and Lance, unpublished). Low social status is associated with large adrenals in *Cnemidophorus* (Bracken, 1978). *Anolis sagrei* males implanted with corticosterone show a decrease in aggression and assume a more subordinate position in territorial competition (Tokarz, 1987). In contrast, Greenberg and Crews (1990) found that corticosterone levels do not differ between dominant and subordinate male *Anolis carolinensis*, however they acknowledge that their experimental design may have been flawed due to lack of animal adjustment time in the laboratory and periodic disturbance from handling the lizards for courtship tests.

The stress response can also be evaluated by the absence or presence of prepathologies. Immunosuppression is an indicator of prepathologies. Corticosteroids during a stress response can pass through lymphoid tissue and bind to lymphocyte cells thus decreasing their proliferation and weakening the acquired immune system via less antibody-antigen binding. Not only is this a response by the hypothalamic-pituitary-adrenal axis, but the cells of the immune system themselves release ACTH and glucocorticoids mediated by cytokine IL-1. The increase in glucocorticoids act in a negative feedback loop to inhibit IL-1 and IL-2 production which decreases T and B lymphocyte growth and proliferation causing immunosuppression by less specific antigen binding (Dantzer and Kelley, 1989).

The Egyptian lizard *Chalcides ocellatus*, a non-hibernator, shows a T- and B-lymphocyte depression of 30-50% in the blood and spleen and a decrease in allogenic and mitogenic lymphocyte stimulation when circulating corticosterone reaches its seasonal peak in mid-autumn (Saad, 1988). This effect which depends on time, length, and frequency of stimulus exposure in birds and mammals (but is unexplored in reptiles) can be measured via the circulating ratio of heterophils or neutrophils to lymphocytes in the blood (Gross and Siegel, 1982; Siegel, 1980; Lance, 1990). Gross and Siegel (1983) demonstrated that heterophils increased and lymphocytes decreased when the domestic chicken was socially stressed, challenged by *E. coli*, or injected with corticosterone. They suggest that corticosterone is a good indicator of short-term distress, but the heterophil/lymphocyte ratio may be a better indicator of long-term distress. The immune system of reptiles is very similar to that of birds and mammals (Frye, 1981; Marcus, 1981; Duguy, 1970; Dessaur, 1970). A description of reptilian cell types is provided in Appendix A.

In birds, mammals, and reptiles, social status, crowding, allocation and availability of food resources, mate competition, and environmental extremes cause changes in the animal's behavioral repertoire necessary to ensure its survival and cope with a potential threat to its well-

being (Dawkins, 1980; Greenberg and Crews, 1990; Greenberg and Wingfield, 1987; Gross and Siegel, 1973; Lance, 1990; Sapolsky, 1990; Suomi, 1990). In reptiles, the most frequent investigations of the behavioral response to stress describe postures and activity levels in relation to temperature extremes (Chan and Callard, 1972; Dessaur, 1953). The fight or flight reactions to predators in agamic lizard is temperature dependent. The lizard flees from predators when body temperature is hot and retaliates defensively when its temperature is low (Hertz et al., 1982). High stocking densities for captive reared juvenile American alligators result in increased injury and mortality due to fighting as a coping mechanism (Elsey et al., 1990).

HANDLING AS A STRESSOR

Humans handle other animal species for a multitude of reasons ranging from companionship to biomedical manipulation to economic incentives through animal agriculture. The animal's psychological, physiological, and behavioral responses to handling can be assessed to determine if handling is perceived as a stressor that could jeopardize its well-being. This can be particularly important if evading the stressor detracts from normal biological activities that are the focus of research using captive or wild animals or results in a decrease in production from agricultural animals.

Hemsworth *et al.* (1981) found that juvenile domestic pigs exposed to unpleasant handling such as a light slap, electric shock from a prodder, or a snout noose maintained higher basal corticosterone levels and showed an immediate increase when a human entered the pen than those exposed to pleasant stroking. Gonyou *et al.* (1986) used the same approach with juvenile pigs as Hemsworth, but added a minimal handling treatment (the pen was only entered by the experimenter for servicing) and a negative handling treatment in which the pig was approached by the experimenter walking erect with gloved hands outstretched towards the head of the pig. Only pigs subjected to aversive handling via electric shock displayed a decrease in weight gain, were less approachable, and had hypertrophied adrenal cortexes indicative of chronic stress.

Hemsworth *et al.* (1987) found that pleasant and minimal handling of young pigs increased feed efficiency, weight gain, and lower basal circulation of cortisol than pigs handled inconsistently (1:5 ratio of unpleasant:pleasant handling). This raises the possibility that animals can habituate to a consistent non-aversive handling regimen.

Dairy cows accustomed to some handling before first calving have lower basal cortisol levels in their milk and are more easily approached by humans than those that have had either no handling experience or extra handling (Hemsworth *et al.*, 1989). This suggests that some handling

is beneficial but there may be a threshold where it becomes distressful.

Birds responses to handling are similar to those observed in mammals. Laying hens handled four times a day for five days show an increase in basal corticosterone levels when compared to non-handled birds. Crating also increases levels (Beuving, 1980).

HANDLING STRESS IN REPTILES

Few studies have investigated the corticosteroid response to handling and restraint in reptiles and the results are conflicting. Wild-caught male tree lizards captured and confined for ten minutes in a black bag show an increase in corticosterone 6.6 times greater than animals sampled immediately following capture (Moore *et al.*, 1991). This effect may not be representative for responses throughout the day, since there were no non-handled controls available for sampling at the same time as the handled group.

While levels increase during routine capture and handling in whiptail lizards (Grassman and Crews, 1990), they do not increase in male or female garter snakes (Krohmer *et al.*, 1987; Whittier *et al.*, 1987). This difference could be due to differences in the time of sampling after manipulation or sampling at the peak time period of the normal diurnal rhythm. Krohmer *et al.* (1987)

found no significant difference between corticosterone levels of animals collected and bled upon capture and those that were transported from the field for two hours before sampling.

A problem with most physiological studies is the failure to investigate longer-term corticosterone elevations from handling. The animal is usually sampled within four minutes from the time of capture which may not be long enough for levels to increase (Krohmer *et al.*, 1987; Grassman and Crews, 1990) or are sampled again several hours later when the response may have returned to normal (Whittier *et al.*, 1987).

The corticosterone response to handling in captive-hatched animals remains virtually unexplored. All studies except one (Lance and Lauren, 1984) used animals in the field or that were wild-caught and brought into the laboratory. Lance and Lauren (1984) found that normal diurnal peaks in corticosterone secretion in captive-hatched American alligators are lower than those of their wild counterparts. The American alligator (Lance and Elsey, 1984) and the viviparous lizard (Dauphin-Villemant and Xavier, 1987) show decreased plasma sex steroids and elevated corticosteroids following restraint.

There are no published studies on corticosterone in the reptile species used in this investigation. There are no published reptilian studies that examine the

heterophil/lymphocyte ratio in relation to acute or chronic distress from handling or restraint except for that of Elsey (Unpublished, 1990) on the American alligator that showed that restraint increased heterophil and decreased lymphocyte percentages within 24 hours. The normal ratio does not differ between freshly caught alligators and those maintained in captivity for long periods.

Behavioral responses to handling in reptiles have rarely been quantified or investigated. Observations have generally been superficial and lack detail or time analysis of the behavioral components. Lack of appetite, decrease in weight, rostral ulcerations, necrotic dermatitis and stomatitis, impaired ecdysis and emaciation have been considered stress symptoms (Marcus, 1981). Food regurgitation in snakes and lizards after handling is termed "internal expression phenomena" by Hediger (1955). Field collection of first year *Crotalus viridis* decreases rate of growth by several weeks as a result of handling and marking according to Fitch (1987). In these instances, glucocorticoids and immune response were not investigated. The only studies to make detailed behavioral observations as they relate to stress were done using green anoles (*Anolis carolinensis*). Perch sites, body posture and color, and fights of males exposed to females were observed and the animals were sampled immediately or 3 weeks after a fight. Corticosterone increased following an agonistic encounter

(Greenberg and Crews, 1990). Site change, posture change, tongue-flicks, and air-licks were observed in males under stress conditions such as handling. Although there was no formal protocol for handling and corticosterone levels and immune response were not investigated, air-licking increased and tongue-flicking decreased after handling. Since both are components of exploratory behavior, stress may arouse some behaviors and depress others (Greenberg, 1985).

The ball python (*Python regius*) and the blue-tongued skink (*Tiliqua scincoides*) are species that are commonly used in zoological parks both on exhibition and for interactive educational programs. They are popular animals in the pet industry due to their non-aggressive nature when handled by people (Marcellini, pers. comm. 1991; Vosjoli, 1990; Ditmars, 1931). Due to their popularity, they are handled frequently for transport from the wild or suppliers, veterinary care (including force-feeding often necessary in ball pythons), environmental education, and personal gratification. Moore et al. (1990) placed tree lizards in a bag suspended from his belt. Grassman and Crews (1990) chased whiptail lizards in a can with their hands. Both investigations labeled these as handling treatments, but there are no studies that evaluate the physiological and behavioral effects of handling treatments on reptiles that are typical of these species used by reptile breeders, exhibitors, and hobbyists. The purpose of the present

investigation is to determine if such handling elicits a stress response in the ball python and the blue-tongued skink using corticosterone secretion, heterophil/lymphocyte ratio, and changes in locomotor activity and consummatory behavior as indicators of distress. Handling effects on corticosterone level can only be evaluated after an appropriate means of corticosterone measurement is validated and basal circadian secretion determined. The objectives of this investigation were to validate the RSL I₁₂₅ Kit for corticosterone in reptiles, determine the circadian rhythm of the study species, and determine the effects of handling and restraint.

CHAPTER II

SHORT COMMUNICATION:

VALIDATION OF A CORTICOSTERONE RADIOIMMUNOASSAY USING A COMMERCIAL KIT

Determination of corticosterone (CS) levels in reptiles can be a lengthy and expensive process. Techniques used involve fluorometry (Mahapatra et al., 1987), chromatography (Duggan, 1981), and competitive protein binding (Chan and Callard, 1972; Lance and Lauren, 1984; Grassman and Crews, 1990; Moore et al., 1991; Greenberg and Crews, 1990). While these methods are reliable and provide levels of various steroids simultaneously, they involve overnight separations and extractions using hazardous reagents. Radioimmunoassay methods eliminate extraction procedures, are less time consuming, and are typically more precise than fluorometric or competitive protein binding methods (Beuving, 1980).

