THE PHYSIOLOGICAL EFFECTS OF RELOCATION ON GOPHER TORTOISES

(*GOPHERUS POLYPHEMUS*)

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# THE PHYSIOLOGICAL EFFECTS OF RELOCATION ON GOPHER TORTOISES (*GOPHERUS POLYPHEMUS*)

Paula Faith Kahn

A Dissertation

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Paula F. Kahn



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# VITA

Paula Faith Kahn, daughter of Michael J. Kahn and Linda F. Kahn, was born November 11, 1970 in Stoughton, Massachusetts. She graduated from Brockton High School, Brockton, Massachusetts in 1988. She entered New York University in New York City, New York and graduated with a Bachelor of Arts degree in Psychology and Spanish in 1992. She lived and worked in New York City for seven years before returning to school, Hunter College, to take introductory courses in science with the goal of attending graduate school in Biology. She entered Auburn University's graduate program in Biological Sciences in August 2001 to earn a Master of Science degree, but instead, by December 2001, she began working toward her Doctor of Philosophy in Biological Sciences.

DISSERTATION ABSTRACT

# THE PHYSIOLOGICAL EFFECTS OF RELOCATION ON GOPHER TORTOISES (*GOPHERUS POLYPHEMUS*)

Paula F. Kahn

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As a result of habitat destruction throughout the southeastern United States, gopher tortoises (*Gopherus polyphemus*) have experienced an 80% decrease in their populations in the past 100 years. To protect the remaining gopher tortoises from extinction, populations are often relocated to safer habitats. However, the long-term evaluations that are necessary to determine relocation success cannot reasonably be conducted for this long-lived species because it would require decades of data collection. Immediate measures of relocation success involving the use of physiological biomarkers are thus required because such indicators can be measured in the short-term and provide a more relevant assessment of the health and viability of relocated populations.

Physiological biomarkers that could contribute to determining relocation success include measures of corticosterone and adrenal competence, reproductive health, and immune function. I first conducted a validation study to establish proper dosages and timings for these biomarkers in gopher tortoises. I then conducted a study which determined that trapping, handling, and various manipulations to gopher tortoises do not cause them stress, as indicated by a lack of change in corticosterone levels and movement patterns between the pre- and post-protocol periods. Subsequently, I relocated tortoises at Ft. Benning, Georgia and assessed each of the biomarkers prior to and again 30 days and 10 months post-relocation.

I found no changes in baseline corticosterone levels as a result of relocation. However, the relocators showed a significantly stronger response to the ACTH challenge post-relocation in spring indicating that they, and not the residents, may have experienced an acute stress response after being relocated in spring, but the response likely occurred prior to the 30 day measure. There was no effect of relocation on sex steroid levels or on immune function. However, I did find that using the ELISA as a measure of disease status is ineffective and unreliable since titers change rapidly and drastically in less than 30 days. Finally, I found that none of the variables that I measured showed any effect from pre- to post-relocation according to habitat quality.

Ultimately, it appears that relocation does not result in direct physiological detriment to gopher tortoises at the specific time points that I measured. However, removing this keystone species from its environment could have irreparable consequences for the commensal and obligate species that are left behind.

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I also want to sincerely thank my advisor and mentor, Mary Mendonça. She accepted me into her lab despite my limited background in science. Thank you, Mary, for believing in me, for holding me to the highest standards, for pushing me to my academic limits, and for teaching me that manuscripts can be more effective if written in under 50 pages. I also want to thank you for your patience with my learning curve, especially when I accidentally contaminated the lab with testosterone. Oops.

Craig Guyer, you sent me to find gopher tortoises in the woods of rural AL during my first field season with a simple hand-drawn map and a compass. Having just arrived in AL from NYC, I had never been in a forest that was not surrounded by Central Park West on one side and 5$^{th}$ Ave on the other. Working alone in Chunchula, AL was

one of the most terrifying experiences of my life. Thank you for scaring the daylights out of me, but ultimately, for leading me to become a confident field researcher.

Much thanks to my labmates, especially to Dr. Chelsea Ward and Dr. Kristen Navara, who were endlessly patient with me in the RIA lab. I also want to thank Roger Birkhead who made me earn my place in the world of gopher tortoise research and gave me some of the most sound advice of my graduate career, "Think like a tortoise."

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# INTRODUCTION TO DISSERTATION

Human domination of the Earth's natural environments as a result of exponential population growth has resulted in a global biodiversity crisis that may be, at least partially, irreversible (Wilson 1989; Vitousek et al. 1997). The major cause of species extinctions is habitat destruction (Wilson 1989), and, more often than not, this is a result of a myriad of anthropogenic activities (Walker et al. 2005a). Rates of recent species extinctions are alarming – approximately 16 million species are being destroyed each year at a rate of 1800 populations per hour in the tropical rainforests alone (Hughes et al. 1997). Even species that are dominant in their habitats, those that are abundant in number and good competitors, are merely experiencing a time-delayed, but inevitable extinction process as a result of not only extensive, but even moderate habitat destruction (Tilman et al. 1994).

Conservation physiology is a newly emerging field that attempts to address the global biodiversity crisis via integrative, comparative, evolutionary, and environmental perspectives. This applied field of physiology examines how organisms cope with changes in their environment and attempts to find causative mechanisms for population declines and prevent species extinctions (Carey 2005). Conservation physiology provides the means to integrate the assessment of stress physiology, reproductive

endocrinology, and immunology in an effort to provide guidance to conservation managers in how best to promote the successful persistence of threatened populations.

The gopher tortoise (*Gopherus polyphemus*) is a threatened species that could benefit from techniques in conservation physiology. This long-lived tortoise is endemic to longleaf pine forests, sand hills, and upland and scrub oak habitats throughout the southeastern United States (Auffenberg and Franz 1982). The gopher tortoise is a keystone species in its environment with as many as 60 vertebrates and 300 invertebrates using the tortoises' burrows for shelter and protection (Jackson and Milstrey 1989). However, much of the tortoises' habitat has been destroyed for the development of real estate and transportation corridors in order to accommodate ever-growing human populations (Auffenberg and Franz 1982). At one time, longleaf pine forests dominated 28 million hectares of the Coastal Plain (Burke 1989), but by 1993, less than 1.3 million hectares remained (Outcalt and Sheffield 1996). As a result, habitat destruction has become the number one reason cited for the precipitous population declines in this species (Diemer 1986; Lohoefener and Lohmeier 1986; Wahlquist 1991; Gibbons et al. 2000).

With the natural habitat of gopher tortoises decreasing at alarming rates, one of the only remaining methods for conserving this species is to relocate entire populations to protected habitats. Generally, gopher tortoises fit the profile as good candidates for successful relocation because they are a herbivorous, wild-caught (as opposed to hand-reared) species that can be relocated to good quality habitat within their historical range (Griffith et al. 1989). However, tortoises are also a k-selected species, characterized by an extended time to sexual maturity and small clutch size (Pianka 1970), both indicators

of poor relocation success (Griffith et al. 1989; Seigel and Dodd 2000). Therefore, it is important that gopher tortoise relocations be carefully documented and evaluated to determine if these animals are suited to relocation and under which specific conditions relocations would be most beneficial to the individuals and the populations.

While several relocation studies have been conducted on gopher tortoises, particularly in Florida, few studies have clearly demonstrated success in terms of establishing stable or thriving populations. In fact, most studies monitored tortoises for a period of less than three years following the relocations, and they merely examined movement patterns and documented the presence of hatchlings and yearlings (Lohoefener and Lohmeier 1986; Burke 1989; Fucigna and Nickerson 1989; Godley 1989; Stout et al. 1989; Tuberville et al. 2005). While these evaluation attempts were made with good intentions, evaluating relocation success is a complex process that encompasses both short-term and long-term measures of mortality, fidelity to the relocation site, season of relocation, disease, and social interactions, among others. Therefore, most researchers agree that relocation success, particularly in long-lived species like the gopher tortoise, can only be determined after many years of follow-up studies (Dodd and Seigel 1991; Gibbons et al. 2000). However, with the current rate of habitat destruction throughout the gopher tortoise's natural range, there is a critical need for effective short-term assessment of relocation success that could be accomplished using techniques associated with conservation physiology.

Changes in an organism's environment, including relocation from one habitat to another, can serve as a potential stressor. It is well established that animals must adapt to changes in their external environment (i.e., stressors) by altering their internal milieu

(Bernard 1865) in order to maintain or return to homeostasis (Cannon 1932; Selye 1937). The physiological stress response, directed by the hypothalamo-pituitary-adrenal axis, is adaptive in the short-term, allowing the organism to respond to changes in the environment (Chrousos 1997). This short-term response involves the release of corticosterone which redirects the organism's physiological functions making oxygen and nutrients available to the central nervous system while simultaneously inhibiting less critical activities related to digestion, growth, reproduction, and immunity (Chrousos 1997). However, if the stressor is persistent, the physiological stress response can become chronic and maladaptive, leading to physiological, endocrinological, and immunological changes throughout these highly integrated systems within the body (Besedovsky and Del Rey 1996; Buckingham 1996).

Ultimately, if relocation is perceived as a stressor to gopher tortoises, and the move from one location to another causes a persistent increase in corticosterone, these relocated animals may suffer hormonal, reproductive, and immunological consequences that would prevent the successful establishment of a viable population within the new habitat. In fact, the potential immunological effects of relocation can be particularly detrimental to gopher tortoise populations because these animals are susceptible to a debilitating bacterial illness, upper respiratory tract disease (URTD). Symptoms include ocular edema, nasal discharge, and respiratory difficulties which can impede basking and foraging activities. As the disease progresses, an infected gopher tortoise can become lethargic and in severe cases, the animal can dehydrate and die.

Upper Respiratory Tract Disease is caused by exposure to the bacterium *Mycoplasma agassizii* (Brown et al. 1999). As a mollicute, *M. agassizii* is one of the

smallest self-replicating prokaryotes on the planet. It has no cell wall so it dessicates rapidly in the environment and can only be transmitted through face to face contact or by sharing water sources. This bacterium uses an attachment organelle to secure itself to the mucosal surface of the ventrolateral depression deep within the host's respiratory tract. Often times, the bacterium exists at a sub-clinical level, presenting no symptoms and avoiding detection by common diagnostic tests.

Little is known about the immune system and general health of reptiles. These animals are the phylogenetic ancestors of both mammals and birds, and as a class, they have changed little morphologically in the millions of years of their existence. With certainty, they have T cells or T-like cells that mature in the thymus and direct cell-mediated immunity (El Ridi 1998), and they have antibody producing cells with surface immunoglobulins IgM and IgY, that represent the humoral immune system (Ambrosius and Hoheisel 1973; Andreas and Ambrosius 1989).

It is well documented that increases in corticosterone as a result of chronic activation of the stress response can result in altered immunocompetence and the increased susceptibility to infectious disease and pathogens that may already be present in the environment (Anderson 1990; Apanius 1998). In fact, when the survival of an animal is threatened, the immune system is downregulated via the regression of the primary lymphoid organs (Apanius 1998). With a full repertoire of immune responses available to them, reptiles also respond to chronic stressors in this way. Therefore, if relocation is a persistent stressor for gopher tortoises, these animals could suffer from suppressed immune function leading to the development and/or progression of URTD, and in severe cases, the demise of infected populations.

Conservation physiology provides us with diagnostic techniques that may elucidate the physiological effects of relocation on gopher tortoises, which will help us to determine if relocation is a successful methodology in the short-term for use with this species. First and foremost, these techniques will allow us to determine if relocation is a stressor as determined by changes in corticosterone levels from pre- to post-relocation. However, if an animal has low corticosterone levels, it does not necessarily indicate a lack of stress. To the contrary, it could indicate adrenal impairment in which the organism has experienced chronic stress and can no longer secrete corticosterone in response to further stressors (Aguilera 1994). In order to ascertain the genuine meaning of baseline corticosterone levels, we can conduct an adrenocorticotropic hormone (ACTH) challenge, the technique most commonly used to measure the adrenal response of the HPA axis in vertebrates. The response to ACTH is an effective biomarker in studies of pesticide use in areas inhabited by fish (Hontela et al. 1992; Girard et al. 1998), birds (Mayne et al. 2004), reptiles (Romero and Wikelski 2002), and amphibians (Hopkins et al. 1999; Glennemeier and Denver 2001). In addition, an ACTH challenge was used as a biomarker in a food deprivation study of Magellanic penguin chicks (Walker et al. 2005b). The results suggested that the HPA response of young birds may be altered if they are hatched and reared in poor quality habitats with little food availability. These studies clearly indicate that low corticosterone levels are not necessarily indicative of low stress levels, but more likely of endocrine dysfunction, and provide support for the use of the ACTH challenge in determining adrenal health.

Techniques in conservation physiology also allow us to examine both reproductive and immune measures. The same blood sample used to measure

corticosterone levels can also be used to measure reproductive hormones to determine if they are in the normal range both pre- and post-relocation. To complete the physiological assessment, we can also conduct several measures of immune function. Multiple measures of immunocompetence are preferred since the immune system is multi-faceted and investment in different immune resources can be unevenly distributed within an individual (Blount et al. 2003; Adamo 2004). To examine immune responses in vertebrate species, at least two standard *in vivo* measures are commonly used: a phytohemagglutinin (PHA) challenge and a sheep red blood cell (SRBC) challenge.

Phytohemagglutinin is a potent T cell mitogen (Geppert 1998), and can be used to determine the responsiveness of the cell-mediated immune system to environmental changes (Smits et al. 1999). For example, northern leopard frogs (*Rana pipiens*) demonstrate a decrease in *in-vitro* T cell proliferation in response to PHA when they are exposed to low temperatures for extended periods of time (Maniero and Carey 1997). Similarly, *Mauremys caspica*, a turtle that hibernates in cold winter months, has shown seasonal variation in *in-vitro* T cell responses to PHA (Muñoz et al. 2000). Furthermore, a study of nestling bluebirds (*Sialia mexicana*) showed that individuals exposed to high levels of lead shot had a significantly lower *in-vivo* T cell response to PHA than birds in low exposure and lead-free treatment groups (Fair and Myers 2002). These studies indicate that the results of a PHA challenge can be used as an important ecological bio-indicator.

Sheep red blood cells are another innocuous antigen used to examine immune response; it stimulates the humoral immune system by activating a B cell antibody response. Many studies using SRBC have focused on providing information to the

poultry industry regarding immunocompetence of chickens (Boa-Amponsem et al. 2000; Yang et al. 2000). However, other studies determined the relationship of the humoral immune response to endocrine measures and life history traits in red-winged blackbirds (Westneat et al. 2003), superb fairy-wrens (Peters 2000), zebra finches (Deerenberg et al. 1997), common voles (Heise and Van Acker 2000), Siberian hamsters (Hadley et al. 2002), and platypuses, echidnas, and rabbits (Wronski et al. 2003). These studies show that the SRBC challenge is a widely used and effective test of humoral immune response to a general antigen.