The RSL I125 Corticosterone Kit (ICN Biomedicals, Inc., 3300 Hyland Ave., Costa Mesa, CA 92626) is a double antibody liquid phase radioimmunoassay, developed for use with rat and mouse plasma, that is used to determine total CS levels in unextracted plasma. It is simple, rapid, precise, and has both high sensitivity and high specificity. We have used it extensively in avian studies and were interested in its potential use in reptiles. We were able to validate this assay for the blue-tongued skink (*Tiliqua scincoides*) and the ball python (*Python regius*) (Jeffcoate, 1981;

Panella and Scaramuzzi, 1970).

The primary glucocorticoid in reptiles is corticosterone (Lance, 1990). The other biologically active steroids in reptiles which are structurally similar to corticosterone are cortisol, progesterone, aldosterone, and desoxycorticosterone (Chester-Jones, 1987). Percent cross reactivity of the antibody with these steroids was determined by ICN Biomedicals, Inc. to be .34, .10, .05, .03, and .02 for desoxycorticosterone, testosterone, cortisol, aldosterone, and progesterone respectively. All other materials tested had a cross reactivity of less than .01%.

Because we were unable to locate normal CS levels for the study species in the literature, expected levels were based on related species (Chan and Callard, 1972; Duggan, 1981). Table 1 shows CS ranges for reptile species in the literature. Variations within the range can reflect time of day, season and photoperiod, sex, nutritional status, or assay method (Moore et al., 1990; Greenberg and Crews, 1990; Chan and Callard, 1972; Dauphin-Villemant and Xavier, 1987). There is a tendency for saurians, chelonians, and crocodylians to have lower levels than squamates. These values suggested that, to fit within the limits of sensitivity of the assay (.125 to 5 ng/ml corticosterone), skink plasma should be diluted between 1:1.25 and 1:10, while python dilutions should be no greater than 1:20 and no

TABLE 1

BASAL CORTICOSTERONE (CS) RANGES FOR REPTILES^a

Sex	Species	CS (ng/ml)	Source	Reference
Crocodylia				
?	American alligator <i>Alligator mississippiensis</i>	2.22 to 8.49	CV	Lance and Lauren, 1984
Chelonia				
M	Soft-shelled turtle <i>Lissemys punctata punctata</i>	15 to 64	A	Mahapatra, et al., 1987
Squamata				
F	Parthenogenic whiptail <i>Cnemidophorus uniparens</i>	2.4 to 6.2	OS	Grassman and Crews 1990
F	Desert iguana <i>Dipsosaurus dorsalis</i>	13.6 to 51	D	Chan and Callard, 1972
F	Viviparous lizard <i>Lacerta vivipara</i>	5.4 to 50	OS	Dauphin- Villemant and Xavier 1987
M	Tree lizard <i>Urosaurus ornatus</i>	1.0	OS	Moore, et al., 1991
M	Green anole <i>Anolis carolinensis</i>	1.45 to 6.82	D	Greenberg and Crews 1990
F	Red-sided garter snake <i>Thamnophis sirtalis parietalis</i>	20 to 80	OS	Whittier, et al., 1987
M	Red-sided garter snake <i>Thamnophis sirtalis parietalis</i>	20 to 120	CV	Krohmer, et al., 1987
?	Banded sea snake <i>Hydrophis cyanocinctus</i>	55 to 130	D	Duggan, 1981
?	Hardwick's sea snake <i>Lapemis hardwickii</i>	260 to >400	D	Duggan, 1981
?	Yellow-bellied sea snake <i>Pelamis platurus</i>	12 to 240	D	Duggan, 1981

Table 1. (Cont.)

Sex	Species	CS (ng/ml)	Source	Reference
?	Slender sea snake <i>Microcephalophis gracilis</i>	39	D	Duggan, 1981
?	Indian cobra <i>Naja naja</i>	<1 to 11.2	D	Duggan, 1981
?	Indo-Chinese rat snake <i>Ptyas korros</i>	29 to 168	D	Duggan, 1981
?	Kowloon tong <i>Enhydris enhydris</i>	14.6 to 34	D	Duggan, 1981
	<i>Enhydris bocourti</i>	35 to >400	D	Duggan, 1981
?	White-lipped pit viper <i>Trimeresurus albolabris</i>	55	D	Duggan, 1981

* CS values represent experimental controls or upon-capture data. Seasonal and daily variations are included in the range.

A= adrenal, CV=caudal vein, D=decapitation, OS=orbital sinus

less than 1:80.

Animal subjects (pythons n=4, skinks n=3) were sampled via cardiac puncture within 15 seconds after removal from the enclosure. One half cc blood was withdrawn at each sampling. Husbandry methods are described in Kreger and Mench (Chapter III).

Samples were dispensed into tubes containing one drop of 11% EDTA and stored in ice for a maximum of 30 minutes prior to centrifugation, which was at 3000 rpm at 11°C for 10 minutes. Samples were frozen at -15°C until they were assayed. Plasma was pooled from both species.

To assess parallelism, sample dilutions were made consisting of 1 part plasma to 1.25 and 10 parts distilled deionized water for the skink and the python, respectively. These initial dilutions were then serially diluted by 1:1 with 100 ul distilled deionized water. The assayed values of the successive dilutions closely paralleled the expected plasma values (Table 2).

To assess recovery, 100 ul of kit standards ranging from 1.25 to 5.0 ng/ml were added to 100 ul of samples diluted as in the serial dilutions. The recovery assay values closely approximated the predicted values (Table 3).

The within assay coefficient of variation was 4.84%. A pool of python plasma ran consistently throughout 8 succeeding assays ($\bar{x}=54.86 \pm 2.24$ ng/ml); the between assay coefficient of variation was 9.5%.

TABLE 2

DILUTION RESULTS USING RSL I₁₂₅ CORTICOSTERONE KIT

<u>Plasma:Water Dilution</u>	<u>Corticosterone (ng/ml)</u>	
	<u>Assayed Value</u>	<u>Plasma Value^a</u>
Blue-tongued Skinks		
1:1.25	2.96	6.66
1:2.5	1.20	5.40
1:5.0	0.55	4.95
1:10.0	0.25	4.50
Ball Pythons		
1:10	4.13	45.43
1:20	3.48	76.56
1:40	1.66	73.04
1:80	0.80	70.40
1:160	0.31	54.56

^a Plasma value is the assayed corticosterone (CS) value multiplied by the dilution factor.

TABLE 3

RECOVERY RESULTS USING RSL I₁₂₅ CORTICOSTERONE KIT

Recovery ^a	Corticosterone (ng/ml)		Difference ^c
	Predicted Value ^b	Assayed Value	
Blue-tongued Skinks			
		2.81	-0.08
1:1.25+2.5	2.73	4.91	0.93
1:1.25+5.0	3.98	1.23	0.00
1:2.50+1.25	1.23	2.05	-0.20
1:2.50+2.5	1.85	3.10	0.17
1:2.50+5.0	3.27	0.58	-0.05
1:5.0+0.5	0.53	0.85	0.05
1:5.0+1.25	0.90	1.57	-0.04
1:5.0+2.5	1.53	2.62	0.16
1:5.0+5.0	2.78	0.49	-0.11
1:10.0+0.5	0.38	0.60	0.15
1:10.0+1.25	0.75	1.03	0.35
1:10.0+2.5	1.38	1.83	-1.20
1:10.0+5.0	2.63		
Ball Pythons			
		2.45	0.54
1:20+2.5	2.99	3.68	0.56
1:20+5.0	4.24	1.22	0.24
1:40+1.25	1.46	1.79	0.29
1:40+2.5	2.08	3.11	0.22
1:40+5.0	3.33	0.54	0.11
1:80+0.5	0.65	0.93	0.10
1:80+1.25	1.03	1.63	0.02
1:80+2.5	1.65	2.83	0.07
1:80+5.0	2.90	0.44	-0.16
1:160+0.25	0.28	0.42	-0.01
1:160+0.5	0.41	0.83	-0.05
1:160+1.25	0.78	1.38	0.03
1:160+2.5	1.41	2.68	-0.02
1:160+5.0	2.66		

^a The ratio represents 100 ul of the dilution ratios in Table 2. The second number is 100 ul of the kit corticosterone standard (ng/ml) that was added to the plasma dilution.

^b Predicted CS values are the sum of half the dilution value from Table 2 and half the value of the calibrator.

^c Difference = Predicted Value - Assayed Value

The optimal dilution for skinks was determined to be 1:5 and 1:80 for the pythons as those values lie in the middle and most accurate area of the curve.

We feel this commercial kit gives an accurate determination of plasma corticosterone levels for the blue-tongued skink and the ball python and would recommend it as a quick and affordable alternative to other commonly used methods of corticosterone determination in reptiles.

CHAPTER III

EFFECTS OF ARTIFICIAL LIGHT ON THE CIRCADIAN RHYTHM OF CORTICOSTERONE IN THE BALL PYTHON (*Python regius*) AND THE BLUE-TONGUED SKINK (*Tiliqua scincoides*)

ABSTRACT Diurnal rhythmicity of corticosterone secretion (CS) was determined under two different photoperiodic conditions for the nocturnal ball python (*Python regius*) (n=4) and the diurnal blue-tongued skink (*Tiliqua scincoides*) (n=3). The first condition was a 12L:12D cycle with a green incandescent lightbulb switched on briefly during the dark period for blood sampling. In the second condition, a red incandescent bulb was kept on throughout dark and light periods, simulating a nocturnal species zoo exhibit. Since CS levels did not significantly differ between light regimens, the data were combined. A significant biphasic CS rhythm was observed in the ball python, with one peak occurring at 1200 hours, corresponding to peak body temperature, and another occurring at 2400 hrs. Although no significant rhythm was determined in the skink, body temperature peaked during midday, when there was also a CS plateau. Red light did not alter the CS rhythm in either species. Neither species showed a trend in heterophil/lymphocyte ratio or day effects that correlated with CS indicating that the restraint and cardiac puncture used in blood collection was

not perceived as a chronic stressor.