Collectively, these physiological measures allow us to examine the integrative nature of stress physiology, reproductive endocrinology, and immunology in order to assess the efficacy of field-based conservation techniques and determine how best to protect our threatened and endangered wildlife from succumbing to the global biodiversity crisis. To that end, I conducted a comprehensive physiological case study of gopher tortoise relocation to examine plasma corticosterone levels, adrenal responsiveness, sex steroid levels, general immune parameters, disease status, and overall health. I compared these variables prior to and again 30 days and 10 months following a relocation event to determine if relocation was successful in these physiological terms. I also compared differences in these parameters by season and habitat quality. Ultimately, my goal is to determine if relocation is an effective technique in the conservation of the threatened keystone species, the gopher tortoise, and to provide support for the use of these physiology-based techniques with species whose survival depend on our immediate conservation actions.

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# CHAPTER ONE

# GEOGRAPHIC AND HABITAT DIFFERENCES IN ADRENAL AND IMMUNE RESPONSIVENESS OF GOPHER TORTOISES (*GOPHERUS POLYPHEMUS*)

## Introduction

Environmental stressors cause increases in glucocorticoids in vertebrate species via the highly conserved response of the hypothalamo-pituitary-adrenal (HPA) axis. Specifically, acute changes in glucocorticoids often result from the presence of predators or short-term anthropogenic activities, such as handling and restraint (Lance and Elsey 1999; Bauer et al. 2001). This temporary increase can be beneficial because it increases available glucose, allowing the organism to exhibit physiological adaptation to a situation (Chrousos and Gold 1992; Sapolsky et al. 2000) which may ultimately promote survival (Cote et al. 2006). However, long-term elevated glucocorticoid levels may be harmful to an animal over the course of time. Chronic elevation can lead to changes in reproduction (Chrousos and Gold 1992; Chrousos 1997; Wingfield and Sapolsky 2003), foraging behavior (Carr 2002), and immune response (Chrousos and Gold 1992; Apanius 1998; Dhabhar and McEwen 1999; Maule and VanderKooi 1999).

One of the most common chronic stressors for animals is the degradation or destruction of their habitats. Animals subjected to adverse changes in their environment

may have to cope with limited access to food and mates, fewer options for shelter, and an increased presence of predators and invasive species (Mitchell and Klemens 2000). Over the long term, these environmental stressors have the potential to lead to chronic, long-term increases in glucocorticoids. For example, daily anthropogenic activities, such as vehicular traffic, have been shown to correlate with higher glucocorticoid levels in elk (Millspaugh et al. 2001; Creel et al. 2002). In addition, spotted salamanders (*Ambystoma maculatum*) exhibit higher corticosterone levels in a habitat that was partially converted to a subdivision, compared with salamanders living in a largely intact forest (Homan et al. 2003). Furthermore, one of the reasons for worldwide amphibian decline is that environmental stressors, such as toxicants and ultraviolet exposure resulting from habitat degradation, chronically activate the HPA axis of amphibians, leading to increased levels of glucocorticoids and immunosuppression (Rollins-Smith 2001). These are just a few of the many studies that have indicated that disturbed habitats can have substantial effects on the glucocorticoid levels of their inhabitants.

Both acute and chronic stress have not only been shown to increase glucocorticoid levels, but they alter immune function in animals as well (Guillette et al. 1995; Bauer et al. 2001). To deal with acute stressors, an individual may downregulate the immune system in an attempt to redirect resources for survival. However, chronic stress may cause primary lymphoid organs to regress, which results in improper functioning of the immune response (Apanius 1998). For example, environmental stressors, such as high levels of phenol in the water, alter the immunocompetence of fish (Anderson 1990). In fact, when fish are subjected to daily stressors, they exhibit increased cortisol levels, as well as impaired function and lower numbers of

lymphocytes, which together contribute to long-term detrimental effects (Barton et al. 1987; Barton and Iwama 1991). Few studies of adrenal-immune interactions have been conducted on reptiles. However, in a recent example, tree lizards (*Urosaurus ornatus*) showed that daily stress increases corticosterone levels and decreases the rate of wound healing (French et al. 2006).

Reptiles are sensitive to changes in their environment due to biophysical constraints, including specific requirements for habitat quality and thermal conditions (Dodd and Seigel 1991). However, little is known about the adrenal and immune responsiveness of reptiles, particularly in terms of the relationship to habitat quality. One reptile species that may be affected by habitat degradation and destruction is the gopher tortoise (*Gopherus polyphemus*), a threatened keystone species associated with longleaf pine forests and scrub oak habitat in the southeastern United States. The well-drained sandy soils of this habitat are ideal for construction of residential and commercial buildings. As a result, loss of habitat is the primary threat to the gopher tortoise's survival (Auffenberg and Franz 1982; Diemer 1986; Lohoefener and Lohmeier 1986).

Little is known about the physiological responses of gopher tortoises. Adrenal and immune challenges have not previously been conducted on these animals; therefore, it is not known if standard protocols are appropriate for use with this species or if the physiological responses may vary by geographic location and/or habitat quality. We conducted two experiments to address these issues: 1) a preliminary validation study to determine the dose-response curves for the use of standard adrenal and immune challenges with gopher tortoises, and 2) a study to determine if the physiological

responses to these measures vary geographically or by habitat quality across the range of *Gopherus polyphemus*. All protocols were approved by the Auburn University Institutional Animal Care and Use Committee, PRN numbers 0303-R-2534 and 2003-0468.

## Materials and Methods

### Protocol Validation

The purposes of the validation study were to determine the appropriate dosages of adrenocorticotropic hormone (ACTH), phytohemagglutinin (PHA), and sheep red blood cells (SRBC) to use in gopher tortoises and to establish dose-response curves for tortoises' responses to these adrenal and immune challenges. In April 2002, 12 gopher tortoises, including 6 males and 6 females ranging in weight from 1.795 kg to 4.786 kg, were trapped at Ft. Rucker Army Base in Alabama (see trapping protocol below). The tortoises were transported within 48 hours of capture in 37.85 liter Rubbermaid® bins to a holding facility at Auburn University. At the holding facility, tortoises were maintained in outdoor, earth-bottom, circular pens measuring 2.5 meters in diameter. Pens were constructed of 50.8 cm x 15.24 m aluminum flashing, with approximately 15 cm buried in the ground. Black mesh was used to cover 1/3 of the top of each pen to provide shaded areas. In addition, plastic dog houses were placed in the pens to serve as covers for makeshift burrows. Two to three tortoises that were captured in neighboring burrows were held in each pen. Tortoises were fed daily with a diet of greens (including kale, endive, and romaine lettuce) and occasional fruits, including a variety of berries.

Water was available to the tortoises *ad libitum* using water dishes placed flush in the ground.

Following are the protocols used in the validation study to determine tortoises' responses to the adrenal and immune challenges. The first full day after arriving at the Auburn University holding facility is considered Day 1 of the study. The study was completed on Day 52, at which time tortoises were returned to the field site and placed at their burrow of capture.

*Stress Associated with Capture, Handling, and Captivity*

Eight tortoises were blood sampled for corticosterone throughout the course of the validation study (at 08:00am on Day 0 (initial day of capture), Day 1, Day 3, Day 11, Day 32, and Day 52) to determine if tortoises experienced either trap/handling stress or captivity stress. On Day 0, after being removed from the trap, tortoises had an average of 2.90ng/ml of corticosterone. On Day 1 at the holding facility, tortoises had a mean of 2.05ng/ml. By Day 3 in captivity, tortoises had approximately 1ng/ml of corticosterone, and that level stayed constant for the remaining sampling days of the study. Although there was a slight decrease in corticosterone from Day 0 to Day 3, this decrease was not significant. According to a repeated-measures ANOVA, tortoises showed no significant difference in corticosterone levels among any of the time periods assessed ($F_{5,35}$=2.11, p=0.09). These data indicate that neither trap stress nor captivity stress were factors in this validation study.