INTRODUCTION

The major glucocorticoid in reptiles is corticosterone (CS) (Lance, 1990). Reptile corticosterone secretion has been shown to have a diurnal circadian rhythm (Grassman and Crews, 1990; Dauphin-Villemant and Xavier, 1987; Chan and Callard, 1972; Lance and Lauren, 1984). It is, therefore, important to consider normal diurnal fluctuations in CS levels in order to interpret CS results from experimental manipulation.

Previous research has indicated that elevations in daily CS secretion tend to coincide with periods of peak activity. Peak corticosterone levels in the diurnal desert iguana (*Dipsosaurus dorsalis*) occur in the late afternoon when the animal is most active (Chan and Callard, 1972), while American alligators (*Alligator mississippiensis*) in the wild and in captivity show peak levels that correspond to their natural biphasic activity pattern (Lance and Lauren, 1984). Whiptail lizards (Grassman and Crews, 1990) and the viviparous lizard *Lacerta vivipera* (Dauphin-Villemant and Xavier, 1987) show peaks at periods of high activity as well as during previtellogenesis, and depressions during vitellogenesis.

To our knowledge, diurnal variation of CS in boids and skinks have not been reported. The ball python (*Python*

regius) and the blue-tongued skink (*Tiliqua scincoides*) are common exhibit and pet species. Future studies of these species that use CS as an indicator of stress or social and reproductive status could use such information as a baseline. It is accepted husbandry among zoos and private herpetoculturists to use red-bulbed basking lamps at night to increase enclosure temperature or observe animals based on the assumption that reptiles cannot see red wavelengths (Demeter, 1989). Zoological parks often use red bulbs to illuminate exhibits that contain nocturnal species to allow visitors to view nocturnal activity. The effects of constant red light on diurnal secretion of CS have not been investigated.

The objectives of this investigation were to: 1) Determine the CS circadian rhythm in pythons and skinks under normal (12L:12D) light; 2) determine the effect of low intensity red light during the dark period on the normal CS rhythm; 3) determine if there is a correlation between CS, body temperature, and heterophil/lymphocyte ratio (H/L) which is used as an indicator of prepathologies and chronic stress (Gross and Siegel, 1983); and 4) determine if repeated cardiac puncture acts as a chronic stressor in reptiles.

METHODS:

Animals, housing, and maintenance: Adult male blue-

tongued skinks (*Tiliqua scincoides*) (n=3, 22.17cm mean snout-to-vent length, 302.7g mean body weight) and ball pythons (*Python regius*) (n=4, 96.4cm snout-to-vent length, 1085.75g mean body weight) were purchased from a commercial supplier. The skinks were captive-born, but the origin of the pythons is uncertain. Animals were housed individually in 60-gallon plastic containers with modified screen lids, and were illuminated by two sets of broad-spectrum fluorescent lights in a room controlled for photoperiod (12L:12D), temperature (27 to 29°C), and mean relative humidity (50.17%). The light period was 0600 to 1800 hours. A thermal gradient (25 to 33°C) for basking was provided by placing the heating pads under half of each enclosure. Newspaper was used as substrate. Each snake enclosure was equipped with a plastic shelter for cover and a water dish. Skink enclosures included a PVC tube cut longer than lizard body length and a water dish. Water for drinking and soaking was available *ad libitum*. All animals were fed once per week and weighed monthly to assure maintenance or increase of body weight. Snakes were fed sacrificed adult mice. Three of the snakes could not be trained to accept non-live food and were force-fed. Skinks were fed sacrificed weanling and juvenile mice. Rodents were germ-free surplus. Feed was vitamin and mineral-dusted. This investigation followed an 8-month animal acclimation period.

Animals were not fed during the light regimen treatment

periods so that results would not be complicated by a feed-on/feed-off factor. This is justified because wild reptiles remain healthy for several weeks or months during breeding seasons or when the food supply is low (particularly ball pythons (Vosjoli, 1990). They were, however, fed during the 30-day readjustment period between changes in light regimen.

Both treatments included full fluorescent broad-spectrum illumination during the 12-hour light period. The normal treatment was completely dark during the dark period except that a green 25 watt bulb was switched on briefly during blood collection times at 2400, 300, 600, and 2100 hours. Green was selected as it was the lowest intensity available that allowed the investigator sufficient illumination for accurate sampling. The bulb was angled lower than enclosures so the range of illumination was reduced. The constant light treatment included a 25 watt incandescent red lamp which remained on 24 hours per day and thus supplemented the normal fluorescent lighting from 0600 to 1800 hours.

Blood sample collection and Radioimmunoassay: Blood was collected by cardiac puncture using a 23 gauge needle. Collection time per animal never exceeded 30 seconds and rarely exceeded 15 seconds. One half cc of blood was collected from each animal per sampling time. There were no obvious ill-effects resulting from sampling. Samples were dispensed into tubes containing one drop of 11% EDTA and

stored on ice for a maximum of 30 minutes. A blood smear was made of each sample for leucocyte counts, and plasma was frozen at -15°C for later radioimmunoassay. Corticosterone was measured using the RSL I₁₂₅ Corticosterone Kit (liquid phase). Based on our previous results (Chapter II), lizard samples were diluted 1:5 with deionized distilled water and snake samples 1:40 for assay. The RSL kit has been validated for parallelism and recovery by the investigators (Chapter II). Within and between assay coefficients of variation were 4.84% and 9.5% respectively. Slides were stained using HEMA 3 (Biochemical Sciences, Inc., Bridgeport, NJ 08014), and heterophil/lymphocyte (H/L) ratios were calculated from a count of 200 leukocytes per slide. Leukocyte classification was based on descriptions by Hawkey and Dennett (1989), Montali (1988), and Frye (1981).

Experimental design: This investigation used an incomplete randomized block design and was blocked by sampling time. Sampling occurred every other day and consisted of 2 sampling times. Each animal was randomly assigned to one of the times and sampled in random order until all selected hours were represented. Each animal was sampled once per time period per light treatment over 3 months. Sampling times were 2400, 300, 600, 900, 1200, 1500, 1800 and 2100 hours. A 30-day reacclimation period separated the two light treatments. Cloacal temperature,

room temperature, and relative humidity were also monitored.

Corticosterone, H/L ratio, and cloacal temperature relationships were analyzed using the SAS General Linear Models Procedure (GLM) (SAS Institute, 1987) and correlation analysis. The model included animal, light treatment, and animal-light interaction. The day effect was treated as a covariate. A residual test indicated that the data met the assumptions of analysis of variance (Sokal and Rohlf, 1981). A regression polynomial curve fitting technique (Little and Jackson, 1978) was used to determine the rhythmicity of the dependant variables. Correlation analysis was run at each time period for each pair of dependent variables.

RESULTS

Body weights of the animals were maintained throughout the experiment.

Light regimen did not effect the pattern of CS secretion in either species. Data from both regimens were therefore combined for further analysis. There was no day effect within or between regimens for the combined data, but a significant rhythm was apparent for the pythons ($P < .01$, $n=8$, $df=2$) with peaks occurring at 2400 and 1200 hours. Data for pythons are shown in Fig. 3. Neither H/L ratio nor body temperature showed a comparable rhythm. Although there was no statistically significant correlation between body temperature and CS (Fig. 3), the temperature peak at 1200

hours coincided with the CS peak.

Skink data are shown in Fig. 4. None of the variables had a statistically significant rhythm, nor were they correlated with one another. Corticosterone levels plateaued between 600 and 1800 hours, and peak body temperatures occurred between 900 and 1200 hours. The H/L ratio showed a non-significant variation that paralleled CS levels.

DISCUSSION

Corticosterone (CS) rhythmicity was observed in the pythons, with peaks occurring at 2400 and 1200 hours. Although not statistically significant, there does appear to be a CS plateau which may be of biological significance for the skinks between 0600 and 1800 hours. Both species demonstrated CS peaks at periods of greatest activity. In the wild, ball pythons are nocturnal (Vosjoli, 1990) and, in our studies, one peak occurred at midnight. Blue-tongued skinks are diurnal in the wild (Capula, 1989), and our study revealed a level peak during the day with a trough occurring at night when the animals were least active. It is evolutionarily advantageous for each species to mobilize its energy resources, which are partially CS dependent, for behavioral activity. It is therefore not surprising that our data coincide with other reptile circadian rhythm studies that show CS peak levels at periods of high activity