*ACTH Challenge*

On Day 3, 0.3ml of blood was taken from each tortoise to be used as a measure of baseline corticosterone in the ACTH Challenge. Tortoises were then randomly placed in low (20µg/ml, n=4), high (100µg/ml, n=5), or control (saline only, n=3) dose ACTH (Sigma-Aldrich, adrenocorticotropic hormone from porcine pituitary) groups. They were injected with a total of 0.1ml ACTH or saline solution using an intraperitoneal (IP) method. The range of doses was extrapolated from doses given to the toad *Bufo terrestris* (Hopkins et al. 1999). A 0.3ml blood sample was then taken from each tortoise at the following time intervals post-injection: 15 minutes, and 1, 2, 4, 8, and 24 hours. Blood was drawn, centrifuged, and frozen as described above. Each sample was ether-extracted and quantified for corticosterone (ng/ml) using a tritiated steroid radioimmunoassays (Mendonça et al. 1996; Ott et al. 2000).

Two corticosterone assays were conducted with a intra-assay variabilities of 6.6% and 9.72%, and an inter-assay variability of 15.25%. Baseline corticosterone levels ranged from 0.4ng/ml to 3.45ng/ml, with a mean of 1.42ng/ml. These levels were well within the range for gopher tortoises indicated by Ott et al. (2000). A repeated measures ANOVA indicated that the tortoises' adrenal responses to the ACTH injection varied by dose ($F_{2,18}$=3.95, p=0.004) (Figure 1a). Specifically, there was a significant difference between the mean corticosterone response of tortoises that received a high dose of ACTH (100µg) and those that received saline (p=0.03), while there was no difference in response between tortoises that received the low dose and saline (p=0.17). Overall, corticosterone began increasing at 1 hour post-injection and continued to increase up to 4 hours post-injection. By 8 hours post-injection, corticosterone

responses decreased to near baseline levels and returned to baseline by 12 hours post-injection. The greatest difference in corticosterone response between low and high dose tortoises occurred at 4 hours post-injection: therefore, the high dose of ACTH was selected as the standard dosage for gopher tortoises undergoing an adrenal challenge with ACTH, with a maximum response time of 4 hours.

*PHA Challenge*

On Day 8, tortoises were randomly assigned to low (1.6mg/ml, n=5), medium (2mg/ml, n=5), and high (3mg/ml, n=2) dose PHA (Sigma-Aldrich PHA-P, lectin from *Phaseolus vulgaris*) groups. The range of doses was extrapolated from doses given to birds (Smits et al. 1999; Smits and Williams 1999). A non-toxic permanent marker was used to make a small marking on the ventral surface of the skin flap that connects the medial aspect of the thigh to the abdominal region. A measurement was taken at the marking using a digital micrometer (W.W. Grainger, Mitsuoyo, 0-25mm) to the nearest thousandth of a millimeter. The measurement was taken three times and an average of the three measurements was recorded. Then 0.5ml of the appropriate dose was injected subcutaneously at the marking where the measurement was taken. As a control, each tortoise received the same protocol in the ventral skin flap on the other medial thigh with a saline injection. The swelling resulting from the PHA and saline injections was measured three times each using a digital micrometer and averaged at the following time intervals post-injection: 3, 6, 12, 15, and 28 hours.

A repeated measures ANOVA indicated that there was a significant difference in the swelling responses between the skin flaps that received PHA versus those that

received saline ($F_{5,100}$=28.47, p<0.0001). The changes in response to PHA were also significant over the dosages and time course of measurements taken ($F_{15,100}$=6.31, p<0.0001) (Figure 1b). The high dose tortoises showed the strongest response to PHA at 3 hours post-injection compared with tortoises in the other groups at that same time point (p<0.0001). Although the high dose swelling response at that time was significantly larger than the swelling responses of tortoises in the other groups, the response occurred at only 3 hours post-injection, which is generally too soon for a T cell response to occur (Abbas et al. 2000). At 6, 15, and 28 hours post-injection, there were no significant differences in the responses of tortoises in the three PHA groups. However, at 12 hours, tortoises in the high and medium dose groups showed a significantly stronger response than tortoises in the low group ($F_{3,20}$=13.47, p<0.0001). In addition, the response of the medium dose group was significantly higher at 12 hours than at any other time point for that group ($F_{5,20}$=13.10, p<0.0001), except for the 6 hour measure (p=0.10). Therefore, the medium dose, with a characteristic dose-response curve, was chosen to serve as the standard dosage for the gopher tortoise PHA challenge, with a maximum response time at 12 hours.

*SRBC Challenge*

On Day 8, while receiving the PHA injection, tortoises also received an intraperitoneal injection with one of three randomly selected 5ml doses of unwashed SRBC (Colorado Serum Company, sheep blood defibrinated) mixed with phosphate buffered solution (PBS): low (5% solution, n=4), medium (10% solution, n=4), and high (20% solution, n=4). The range of doses was extrapolated from doses given to chickens (Boa-

Amponsem et al. 2000). A 1ml blood sample was taken post-injection on days 11, 17, 22, 27, and 32 to measure tortoises' antibody responses to SRBC. We conducted a hemagglutination titration assay to determine tortoises' antibody titers to SRBC (Wegmann and Smithies 1966; Hay et al. 2002). Control tortoises were not used in this challenge because a previous preliminary study using both positive and negative controls, as well as blood samples from tortoises that were never exposed to SRBC, indicated that unexposed tortoises do not have an antibody response to this antigen.

A repeated measures ANOVA revealed that there was a statistically significant difference among the antibody responses to SRBC during the time course of measurements ($F_{5,35}$=14.42, p<0.0001), although there was no interaction effect with dose ($F_{10,35}$=1.57, p=0.15) (Figure 1c). According to a Fisher's PLSD test, the antibody response to SRBC was the same from Day 4 to Day 11 (p=0.71), from Day 11 to Day 17 (p=0.07), and from Day 17 to 22 (p=0.46). However, the antibody response had increased significantly from Day 22 to Day 27 (p=0.0008) and continued to be significantly higher on Day 32 (p=0.005). Although dose was not significant, the curve of the medium dose showed a peak at Day 27 and then began to decline, whereas the response to the high dose peaked at Day 27 but remained high, even at 32 days post-injection. Therefore, we chose the medium dose as the standard for the subsequent gopher tortoise SRBC challenge, with a maximum response time of 22-27 days.

*Trapping and Blood Collection*

Wire live traps (Tomahawk Live Trap Company, custom order) were placed at the mouth of active gopher tortoise burrows. Active burrows were identified by the

presence of fresh soil at the mouth of the burrow, a clean burrow entrance free of plants and debris, and tortoise tracks on the burrow apron (Witz et al. 1991; Aresco and Guyer 1999a). The floor and foot pedal trip mechanism of each trap were partially covered with sand from the burrow apron. The entrance to the burrow and the trap were covered with a $1m^2$ piece of burlap. The burlap made the trap appear to be an extension of the burrow to encourage the tortoise to enter, and it provided a shady area to prevent trapped tortoises from overheating. Gopher tortoises do not experience a significant increase in corticosterone when left in a trap for up to 12 hours (Ott et al. 2000), so traps were set no later than 08:00am and they were checked at least twice daily, 12 or fewer hours apart, until tortoises were caught.

Upon capture, the tortoise was removed from the trap and 1ml of blood was immediately sampled from the tortoise's femoral vein using a 1ml heparinized syringe and 25 gauge needle. Blood samples were placed in a cooler for transport. Tortoises were each given an individual identification number written on the carapace in a non-toxic white paint pen. They were then placed in a 10 gallon Rubbermaid® bin for transport. The cover and sides of the bin were punctured with numerous 1.5 cm air holes, and the bottom of the bin was filled with 5 cm of sand from the burrow apron. The tortoises and blood cooler were transported to the field lab after all traps were checked (within 4 hours of the first trap check).

At the field lab, the blood was centrifuged in a tabletop centrifuge, and the plasma was removed with a pipette. Both the plasma and the red blood pellet were frozen in 1ml microcentrifuge tubes at -20C until radioimmunoassays could be conducted (see details below). Tortoises in the validation study were then transported to

the Auburn University holding facility (described below) within 48 hours of capture, while tortoises in the geographic/habitat study were taken to a local, temporary holding facility where they were kept in their sand-lined Rubbermaid® bins in a temperature-controlled room (25.5C) until release the following day.

## Geographic/Habitat Study

### *Study Sites*

The geographic/habitat study was conducted during the early part of the active season, April and May. A total of 87 gopher tortoises were studied from three geographically distant sites across their natural range: Georgia, Alabama, and Mississippi. We visually examined each site by examining key characteristics of quality gopher tortoise habitat, looking most specifically for an open canopy and abundant ground-cover vegetation (Aresco and Guyer 1999b; Aresco and Guyer 1999a). Although habitat characteristics were not directly measured, on consultation with experienced gopher tortoise researchers, we constructed a rating system based on the following variables: predominant habitat at the site, canopy cover, ground vegetation, land management, and anthropogenic activity. The sites were ultimately categorized as high or low quality. Table 1 presents the specific habitat qualities that we reviewed at each site. Briefly, two high quality sites were located at Army Installations: Fort Benning, Georgia (compartment F03) and Camp Shelby, Mississippi. Both were characterized by predominantly longleaf and mixed pine forests that underwent periodic controlled burns. As a result of land management, both also had open canopies and abundant ground-cover vegetation as food sources. In addition, neither was subject to frequent foot or

vehicular traffic. The third high quality site was a privately owned 92 hectare property in Chunchula, Alabama (formerly owned by the International Paper Company [IP]). Tortoises on that land inhabited a variety of longleaf and scrub oak habitats that had both open and closed canopies. All animals had free access to the entire site and food sources were available. However, the site did not undergo any type of land management, and there was occasional anthropogenic disturbance, including hunting and off-road vehicles. These three geographically distant sites were all considered to be of relatively high quality.

In order to determine if differences among the sites were related to population effects or habitat quality, we also sampled a total of 15 gopher tortoises inhabiting low quality habitats in Georgia and Mississippi. Ft. Benning's D06 compartment in Georgia was only 3.25 km away from the F03 high quality site, and was therefore considered part of the same metapopulation. However, the habitat quality at the two sites differed greatly. D06 had a wide open canopy in some locations, but the middle story brush was thick with hardwoods. In addition, the majority of the herbacious vegetation at the site was either nonexistent or inedible. In other areas of the site, there were desert-like sandy hills, and little to no food sources available. Furthermore, there was heavy foot and vehicular traffic, both in the form of wheeled and track vehicles, on a daily basis. The site was used extensively for military practices. In fact, Guyer et al. (1996) used the site as an "impacted" habitat in a study of the long-term effects of track vehicles on gopher tortoises and reported significant findings in terms of behavior and movement.

The second low quality site was located in Saucier, Mississippi, approximately 82 km from the higher quality Camp Shelby, MS site. Unlike the tortoises at Camp

Shelby, the tortoises at this second site lived in nearly uninhabitable forests with dense, nearly impassable hardwood forests, completely closed canopies, and, according to our visual assessments, few or no available food sources.

*Experimental Design*

Using the doses determined in the validation study, we carried out experiments to determine if responses to adrenal and immune challenges vary across the tortoises' geographic range and/or by habitat quality. Several of the gopher tortoises at the study sites were subjects in previous studies that documented home range and movement patterns, or they were control subjects in relocation projects. As such, their carapaces were already affixed with radiotransmitters. Additional tortoises at each site were located in and around active burrows during the study and were also fitted with radiotransmitters (American Wildlife Enterprises, Model AWE-GA). All tortoises were tracked once every 2 to 5 days and their burrow locations were marked with a GPS (Garmin GPS 12 Personal Navigator) and recorded on field data sheets. As explained previously, tortoises at each site were trapped and a 1ml blood sample was drawn to measure baseline circulating corticosterone. The tortoises were then brought to a temporary holding facility where they were administered the adrenal and immune challenges using the dosages, timing, and methods determined in the validation study. The tortoises were held overnight in individual sand-lined 37.85 liter Rubbermaid® bins in a climate controlled environment and returned to their burrow of capture the following morning.

*Data Analyses*

A total of 11 radioimmunoassays were conducted to analyze baseline corticosterone levels and the corticosterone response to the ACTH challenge. These assays had an inter-assay variability of 18.11%. To correct for heterogeneity of variance and normalize the data, corticosterone levels were log transformed and subjected to a two-way analysis of variance (ANOVA) to analyze the effects of site or habitat. Responses to the ACTH and PHA challenges were analyzed using percent change from baseline. These data were analyzed using two-way ANOVAs, also by site or habitat. For the ACTH challenge, percent change was calculated using the following equation:

$$\frac{((4 \text{ hour post - corticosterone level}) - (\text{baseline corticosterone level}))}{\text{baseline corticosterone level}}$$

A constant (1.1) was added to all percents to eliminate negative numbers and then the data were log transformed for normality. Similarly, for the PHA challenge, percent change was calculated using the same equation indicated above for the ACTH challenge, but using the 12 hour post-injection skin flap measure in millimeters and the baseline skin flap measure. Again, a constant (1.1) was added to all percents to eliminate negative numbers, and then the data were log transformed for normality. Finally, we used a one-way ANOVA by site to analyze log-transformed SRBC titers. Additional planned post-hoc comparisons were made using Fischer's PLSD to determine differences between individual sites.

A one-way ANOVA showed significant differences in body condition index among the three primary high quality sites ($F_2$=4.07, p=0.02) so data comparing these

sites were analyzed with ANOVAs using body condition index as a covariate. Body condition index was calculated using the residuals from the following equation:

$$\frac{\text{mass}}{\text{straight carapace length}}$$

We found that body condition index did not vary significantly between the high and low quality GA sites ($F_1$=1.85, p=0.18) or between the high and low quality MS sites ($F_1$=3.04, p=0.08) so we did not use body condition index as a covariate in our analyses of data by habitat quality. Differences were considered significant when $p<0.05$.

**Results**

*Baseline Corticosterone*

Circulating baseline corticosterone levels were significantly different in the three geographic populations of gopher tortoises ($F_{2,72}$=5.30, p=.007, Figure 2). Specifically, tortoises at the MS site exhibited significantly lower baseline corticosterone levels than tortoises at the AL site (p=0.008) and those at the GA site (p=0.006). This effect was not related to sex ($F_{1,72}$=0.10, p=0.74) and body condition index was not a factor ($F_{1,64}$=0.19, p=0.66). In addition, a one-way ANOVA indicated that the low quality site in MS (Saucier) showed significantly higher corticosterone levels than tortoises living in the high quality MS habitat (CS) ($F_{1,32}$=3.95, p=0.05, Figure 3a). Tortoises at the low quality site in GA (D06) did not show significantly higher corticosterone levels than the tortoises living in high quality GA habitat (F03), but their mean baseline corticosterone level of 10ng/ml is at the higher end of what is considered normal for gopher tortoises (Figure 3b). In addition, there were only 5 tortoises living at the low quality GA site and

they showed great variation in their baseline corticosterone levels, with 3 of the 5 tortoises having higher than normal baseline corticosterone levels ranging from 11.75ng/ml to 17.66ng/ml.