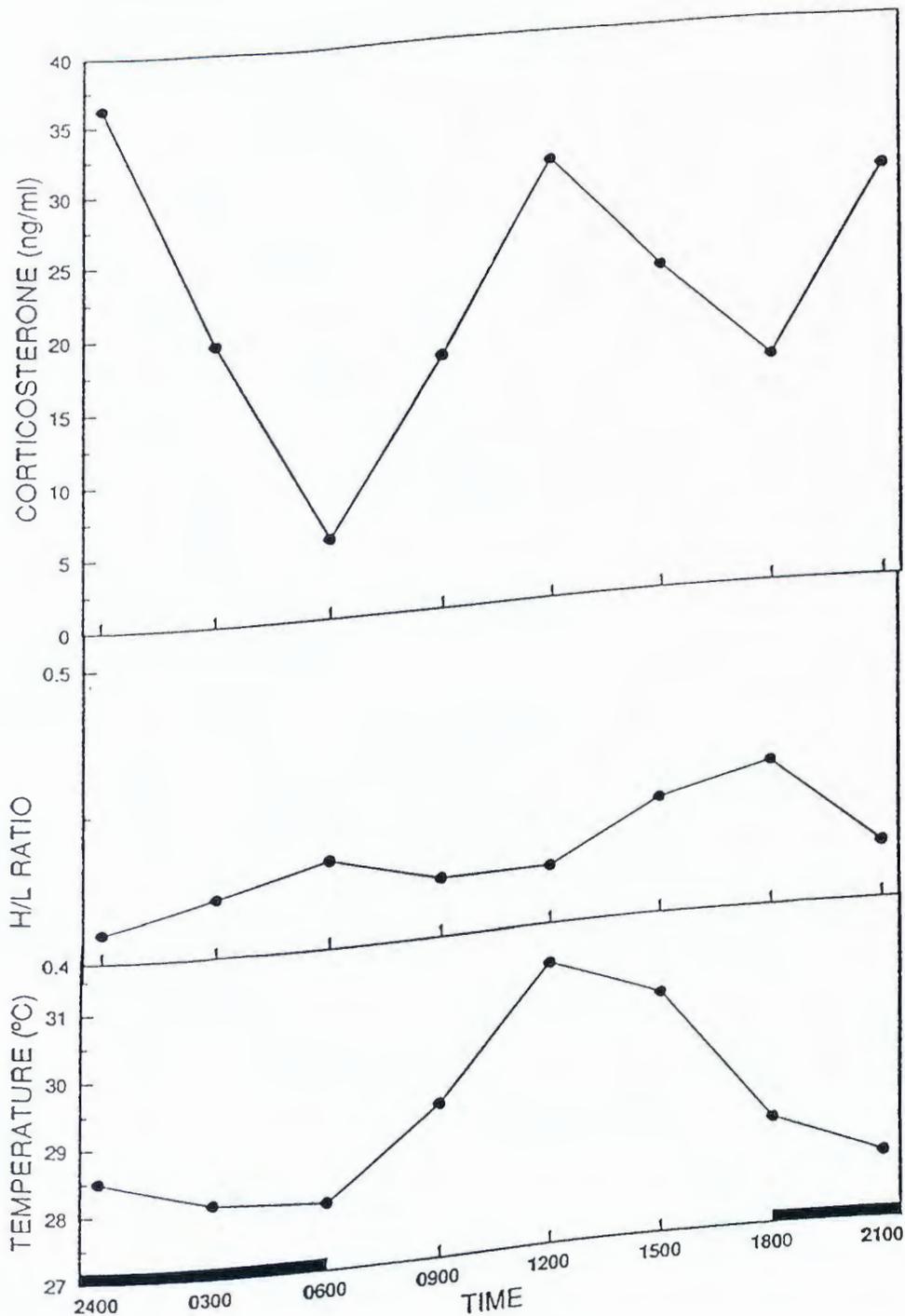


FIG. 3. Circadian variations in plasma corticosterone levels (top graph), heterophil/lymphocyte ratio (middle graph), and body temperature (bottom graph) in the nocturnal ball python. The dark phase is represented by the shaded bar on the abscissa.

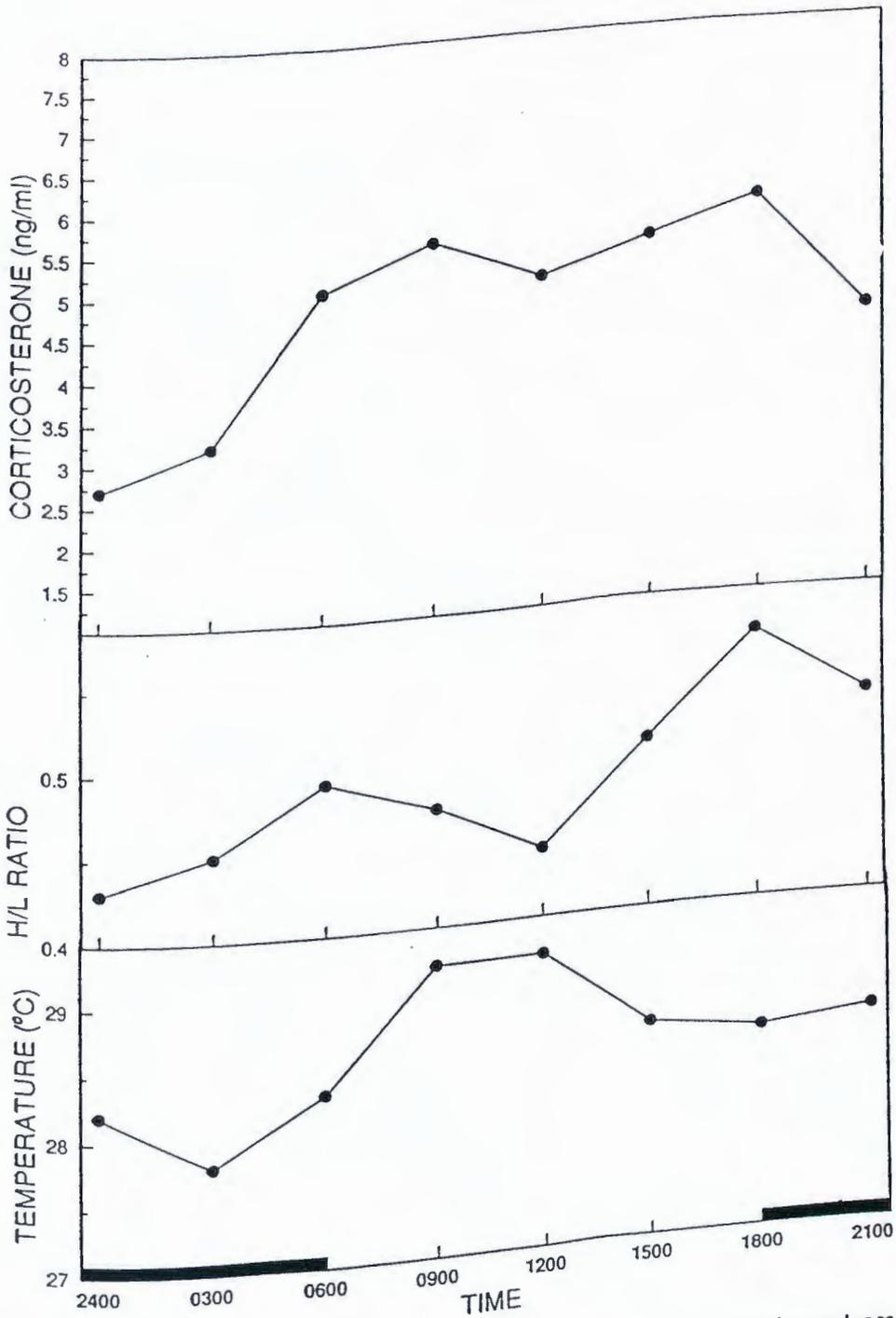


FIG. 4. Circadian variations in plasma corticosterone levels (top graph), heterophil/lymphocyte ratio (middle graph), and body temperature (bottom graph) in the diurnal blue-tongued skink. The dark phase is represented by the shaded bar on the abscissa.

(Lance and Lauren, 1984; Chan and Callard, 1972).

Locomotor activities such as shuttling between sun and shade to attain optimal body temperature for maintaining normal metabolic processes, foraging, territoriality, and courtship require energy. Corticosterone promotes hyperglycemia and glycogen deposition in reptiles (see review in Gapp, 1987). Exogenous glucocorticoids in the American alligator increase blood glucose levels (Lance and Lauren, 1984). Choudhury et al. (1982) measured blood glucose and catecholamines in soft-shelled turtles (*Lissemys punctata punctata*) at 0600, 1200, 1800, and 2400 hours and found that blood glucose increased and peaked between 0600 and 1800 hours and troughed at 2400 hours. Epinephrine and norepinephrine paralleled the peak. The turtles are diurnally active and rest at night. Jacob and Ooman (1992) found that exogenous corticosterone administered to the Indian bloodsucker lizard raised blood glucose levels and decreased hepatic cholesterol, indicating lipolysis. There was also evidence of protein catabolism which favors metabolic energy production rather than protein synthesis.

Although the least amount of daily locomotor activity (exploration and drinking) in the ball python occurred between 0900 and 1500 hours in another study using normal 12L:12D lighting (Kreger and Mench, in preparation), lack of activity during the midday peak in diurnal CS secretion suggests that peaks may be endogenous (Lance and Lauren,

1984; Underwood, 1977). It is evolutionarily advantageous for nocturnal reptiles to bask during the hottest part of the day to conserve energy for evening foraging when ambient temperature is low (Heatwole and Taylor, 1987).

Chan and Callard (1972) showed that CS levels in the desert iguana peaked during the animal's most active period in the laboratory. Their enclosures, included a thermal gradient, but activity and CS peaked at the time of the day that would be chronologically equivalent to the hottest time of the day in the wild. Body temperature was not monitored.

There was no evidence in this study of a light regimen effect for either species. This is either further evidence that the rhythm observed is partially endogenous or indicates that the red light was not perceived. We were unable to locate any studies that examined the effects of light color on reptile activity or CS secretion, but Underwood (1977) observed that Texas spiny lizards (*Sceloporus olivaceous*) did not alter their activity rhythm under continuous illumination unless both the parietal eye and the pineal gland were removed. Although its function is unclear, the parietal eye is influenced by the pineal gland and appears to play a role in photosensitivity, particularly as it pertains to day length and seasonal behavior (Underwood, 1977; Haldar and Thapiyal, 1977).

Lance and Lauren (1984) showed that alligators, which lack a pineal, display a CS diurnal rhythm that corresponds

with the natural light cycle. The effect of alterations in light intensity or length were not explored in this study. While there is evidence that alterations in photoperiod modify diurnal activity cycles in some species such as Australian skinks, African skinks, and geckos (Heatwole and Taylor, 1987), the occurrence of similar changes in CS secretion have not been investigated.

In the ball python and the blue-tongued skink, it appears that CS rhythm is at least partially correlated with temperature, although experimentation with suboptimal substrate temperatures would be necessary for confirmation. Activity in ectotherms is controlled by light and temperature. Light temporally synchronizes the activity cycle of the sand boa (*Eryx conicus*), but ambient temperature influences the intensity of activity (Griffiths, 1984). Cloudsley-Thompson (1965) demonstrated that activity rhythms remain normal when a diurnal skink and a nocturnal gecko are put into complete darkness, but temperature reversal (low during the normal daylight hours and high during normal night hours) reverses the activity periods. When the normal light/dark cycle is reestablished but temperature regimen remains the same, the animals entrain on the light.

Because CS is necessary to aid in energy-requiring behavioral activities and activity is partially thermally controlled, it is not surprising that skink peak body

temperature in our study occurred between 0900 and 1200 hours, coinciding with the CS plateau which occurred between 0600 and 1800 hours. The small sample size may have been responsible for the lack of statistical correlation.