*ACTH Challenge*

Tortoises at all five study sites combined showed significantly greater adrenal responses to the ACTH injection versus the saline injection ($F_{1,54}$=9.48, p=0.003). Furthermore, there was a significant difference in the adrenal responses of tortoises specifically to the ACTH injection among the three primary geographic populations ($F_{2,22}$=4.74, p=0.01). This change was not related to body condition index ($F_{1,22}$=0.23, p=0.63). According to percent change in corticosterone from baseline levels, tortoises at the AL site exhibited a significantly weaker corticosterone response compared with tortoises in MS (p=0.009) and GA (p=0.02, Figure 4). However, adrenal responses to the ACTH challenge did not differ significantly between tortoises living in high and low quality habitats in either MS ($F_{1,4}$=1.65, p=0.26) or GA ($F_{1,13}$=1.88, p=0.19) (Figure 5).

*PHA Challenge*

Gopher tortoises among all 5 sites combined showed a significantly greater T cell swelling response to the injections of PHA versus saline injections ($F_{1,71}$=27.38, p<0.0001). There was also a significant difference in the responses among the three primary sites to the PHA injection specifically ($F_{2,40}$=4.80, p=0.01). When we examine the data using percent change in skin flap thickness from pre- to post-injection in order to account for slight differences in baseline skin flap measurements, we find that

tortoises from the GA population showed a significantly weaker T cell response than tortoises from either that AL (p=0.006) or MS (p=0.01) populations (Figure 6). However, there was no difference in the T cell swelling response of tortoises living in high quality versus low quality habitat within either of the two MS ($F_{1,16}$=0.09, p=0.76) or GA ($F_{1,16}$=0.40, p=0.53) populations (Figure 7).

*SRBC Challenge*

There was a significant difference in antibody response among tortoises living in the three geographically distant locations ($F_{2,20}$=6.70, p=0.005). Specifically, gopher tortoises at the GA site showed a significantly weaker antibody response to the SRBC challenge than tortoises at either the MS site (p=0.02) or the AL site (p=0.0003, Figure 8). Body condition index was not a significant covariate ($F_{1,20}$=0.74 p=0.39), and habitat differences were not examined using this immune measure.

**Discussion**

Determining the variation in physiological responses of gopher tortoises across their natural geographic range and in different quality habitats may be a vital step in aiding in their conservation. Establishing appropriate dose-response curves for adrenal and immune function in this species is critical since this study indicates that gopher tortoises do not have the same response times as many other previously studied animals. For example, we found the appropriate ACTH dosage for gopher tortoises weighing less than 5kg was 0.1ml of 100µg/ml, and a blood sample must be drawn at 4 hours post-injection to evaluate a near maximum response. This timing is different than that of other species

that have undergone an ACTH challenge, such as the Southern toad *Bufo terrestris*, which experiences a maximum corticosterone response to ACTH 10 hours post-injection with a dose of only 1µg (Hopkins et al. 1999). Yellow perch (*Perca flavescens*), on the other hand, experience a maximum response to an ACTH challenge at only 2 hours post-injection with a dose of 4 IU/100g of body mass (Girard et al. 1998). Other birds, such as the Smith's longspur (*Calcarius pictus*), exhibit a maximum response to an ACTH challenge within 60 minutes of the injection using a dose of 100 IU/kg (Meddle et al. 2003).

We found similar results using the PHA challenge. In many species, a near maximum T cell swelling response to a PHA challenge occurs in approximately 24 hours, but we found that gopher tortoises reach a peak in response in only 12 hours. A review of the use of PHA in birds showed that 7 studies using birds that ranged in total body mass from 9g to 800g conducted measurements of swelling at 24 hours post-injection (Smits et al. 1999). In addition, although we found that a maximum swelling response at 12 hours resulted from the medium dose of PHA, the high dose caused a significantly greater swelling response only 3 hours post-injection. The swelling response at this time interval was greater than either the low ($p=.036$) or the medium ($p=.004$) doses. However, we chose the medium dose as our standard since it presented a standard response curve in a more realistic time frame. We are currently conducting further studies to examine the cellular versus general edema responses to PHA in gopher tortoises at 12 and 24 hours post-injection.

Gopher tortoises also present a different timeline of antibody response to a SRBC injection than other species. Gopher tortoises in this study mounted a significant

response to SRBC at 22 days, and they maintained the response through Day 32. Therefore, we established a critical time period of antibody response at approximately 20-30 days post-injection. This timeline is different from that previously established for birds. In particular, male white leghorn chickens show a peak in antibody response to SRBC only 5 days post-injection, with levels returning nearly to baseline by approximately Day 14 (Boa-Amponsem et al. 2000). Zebra finches show similar antibody response times, from 5 to 10 days post-injection (Deerenberg et al. 1997). Antibody responses of house finches also fall within this range, with the maximum response occurring at 8 days post-injection (Hawley et al. 2005). Mammals, such as mice and voles, exhibit a near maximum response to SRBC at 5 to 12 days (Heise and Van Acker 2000), still far fewer days than that for gopher tortoises.

We used these newly developed dose-response curves to implement protocols in the field to determine if we can use these measures to conduct physiological assessments of gopher tortoises for conservation purposes. In examining physiological responses, we found that tortoises' baseline levels of corticosterone and their responses to the adrenal and immune challenges varied significantly according to geographic location and habitat quality. For example, tortoises in the MS population had the lowest baseline levels of circulating corticosterone and the strongest adrenal response to the ACTH challenge when compared with tortoises in the AL and GA populations. This is important information because the low baseline corticosterone in the MS tortoises could have been mistaken for adrenal exhaustion without the supporting data of the ACTH challenge which indicates a strong, and presumably healthy, adrenal response.

We found that the differences in baseline corticosterone were not simply geographical, but they varied by habitat quality as well. For example, tortoises in habitat characterized as low quality in MS showed significantly higher baseline levels of circulating corticosterone compared with their nearby counterparts living in high quality habitats. Furthermore, tortoises in the low quality habitat in GA had higher corticosterone levels than what we consider to be within a normal range (Ott et al. 2000). Other studies have also shown that animals in disturbed habitats have high corticosterone levels. For example, in studies conducted in South Carolina, researchers found that toads exposed to polluted habitats containing coal fly ash had significantly higher levels of baseline corticosterone than toads at a nearby reference site (Hopkins et al. 1997; Hopkins et al. 1999). In addition, corticosterone levels were cited as more effective in predicting survival of Galápagos marine iguanas during El Niño events than body condition, with high corticosterone levels correlating with mortality (Romero and Wikelski 2001). These high corticosterone levels were also present in the iguanas during a low-level oil spill in their habitat, during which time high corticosterone levels were again effective in predicting mortality in the newly disturbed habitat (Romero and Wikelski 2002b).

We did not find statistically significant differences in adrenal response to the ACTH challenge by habitat, but we did notice a trend in which tortoises in the low quality habitats in both MS and GA appeared to show a lower corticosterone response to the challenge than tortoises in high quality habitats nearby. With larger sample sizes, we believe these data will show significance, similar to the habitat findings for other species. For example, spotted salamanders (*Ambystoma maculatum*) showed

significantly higher stress-induced corticosterone levels at undisturbed sites than at disturbed sites (Homan et al. 2003). Toads and fish also show similar responses resulting from the administration of ACTH; those living in polluted habitats showed little or no increase in corticosterone after an ACTH injection (Girard et al. 1998; Hopkins et al. 1999). These responses could be the result of adrenocortical modulation in which organisms have the ability to alter their adrenal response according to environmental factors, such as habitat quality (Moore and Jessop 2003).