The ball python rhythm differed from that of the skink in that CS showed a midday peak that paralleled body temperature and a midnight peak that did not. The animals were maintained under a constant ambient temperature and relative humidity and had use of a substrate thermal gradient. We observed that during evening locomotion, pythons were often in constant vertical motion against cooler enclosure walls or furniture such as the top of the hidebox which may have resulted in a drop in body temperature. In the wild, snakes bask when midday environmental temperatures peak. This might serve to increase CS secretion for glucose mobilization which can be utilized at lower temperatures later in the evening during nocturnal foraging, possibly accounting for the second peak.

Neither study species demonstrated a cycle or trend in the heterophil/lymphocyte (H/L) ratio. During a chronic stress response, CS binds to the cytoplasm of lymphoid cells, enters the nucleus as a steroid-receptor complex, and causes suppression of Interleukin II (Gillis et al., 1979) and thus suppression of lymphocyte production.

Not all species have CS-sensitive immune systems. Chickens (Gross and Siegel, 1982), hamsters, mice, rats, and

rabbits are sensitive species while ferrets, nonhuman primates, guinea pigs, and humans are resistant (Claman, 1972). The only report on H/L ratio in reptiles is mentioned by Lance (1990), who indicates that R.M. Elsey found an increase in the ratio at 24 hours post-restraint in alligators (unpublished data).

The reptilian immune and endocrine systems are more similar physiologically to those of birds than mammals (Chester-Jones, 1987) and available avian and reptilian data suggest both classes have immune systems that are CS sensitive. Therefore, an increase in the H/L ratio for the python and skink would be expected over time as the result of repeated capture and cardiac puncture if these represented chronic stressors. However, neither species had a significant animal or day effect for H/L ratio.

Elevations in basal CS level have been implicated as a physiological indicator of stress in birds and reptiles (Seigel, 1980; Lance and Lauren, 1984; Greenberg and Wingfield, 1987). Investigation and recognition of the normal daily rhythm of circulating corticosterone is important in order to choose the best sampling time and to avoid confounding results in studies that evaluate stress in reptiles. Our investigation determined that there was a bimodal rhythm in the ball python and a unimodal rhythm in the blue-tongued skink. Optimal blood sampling times would therefore be during the troughs in the late evening/early

morning for the skink and either early morning or late afternoon for the python when elevations in CS resulting from treatment effects would be most obvious. Use of low-intensity red bulbs by zoos or investigators during the normally dark phase of the photoperiod do not alter this rhythm. The CS rhythm may have evolved to parallel periods of maximum activity and ambient temperature since a parallel body temperature rhythmicity was found in the lizard and for one of the CS peaks in the snake. Finally, when other methods of blood sampling are unavailable, cardiac puncture is an alternative that is quick and does not represent a chronic stressor.

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CHAPTER IV
THE PHYSIOLOGICAL AND BEHAVIORAL EFFECTS OF
HANDLING AND RESTRAINT ON THE
BALL PYTHON (*Python regius*)
AND THE
BLUE-TONGUED SKINK (*Tiliqua scincoides*)

ABSTRACT

Four handling techniques commonly used with snakes and lizards in zoological park reptile collections were evaluated with respect to animal well-being. Adult male blue-tongued skinks (n=3) and ball pythons (n=4) were either handled gently, restrained manually, or container restrained for 10 minutes, released into their enclosures for 15 minutes, and blood sampled. Unhandled animals served as controls. Plasma corticosterone levels (CS), pre- and post-treatment locomotor and consummatory activities, and heterophil/lymphocyte (H/L) ratios were determined. None of the parameters evaluated differed among treatments, with the exception of CS levels in container restrained pythons (56.2 ± 6.7 ng/ml), which were significantly higher ($P < .05$) than those of controls (37.6 ± 6.1 ng/ml). Brief periods of handling in captivity thus do not appear to cause chronic stress in the study species, although short-term stress was associated with container restraint in pythons.

INTRODUCTION

Reptiles are handled during routine maintenance of zoo exhibits, educational demonstrations, for venom extraction, examination and forcefeeding, and as companion animals. Reptiles have also become attractive, unusual, inexpensive, and quiet pets for a growing population of apartment dwellers. Current concern about animal welfare within the private and public sectors has prompted those who handle animals to evaluate the effects of their methods on the general well-being of their charges (Hemsworth et al., 1987; Gonyou et al., 1986).

In birds and mammals, aversive and infrequent handling results in increased basal levels of circulating glucocorticoids, and decreased feed efficiency and impaired growth rate (Hemsworth et al., 1987 and 1981; Gonyou et al., 1986; Beuving, 1980). A combination of handling and blood sampling results in elevations of corticosterone in the American alligator (*Alligator mississippiensis*) (Lance and Lauren, 1984), the skink (*Tiliqua rugosa*) (Bradshaw, 1978), and the tree lizard (*Urosaurus ornatus*) (Moore et al., 1991). We have found no systematic studies in the literature of the behavioral and physiological effects of handling and restraint in reptiles that provide a comparison of different handling routines.

This experiment was designed to provide information about physiological and behavioral responses of reptiles to

different types of handling and restraint. Three handling techniques were selected for evaluation based on their application to current herpetoculture. Gentle handling of reptiles is frequently used by environmental educators to increase audience awareness and appreciation of reptiles. Manual restraint is used by veterinarians and researchers for close examination of the animal or for force-feeding. Container restraint was intended to represent placing the animal in a tube-like shipping container that restricts some movement or allows handling of the posterior of the animal without the hazard of bite infliction to the handler.

Measurement of corticosterone was supplemented by analysis of the heterophil/lymphocyte ratio, which has been used as an indicator of chronic stress in domestic poultry (Gross and Siegel, 1983) and American alligators (Elsley, unpublished), and by changes in activity behaviors which may be indicative of stress-related pre-pathologies (Moberg, 1985; Marcus, 1981; Hediger, 1955). Pre- and post-handling locomotor and consummatory behavior levels were compared.

The species selected for the study were the nocturnal ball python (*Python regius*) found in west and central African grasslands (Vosjoli, 1989) and the diurnal blue-tongued skink (*Tiliqua scincoides*) found in coastal woodlands, montane forests, and grasslands in north, east, and southern Australia (Capula, 1989). Their selection was due to their large size which would allow multiple blood

sampling without hemodilution or anemia, their generally tractable nature, their frequency in zoos, and their popularity in the pet industry.

METHODS

Animals, housing, and maintenance: Adult male blue-tongued skinks (n=3, 22.17cm mean snout-to-vent length, 302.7g mean body weight) and ball pythons (n=4, 96.4cm snout-to-vent length, 1085.75g mean body weight) were purchased from a commercial supplier. The skinks were captive-born, but the origin of the pythons is uncertain. Animals were housed individually in 60-gallon plastic containers with modified screen lids, and were illuminated by two sets of broad-spectrum fluorescent lights in a room controlled for photoperiod (12L:12D), temperature (27-29°C), and mean relative humidity (50.17%). A thermal gradient (25-33°C) for basking was provided by the placement of heating pads under half of each enclosure. Newspaper was used as substrate. Each snake enclosure was equipped with a plastic shelter for cover and a water dish. The skink enclosure included a PVC tube cut longer than lizard body length and a water dish. Water for drinking and soaking was available *ad libitum*. All animals were fed once per week and weighed monthly to assure maintenance or increase of body weight. Snakes were fed sacrificed adult mice. Three of the snakes would not accept dead food, so force-feeding

was necessary. Skinks were fed sacrificed weanling and juvenile mice. Feed was vitamin and mineral-dusted. This investigation followed an 8-month animal acclimation period.

Experimental design: The experiment was run from October through December, 1991. The experimental design was an incomplete randomized block with each individual serving as its own control. Each animal was sampled twice per treatment. A control treatment was included for each sampling day. Because only three lizards were used, several received a treatment three times in order that each received all treatments twice.

A previous investigation (Chapter III) showed that there was a trough in corticosterone at 1700 hours in the ball python. No statistically significant rhythm was determined in the skink. Filming and sampling thus began for each species every 4 days at 1700 hours so that corticosterone elevations would be more indicative of treatment rather than time of day.

On the sampling day, each individual of one species was assigned to one of four treatments. Gentle handling (G) consisted of the animal being placed on the experimenter's upright palm, free to move along the hand and arm unrestrained. Manual restraint (M) consisted of the experimenter placing hands on the animal dorsally, with one hand near the head and the other anterior to the vent, thus minimizing the animal's ability to move freely. Container

restraint (C) consisted of the animal being placed inside a PVC tube which was sealed on the side the animal faced, allowing some lateral movement but not the ability to turn around. The non-handle treatment (N) in which the animal was removed from its enclosure for immediate sampling served as the control. All treatments except N lasted 10 minutes, after which the animal was placed back in its enclosure for 15 minutes prior to sampling. Personal interviews with herpetologists, educators, and veterinarians who handle reptiles regularly indicated that 10 minutes is a typical handling period. Because corticosterone elevation from handling shows a rapid increase at 10 minutes and begins to plateau at 1 hour in the tree lizard (Moore et al., 1991), it was decided that 15 minutes post-treatment for sampling would reflect elevations due to handling. Following sample collection, cloacal temperature was measured and the animal was returned to its enclosure. Room temperature and relative humidity were also monitored.

Behavior: Two weeks prior to the experiment, each species was videotaped for six days using 2 cameras connected to a sequential switcher and video cassette recorder. Two enclosures were filmed simultaneously with each camera. Filming was alternated between cameras every minute. Each camera filmed 2 enclosures simultaneously. A 25 watt green incandescent light bulb produced low intensity light during the 12-hour dark period for filming of

nocturnal activity. Although statistical units varied, a comparison of activity levels could be made between the pretest animals which were not sampled and those that received the non-handle treatment during the experiment in order to assess the effect of cardiac puncture on behavior.

During the experiment, the behavior of the species to be sampled was filmed for 24 hours pre-treatment and 24 hours post-treatment, after which the other species was filmed using the same routine. Thus, each species was filmed for 48 hours every other 48 hours.