Similar to our findings with the adrenal response, our data indicate that there are significant differences in both cell-mediated and humoral immune responses of tortoises in the three geographical locations of our study sites. The tortoises from the high quality GA population showed a significantly weaker T cell swelling response and a significantly weaker antibody response than tortoises in the high quality AL and MS populations. These variations in corticosterone levels and adrenal and immune responses may be related to slight variations in genetic composition of tortoises among the three sites, and to variations in the pathogens to which the tortoises are exposed at each site.

Unlike the adrenocortical response, we found no indication that there was any difference in the cell-mediated immune response according to habitat quality. We predicted that the tortoises at the low quality GA site, that had higher than normal baseline corticosterone levels, would have shown an impaired T cell response since other studies have demonstrated that high corticosterone levels suppress immune activity. For example, Galápagos marine iguanas with naturally high corticosterone levels have been shown to exhibit significantly lower immune responses to PHA compared with iguanas

with naturally low corticosterone levels (Berger et al. 2005). However, this was not the case with the gopher tortoises. The T cell responses of tortoises with high corticosterone levels that inhabit low quality sites were similar to those of tortoises with low corticosterone levels living in nearby high quality habitats.

It is interesting to note that the tortoises in the GA population showed a significantly weaker T cell swelling response and a significantly weaker antibody response than tortoises in the AL and MS populations. Perhaps these GA tortoises, living in what was rated the highest quality habitat in this study, were in better condition than the tortoises at the other sites so they did not need to mount a substantial immunological response to our general immune challenges in order to remain healthy. In addition, the tortoises at the AL site, living in the lowest quality of the three primary study sites, showed a stronger T cell immune response than either of the other sites, perhaps because their immune defenses were already primed. A study of loggerhead sea turtles suggests that there is a trade-off among the different immune responses. Specifically, sea turtles that are chronically exposed to toxicants, like organochlorine contaminants, show enhanced lymphocyte function, but suppressed innate immune function (Keller et al. 2006). Perhaps we would see similar results in gopher tortoises if future studies incorporate measures of innate immunity as well as cell-mediated and humoral immune responses.

In addition, although all of the tortoises in this study appeared to be healthy, they were not tested to determine if they were infected with any disease-causing agents, including *Mycoplasma agassizii*, the bacterium that causes URTD. Therefore, the tortoises at each site may have been exposed to and/or infected with various pathogens

that could be site-specific, and could alter their immune responses to our challenges. Such infections also have the potential to be associated with corticosterone levels, as indicated by a study of house finches (*Carpodacus mexicanus*) which showed that symptomatic *Mycoplasma* infection was associated with high baseline and stress-induced corticosterone levels (Lindstrom et al. 2005).

It is important to note that the sample sizes at the low quality sites were small: n=5 at D06 in GA, and n=10 in Saucier, MS. This is not surprising since tortoises often leave low quality habitats in search of areas with open canopy and abundant ground vegetation. Studies of gopher tortoise burrow abandonment have shown that permanent abandonment of burrows most frequently occurs as a result of canopy closure due to successional changes in the overstory (Aresco and Guyer 1999a). Other studies have shown that tortoises avoid creating new burrows in such poor habitat (Boglioli et al. 2000). Despite the low sample sizes, some of the differences we observed with relation to low quality habitats were significant or approaching significance, suggesting that habitat may play a role in physiological responses to adrenal and immune challenges.

The habitats that we used in this study not only varied by quality in terms of canopy cover and ground vegetation, but they also varied by type and frequency of anthropogenic activities. Human disturbance has been cited as a crucial factor affecting the successful existence of many wildlife species. For example, studies have found that human activities significantly affect breeding success in bearded vultures by decreasing their nest attendance (Arroyo and Razin 2006). In addition, glucocorticoid levels in elk and wolves at three national parks correlated with snowmobile usage and it has been suggested that these levels may result in direct fitness costs in the long-term (Creel et al.

2002). Similarly, Galápagos marine iguanas that live in areas heavily exposed to tourism show lower adrenal responses to capture and restraint than their undisturbed counterparts, indicating that they are physiologically affected by tourism, although the long-term effects are not yet known (Romero and Wikelski 2002a).

These studies lead us to believe that anthropogenic activities also play a significant role in the physiological responses of gopher tortoises. The high quality site in GA, which showed a typical adrenal response and low immune responses, is subject to occasional foot traffic from military activity and little to no daily vehicular traffic. The other higher quality sites experience more extensive anthropogenic activities and showed variation in their responses to our challenges. Therefore, the extent and type of anthropogenic activities that are present may be equally or more critical than actual habitat quality in determining why tortoises exhibit varying levels of adrenal and immune responsiveness.

We have determined that ACTH, PHA and SRBC challenges are useful tools for measuring adrenal and immune responsiveness in gopher tortoises. In addition, we found that there are geographic differences in tortoises' physiological responses to these challenges that also may be related to population effects. Furthermore, we documented habitat effects, specifically demonstrating differences between two groups of tortoises from a single metapopulation at Ft. Benning, GA that represented both our highest and lowest habitat quality categories. Therefore, if physiological responses are related to habitat quality, but also can be population-specific, as we suspect, then identifying the types of habitats across the geographic range that negatively affect these responses may

improve our ability to implement more appropriate conservation efforts and management practices to ensure the successful continuation of this keystone species.

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| State | Site | Predominant Habitat | Canopy Cover | Ground Vegetation | Land Management | Anthropogenic Activity | Overall Quality |
|---|---|---|---|---|---|---|---|
| GA | **F03, Fort Benning** | Long leaf and mixed pine/ turkey oak | Open | Abundant | 3 year burn cycle | Minimal foot and vehicular traffic | High |
| | **D06, Fort Benning** | Sandy desert/ hardwoods/ scrub oak | None | Insufficient | 3 year burn cycle (rain during last burn in 2004, insufficient) | Extensive daily foot and vehicular traffic (track and wheeled), common gunfire | Low |
| MS | **Camp Shelby** | Longleaf and loblolly pine/ young mix of oaks/ hardwoods | Partly Open | Abundant | USFS - 10 year burn cycle, timber/wildlife management | Minimal foot and vehicular traffic | High |
| | **Saucier** | Hardwoods/ mixed pine/ scrub oak | Closed | Insufficient | None | Frequent hunting and/or recreation | Low |
| AL | **Chunchula** | Planted slash and natural longleaf pine/ hardwoods/ mixed oaks | Varies | Ample | None | Occasional hunting and/or recreation | High |

Table 1. Assessment of habitat quality. The tortoises used in this study were from varying quality habitats. Each habitat was assessed as high or low quality according to the presence of key qualities essential for supporting gopher tortoise populations, including an open canopy and abundant ground level vegetation as food sources. Anthropogenic activities and land management, which are important to overall habitat and ecosystem health, were also considered in classifying each habitat. The three primary sites used in this study were all high quality habitats. The two low quality sites were compared with their high quality counterparts to determine differences in tortoises' physiological responses by habitat quality.