A total of 672 hours of behavior were analyzed during the pre-test period and 912 hours during the experiment. Behavior was analyzed by comparing the percent time the animal was active and inactive for pre- and post-treatment periods. Activity was considered to consist of feeding, drinking, and locomotor movements that exceeded 10 seconds and daily pre- and post-treatment activity were summed separately for statistical comparisons within and among treatments. Due to unequal statistical units, comparisons of pre- and post-treatment activity used least squares means.

All motionless activities such as basking or remaining in the shelter were considered inactivity. Activities such as a change in body position or a head nod that lasted less than 10 seconds were not included in the analysis. Drinking episodes were recorded as one continuous event as long as

the animal dipped its head in the water at least once every 10 seconds. If additional time elapsed without drinking, the episode ended. Further drinking was counted as a separate episode. Snake locomotor activity was only considered when the head of the animal moved away from the body indicating that the motion was not a shift in body position for basking or resting purposes.

For statistical analysis, total activity was divided into locomotor activity and consummatory behavior. However, unlike the lizards which fed normally throughout the experiment, force-feeding was required for three of the snakes. This species is well-known to go off feed in captivity (Vosjoli, 1990). Because of forcefeeding as well as the difficulty in differentiating drinking from locomotor activity above the water dish due to camera placement, consummatory behavior for snakes was not analyzed.

Blood sample collection and Radioimmunoassay: Blood was collected by cardiac puncture using a 23 gauge needle. Collection never exceeded 30 seconds and rarely exceeded 15 seconds. Each animal was sampled once every 4 days with 0.5 cc blood collected per animal per sampling time. There were no obvious ill-effects resulting from sampling. Samples were dispensed into tubes containing one drop of 11% EDTA and stored on ice for a maximum of 30 minutes. A blood smear was made of each sample for leucocyte counts, and plasma was frozen at -15°C for later radioimmunoassay.

Corticosterone was measured using the RSL I₁₂₅ Corticosterone Kit (liquid phase) which was validated for parallelism and recovery in the study species by the investigators (Chapter II). Plasma samples were diluted with deionized distilled water using 1:5 for the lizard samples and 1:40 for snakes. Within and between assay coefficients of variation were 4.84% and 9.5% respectively. Slides were stained using HEMA 3 (Biochemical Sciences, Inc., Bridgeport, NJ 08014), and heterophil/lymphocyte ratios were calculated from a count of 200 leukocytes per slide. Leukocyte classification was based on descriptions by Hawkey and Dennett (1989), Montali (1988), and Frye (1981).

Statistical Analysis: Corticosterone, heterophil/lymphocyte ratio, and behavioral relationships were analyzed using SAS General Linear Models Procedures and pairwise contrast analysis for mean comparisons among treatments. Multiple contrasts were used to determine within treatment differences and to compare the control treatment with the other treatments. Dependant variables were corticosterone (CS), heterophil/lymphocyte ratio (H/L), and percent activity change (the difference between the daily percent of pre- and post-treatment activity for total, consummatory, and locomotor behavior). Class variables were animal, handling treatment, and whether the animal was fed or not on each sampling day. Date was treated as a covariate and adjusted for interaction with treatment and

feed. Because of the adjustment, least squared means are presented rather than arithmetic means. Residual analysis revealed that corticosterone, heterophil/lymphocyte and behavioral data did not need to be transformed since variance was homogeneous (Sokal and Rohlf, 1981).

RESULTS

All animals maintained or increased their body weight during the course of the study. With the exception of manipulation during routine enclosure maintenance, skink locomotor and consummatory activity occurred only during the lights-on period while python activity was exclusively nocturnal.

Skinks. During the pretest period, there was a significant difference in daily total activity levels among individuals ($P < .04$). Both locomotor activity ($P < .04$) and consummatory behavior ($P < .02$) also differed among individuals. Pairwise contrast analysis revealed that one of the three animals was less active than the other two ($P < .04$). A mean of 84% of the total pretest activity occurred during the lights-on period. There were no date effects. During the experiment, there were no significant differences in the animal's corticosterone, heterophil/lymphocyte ratio, or total, consummatory, and locomotor activity as a result of handling treatments, feed, day, or animal. Results are shown in Figures 5 and 6.

Pythons. Total daily activity varied among individuals ($P < .005$) and possibly by date ($P < .06$) during the pretest period. Pairwise contrast analysis demonstrated greater total activity in the snakes housed on either end of the row of enclosures ($P < .04$). A mean of 72% of the activity occurred during the dark period.

There was a significant handling treatment effect on corticosterone secretion ($P < .01$). The pairwise contrast analysis revealed a difference between the control treatment and the container-restraint treatment ($P < .05$). There were no treatment differences in heterophil/lymphocyte ratio or activity in response to handling treatment, nor were there feed or day effects. Results are presented in Figures 7 and 8.

DISCUSSION

During the pretest period, skinks showed the most total activity during the light period while the pythons showed the most during the dark period. This was consistent with activity data throughout the experiment as well as information in the literature (Cappula, 1989; Vosjoli, 1990).

There are few investigations in the literature of the effects of handling on activity except for anecdotal information about species that become "tame" in that they do not resist handling or become aggressive (Ditmars, 1931;

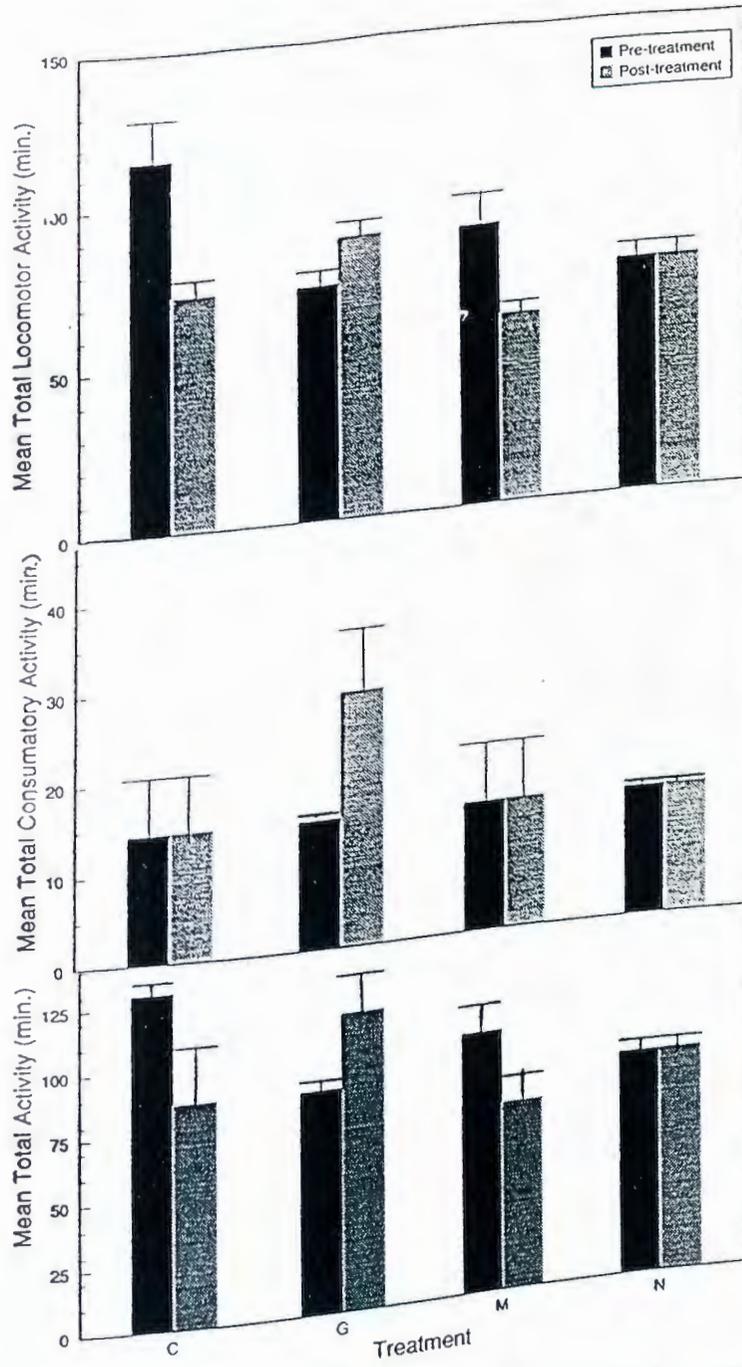


FIG. 5. LS Means of locomotor (top graph), consummatory (middle graph), and total activity (bottom graph) under different handling treatments in the blue-tongued skink (n=3). C=container restraint, G=gentle restraint, M>manual restraint, N=no restraint.

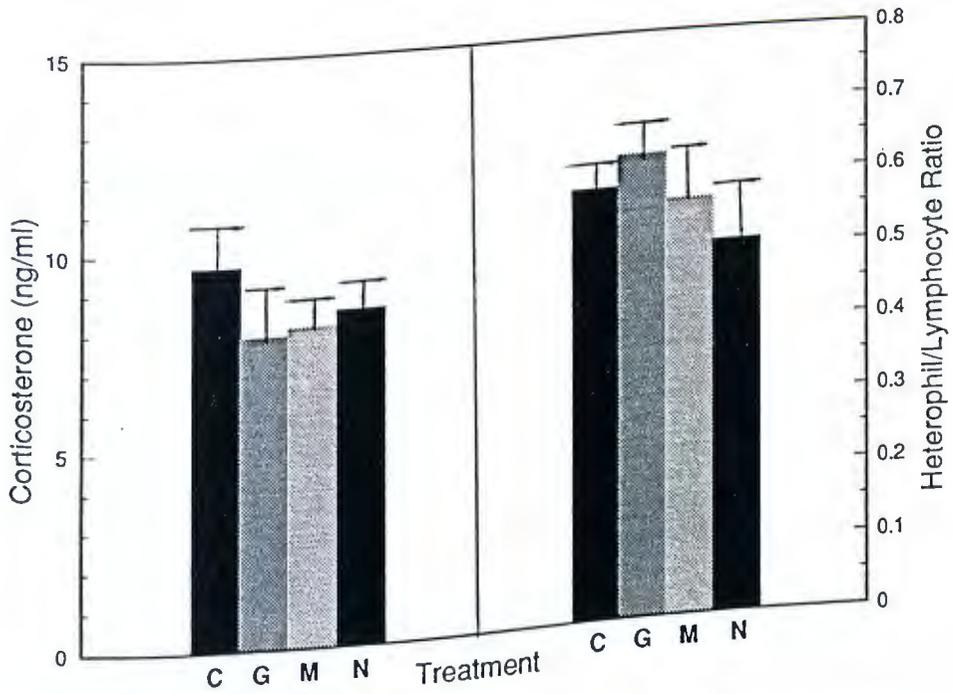


FIG. 6. LS Means of corticosterone and heterophil/lymphocyte ratio under different handling treatments of the blue-tongued skink (n=3). C=container restraint, G=gentle restraint, M>manual restraint, N=no restraint.

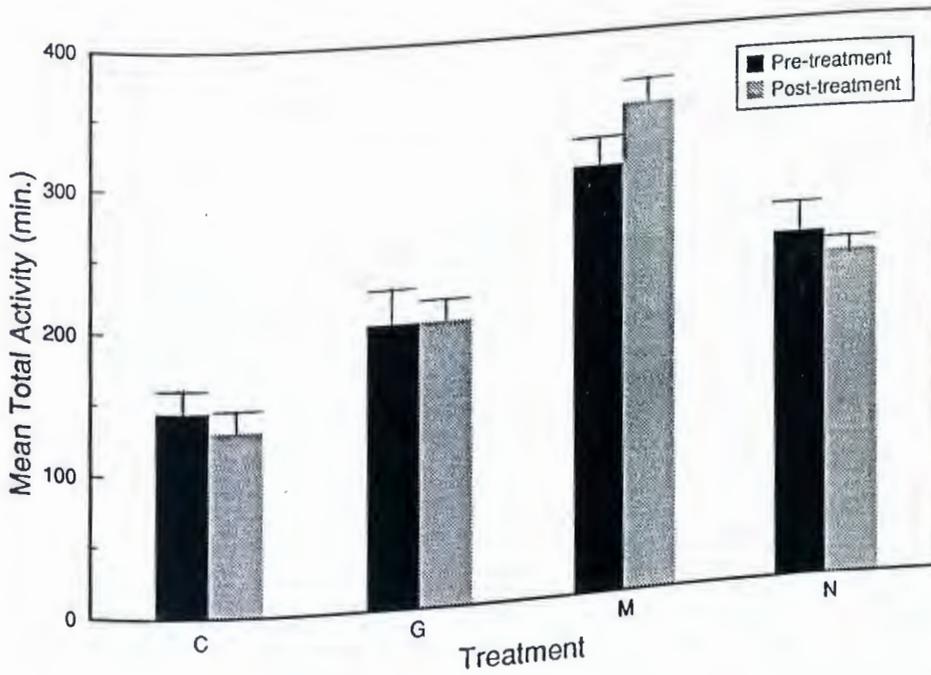


FIG. 7. LS Means of total activity of the ball python (n=4) under different handling treatments. C=container restraint, G=gentle restraint, M>manual restraint, N=no restraint.

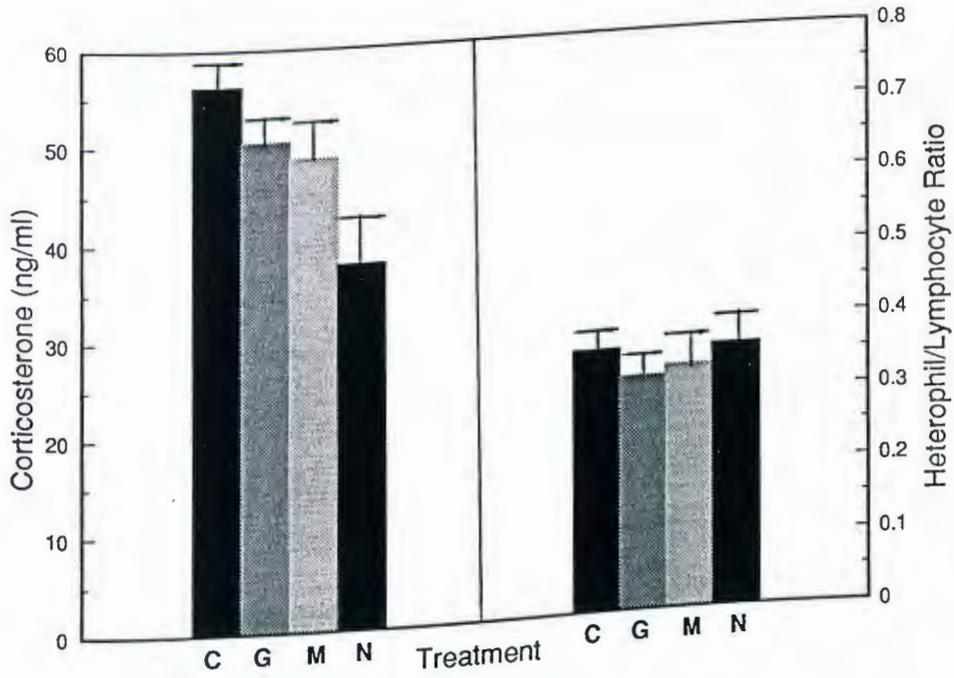


FIG. 8. LS Means of corticosterone and heterophil/lymphocyte ratio of the ball python (n=4) under different handling treatments. C=container restraint, G=gentle restraint, M>manual restraint, N=no restraint.

Wiley, 1930; Vosjoli, 1990). Our study showed no difference in total, consummatory, and locomotor activity levels before and after treatments regardless of time of day, treatment, or feeding. It could be argued that the animals were exposed to a chronic stress situation and simply maintained a high activity level as a result. However, the study species are recommended as pets and education species because of their docility (Marcellini, pers. comm., 1991; Vosjoli, 1990). The investigators did not observe typical threat behavior such as body inflation and a gaping mouth threat exposing the blue tongue in the skinks (Capula, 1989) or hissing, striking, or compact coiling of the ball python (Ditmars, 1931) during the experiment. All of these behaviors were observed during the first month of captivity in the laboratory, and hissing and striking were noted in some of the pythons during ecdysis. None of the individuals in this study threatened the handler during a treatment session or following treatment when the animal had to be re-collected from its enclosure for sampling.

Container restrained pythons had a significantly greater corticosterone level than non-handled/non-restrained animals. The difference may be due to the force that was sometimes necessary to extract the animal from the container following treatment. Unlike the skink, the snake is able to attain body positions that keep it wedged tightly within a small space. The corticosterone increase indicates that

container restraint induced an acute stress response (Moberg, 1985; Lance, 1990).

The few studies of reptile corticosterone secretion in response to handling have produced varying results. Although our methods differ from previous studies, our results agree with Dauphin-Villemant and Xavier (1987) who found no increase in corticosterone after 8 minutes of continuous restraint and blood sampling in the viviparous lizard (*Lacerta vivipara*). Conversely, our results do not agree with Moore *et al.* (1991) who showed that corticosterone levels increase from 10 minutes to 4 hours after capture and placement of the tree lizard (*Urosaurus ornatus*) in a bag suspended from the investigators belt while the investigator continues to walk. Grassman and Crews (1990) placed whiptail lizards (*Cnemidophorus uniparens*) in a bucket for 30 minutes and chased the animals by hand every 5 minutes and found a significant elevation in plasma corticosterone. Such forced activity may be as stressful as forcing the python out of the tube during container restraint so an elevation would be expected. Both Moore *et al.* (1991) and Dauphin-Villemant and Xavier (1987) found increases in corticosterone when wild lizards were taken into captivity for 1 hour to 3 weeks. Since our animals were housed in the laboratory for 8 months, elevations are not likely to be due to insufficient time for acclimation.

There were also no differences in the H/L ratios within each species with regard to feed or treatment. During a chronic stress response, corticosterone binds to the cytoplasm of lymphoid cells, enters the nucleus as a steroid-receptor complex, and causes suppression of Interleukin II (Gillis *et al.*, 1979). The only report on H/L ratio in reptiles is mentioned by Lance (1990), that R.M. Elsey (unpublished data) found an increase in the ratio at 24 hours post-restraint in alligators. If handling and sampling represented stressors to the study animals, an increase in the H/L ratio would be expected. Although pythons showed elevated corticosterone with container restraint, activity and H/L ratio remained constant indicating that container restraint is not a chronic stressor. It is important to note that three of the four pythons required routine force-feeding, yet the parameters examined showed no Feed X Date or animal effects which indicates that they may have become habituated to the process.

The skinks showed no difference among or within treatments in CS secretion. The stability of this parameter combined with the stability of the H/L ratio and lack of activity change demonstrates that the treatments were not chronically stressful. Furthermore, they continued to feed (sometimes immediately after sampling) and maintained body weight throughout the study.

The non-significant results in this study may, in part, be due to habituation to human presence and sampling. As would occur in facilities that maintain reptiles such as zoos, the animals in this study were regularly exposed to humans. The primary investigator provided daily maintenance, force-fed snakes, and weighed each animal monthly during the 8-month acclimation period, throughout a photoperiod study which included blood sampling (Chapter III), and for the duration of this experiment. Reptile habituation to humans was demonstrated by McKnight *et al.* (1978) using tonic immobility (TI) as an indicator of fear in the anole (*Anolis carolinensis*). Tonic immobility is believed to be an innate response to fear (Beuving *et al.*, 1988). In TI, an animal is exposed to a stimulus, and is restrained, inverted, and released. The animal will remain immobile for a period of time depending on the magnitude of the fear. In the anole, TI is lower in animals that are routinely exposed to people as opposed to those that receive less than daily maintenance. Habituation may be a contributing factor in the current investigation.

The present study showed that 10 minutes of handling and restraint of the male ball python and blue-tongued skink followed by a 15 minute rest period before sampling may not be perceived by the animals as stressful. Total locomotor and consummatory activity levels before and after handling treatments as well as H/L ratios were maintained throughout

the study regardless of treatment. Skinks did not show a difference in plasma corticosterone levels, while acute stress was indicated by elevated corticosterone levels in pythons held in a container.

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SUMMARY DISCUSSION

Reptile husbandry, transport, and exhibition involves handling and restraint. Current concern about animal welfare within public and private sectors has prompted those who handle animals to evaluate the effects of their methods on the general well-being of their charges. In this investigation, we addressed the effects of artificial light on the circadian rhythm of corticosterone (CS) as well as CS levels, heterophil/lymphocyte ratio (H/L), and activity as indicators of stress resulting from handling and restraint in the ball python (*Python regius*) (n=4) and the blue-tongued skink (*Tiliqua scincoides*) (n=3).

Low intensity red light kept on throughout the 12L:12D photoperiod did not differ in H/L ratio or CS from that of the normal light cycle when red light was absent. Thus, the combined data indicated significant peaks in python CS rhythm at 1200 and 2400 hours. Although not statistically significant, skink CS levels plateaued between 600 and 1800 hours. These data agree with avian, mammalian, and reptilian studies that show peak CS secretion coinciding with periods of greatest activity (Chan and Callard, 1972; Lance and Lauren, 1984; Dauphin-Villemant and Xavier, 1987). Our results show that zoos can use low intensity red lamps in nocturnal exhibits or as heating lamps without altering the CS rhythm in the study species. Future studies which evaluate CS can use the determined troughs in the circadian

rhythm as the optimal time for blood sampling when treatment effects will not be masked by daily fluctuations.

Due to H/L ratio stability which showed no trend or day effect that paralleled CS, immunosuppression did not occur as a result of blood sampling. Therefore, cardiac puncture did not induce a chronic stress response and is a quick alternative when other sampling methods are unavailable.

In a separate experiment, the animals were handled gently, manually, or container-restrained and served as their own controls. The handling treatments were typical of methods used in herpetoculture and exhibition. Total, locomotor, and consummatory behavior and H/L ratio did not differ among treatments. The only significant effect of treatment on CS secretion was shown in container-restrained pythons which had greater levels than controls. Thus, with the exception of container-restrained pythons, brief periods of handling do not appear to be chronically stressful to the study species. Snakes that required forcefeeding did not differ by feed day, animal, or date from the snake that fed without human assistance for any of the parameters investigated which indicated that forcefeeding may not induce long-term stress. Although previous studies have used hand-chasing the animal in a container or restraining a wild animal in a bag to show the effects of handling and restraint on CS, this investigation is the first to examine typical handling routines used in herpetoculture and

exhibition.

The lack of treatment effects in this study suggests that there may be habituation to human presence, sampling, and forcefeeding which occurred during an 8-month acclimation period.

Future studies should consider a greater animal sample size and greater variety of species. There is evidence that CS elevation in response to handling or aggressive interactions with conspecifics decreases testosterone levels in reptiles (Moore et al, 1991). It is in the interest of the conservation community to research the handling routines used in our study to determine if long-term or longer handling periods cause reproductive suppression. Switching handlers or comparing animals that experience human presence only at sampling time with those that receive daily maintenance would determine the extent of animal acclimation to handling or human presence. The combination of CS, H/L ratio, and activity can also be used to determine the stress effects of exhibit size, social or solitary housing, and visitor interactions with the exhibit in terms of noise level or banging on the enclosure.

Our study has determined the CS circadian rhythm in the ball python which is not changed, in either species, by low intensity red light. Cardiac puncture, brief periods of handling, and forcefeeding do not appear to be chronically stressful in the ball python or the blue-tongued skink.

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APPENDIX A

DESCRIPTION OF REPTILE CELL TYPES

The Leukocytes

Lymphocytes: Lymphocytes can be large or small. The large nucleus stains dark purple in Wright's Stain due to the great quantity of RNA used for antibody synthesis in its endoplasmic reticulum (Roitt et al., 1989). The nucleus is circular small and centrally located in the cytoplasm in small cells and large, irregular, and on the periphery in large cells (Pienaar, 1962). Like mammals, it is mononuclear. The cytoplasm often appears as a crescent of halo around the nucleus and stains light blue (azurophilic).

Age, sex, pathology season, and temperature affect lymphocyte numbers. It is the only leukocyte to vary during ecdysis (Duguy, 1970). Lymphocytes numbers are higher in young the (*Cordylus vittifer*) than older animals (Pienaar, 1962). They are greater in females than males for the asp viper (*Vipera aspis*) (Duguy, 1963a) the common wall lizard (*Lacerta muralis*) (Duguy, 1970) and the adder (*Viper berus*) (Saint Girons, 1961). They are the same in both sexes in slow worm (*Anguis fragilis*) (Duguy, 1963b). In temperate climates, lymphocytes peak in the summer and are at their minimum in the winter (Marcus, 1988; Duguy, 1970).

Heterophils: The heterophil is a morphonuclear granulocyte

that contains peroxidase to inactivate peroxide generated during phagocytosis (Roitt et al., 1988). It's multilobed nucleus stains dark purple and the acidophilic granular cytoplasm stains light purple to muddy brown. They can be mono or polynuclear and are referred to as polymorphonuclear eosinophils by Pienaar (1962). If there is any seasonal variation within species, it is masked by variability between individuals (Duguy, 1970). Among temperature species, the numbers peak in the summer and are at their minimum in winter (Saint Girons, 1970). They increase during pregnancy in *Vipera aspis* (Duguy, 1963a). They comprise 30 to 45% of the total leukocyte count (Frye, 1981).

Eosinophils: Often confused with heterophils, the eosinophil usually has a bilobate nucleus that stains purple or blue with bright orange red stained granules. Their size varies between and within individuals. Lizard eosinophils are smaller than crocodile and turtle which are smaller than these of snakes. In mammals and presumably in reptiles, they are attracted to the invasion site by basophil and T-cell products. Although they phagocytize invaders, they also release histaminase and arylsulphatase to inactivate most cell products like histamine to slow the inflammation response by decreasing granulocyte migration to the invasion site (Roitt et al., 1988). Eosinophils seem to be inversely

proportional to lymphocytes and peak in the winter with the minimum count in the summer (Duguy, 1970). They increase when an infection is due to parasites (Pienaar, 1962) particularly in the lower gastrointestinal tract. They comprise 7 to 20% of the count (Frye, 1981).

Monocytes: As in mammals, reptilian monocytes are usually as large or larger than the erythrocyte and lymphocyte is azurphilic and may have vacuoles. The cytoplasm stains gray or pale blue. Like the heterophil and neutrophil, it is a phagocyte that contains peroxidase. Monocytes present the antigen to lymphocytes.

Basophils: The circular basophil stains dark blue. It is small and has densely packed granules in snakes and lizards; large with loose granules in crocodiles and turtles (Saint Girons, 1970). Often it is too densely packed with spherical and rod-shaped granules that the cytoplasm is not visually distinguishable from the nucleus. It may contain heparin, as in mammals, and play a role against parasitic infections. It decreases with age in *Vipera aspis* (Duguy, 1963a). They are least abundant during hibernation (Duguy, 1970). Basophils comprise 10 to 25% of the total leukocyte count (Frye, 1981).

Neutrophils: Except for the scallop-edged nucleus, neutrophils look and stain like heterophils (Frye, 1981). They increase with parasitic infections in *Lacerta muralis* (Duguy, 1967) and are more prevalent in females than males in *Cordylus vittifer* (Pienaar, 1962). While most investigators count them with heterophils or prefer one cell type name to the other, they compromise 3 to 7% of the leukocyte count (Frye, 1981).

Auxiliary Cells: Thrombocytes are elliptical, similar in size to erythrocytes, stain dark blue, and tend to be in small aggregations. Plasmacytes appear as lymphocytes with a complete halo. Their function is unknown in reptiles and comprise .5% of the leukocyte count.

APPENDIX B

Brief Biology of Study Species

1. Ball Pythons (*Python regius*)

Order: Squamata
Suborder: Serpentes
Family: Pythonidae

The ball python is the smallest of the pythons. It is named for its defensive posture which prompted R.L. Ditmars, then curator of reptiles at the Bronx Zoo, to write in 1931, "The head and neck are so tucked into spaces between the folds that the compact creature, squeezed into the size of a large bowling ball may be rolled ten to twelve feet with a fair push of the hand".

The ball python is a grassland species found in clearcut or cultivated areas of Sudan west of the Nile, southeastern Sudan, Senegal to Sierra Leone and the Ivory Coast to West Central Africa. It obtains a length of 150 cm, is a nocturnal terrestrial ambush hunter that feeds primarily on jumping mice which it kills by constriction (Vosjoli, 1990). It is a solitary animal except when pairing during mating season (Gerhardt, 1988). Sexual maturity is reached at 3 to 4 years.

Ball pythons breed less frequently than other pythons; every two to three years. They are oviparous. Females do not eat when gravid and coil around the eggs following oviposition for over two months until hatch. Unlike other pythons, they are unable to increase body temperature and egg temperature by shivering and coiling. Due to their small size and rapid heat loss, they frequently leave the eggs to bask, return, and increase egg temperature through conduction and their higher body temperature (Ellis and Chappel, 1987; Van Mierop et al., 1981). Coiling likely serves for egg protection from predators rather than for thermal benefits (Ellis and Chappell, 1987).

They are a popular pet species. In 1989, 18,000 ball pythons were legally imported into the U.S. All legal imports came from Togo and Ghana. Their size and low cost place them in high demand although their reluctance to feed in captivity is greater than other snake species (Vosjoli, 1990).

2. Blue Tongued Skink (Tiliqua scincoides)

Order: Squamata

Suborder: Sauria

Family: Scincidae

The blue-tongued skink is found in coastal woodlands,

montane forests, and grasslands of north, east, and southeastern Australia. Its adult length is 36 to 56 cm. It is diurnally active and feeds on insects, gastropods, and, to a lesser extent, berries and flowers (Capula, 1989). Its defense posture includes swelling the body, gaping the mouth, hissing, and protruding its blue tongue which it uses as a threat by rolling it to increase the size and fill the pink mouth cavity with even more blue (Carpenter, 1978). It is one of few lizards that are known to pant which it does by opening the mouth to inhale and closing for exhale (Heatwole and Taylor, 1987). The parietal eye which interacts with the hypothalamus is known in this species to be very light sensitive.

Unlike most skinks, they are ovoviviparous (eggs are retained internally until hatching) and produce 5 to 25 young annually (Capula, 1989).