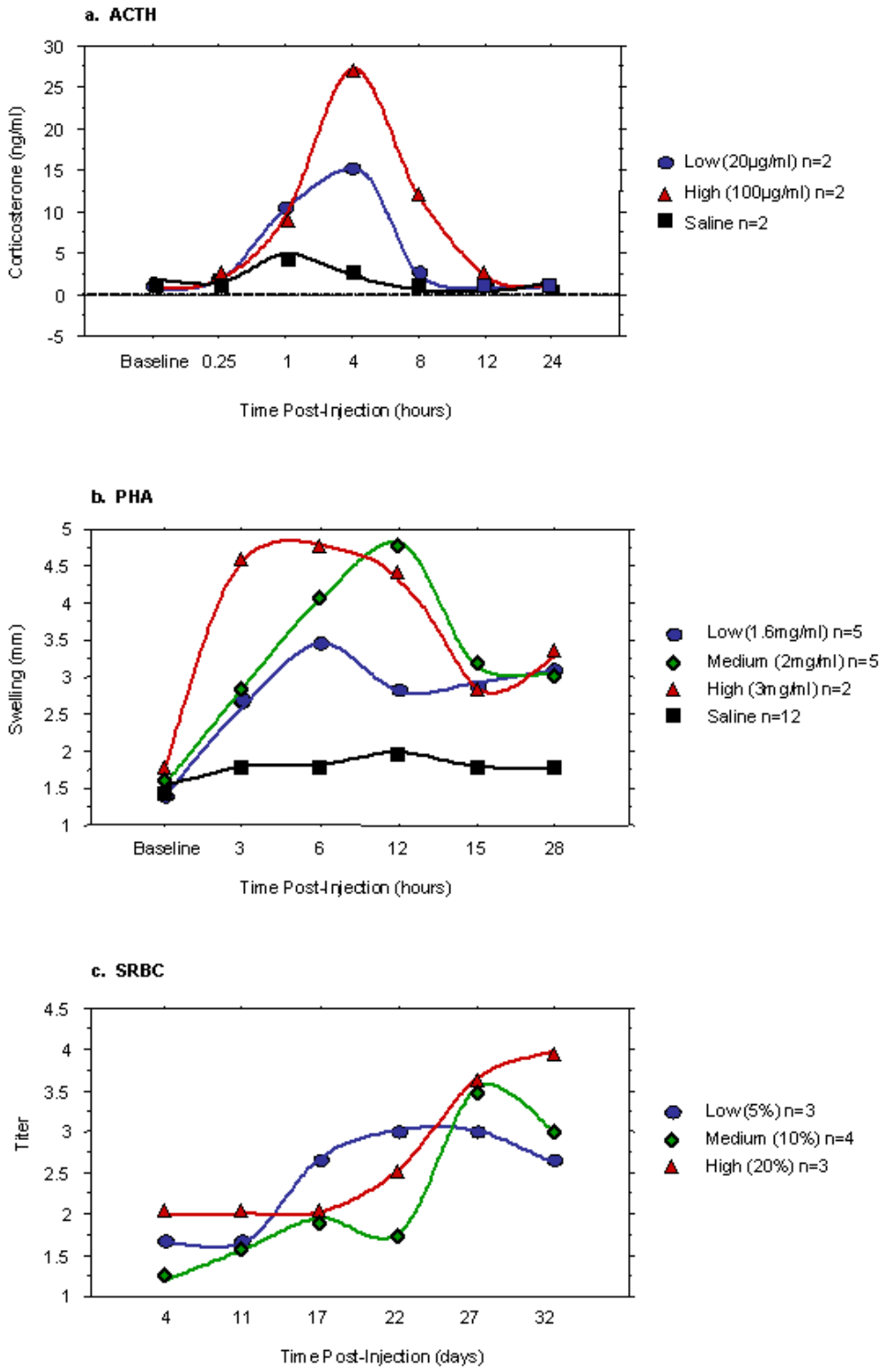


Figure 1. Validation study: Dose response curves for challenges with (a) ACTH, (b) PHA, and (c) SRBC. (a) Tortoises were injected intraperitoneally with 0.1ml of either

low (20µg/ml) or high (100µg/ml) doses of ACTH or a standard dose of saline and their responses were examined by measuring circulating plasma corticosterone. There was a significant dose x time interaction ($F_{2,18}$=3.95, p=0.004), with a peak in response at 4 hours post-injection. At this time point, the high dose of ACTH gave a significantly greater corticosterone response than either the low or the saline dose (p=0.006). (b) Tortoises were injected subcutaneously with 0.5ml of either low (1.6mg/ml), medium (2mg/ml), or high (3mg/ml) doses of PHA or a standard dose of saline and their responses were examined by measuring the swelling at the site of injection which is the result of the T cell response. There was a significant dose x time interaction of the responses ($F_{5,100}$=28.47, p<.0001) with a peak in swelling at 12 hours post-injection using the medium dose. Note that similar peaks were reached by the high dose at 3 and 6 hours, but these peaks did not coincide with appropriate timing for a T cell response. (c) Tortoises were injected intraperitoneally with 5ml of either low (5% unwashed solution), medium (10% unwashed solution), or high (20% unwashed solution) doses of SRBC and the antibody titer was subsequently measured using a hemagglutination titration assay on a blood sample. There was no significant dose x time interaction ($F_{10,35}$=1.57, p=0.15), but all groups reached a peak in response within approximately 20-30 days post-injection.

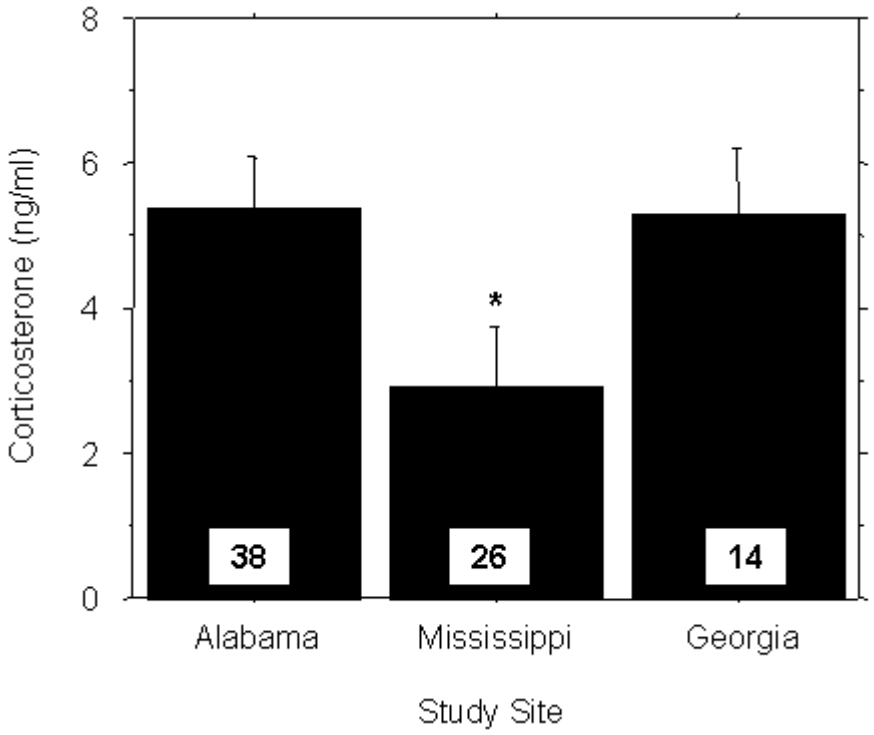


Figure 2. Baseline circulating plasma corticosterone by geographic location. Corticosterone levels are presented in ng/ml, but analyses were conducted on data that were log transformed to prevent heterogeneity of variance. There was a significant difference in corticosterone levels among tortoises in the three high quality geographically distant populations ($F_{2,72}$=5.30, p=0.07). Tortoises in the Mississippi population had significantly lower baseline levels of corticosterone than tortoises in either the Alabama (p=0.008) or Georgia (p=0.006) populations. Note that all levels were within a normal range of 1 to 10 ng/ml.

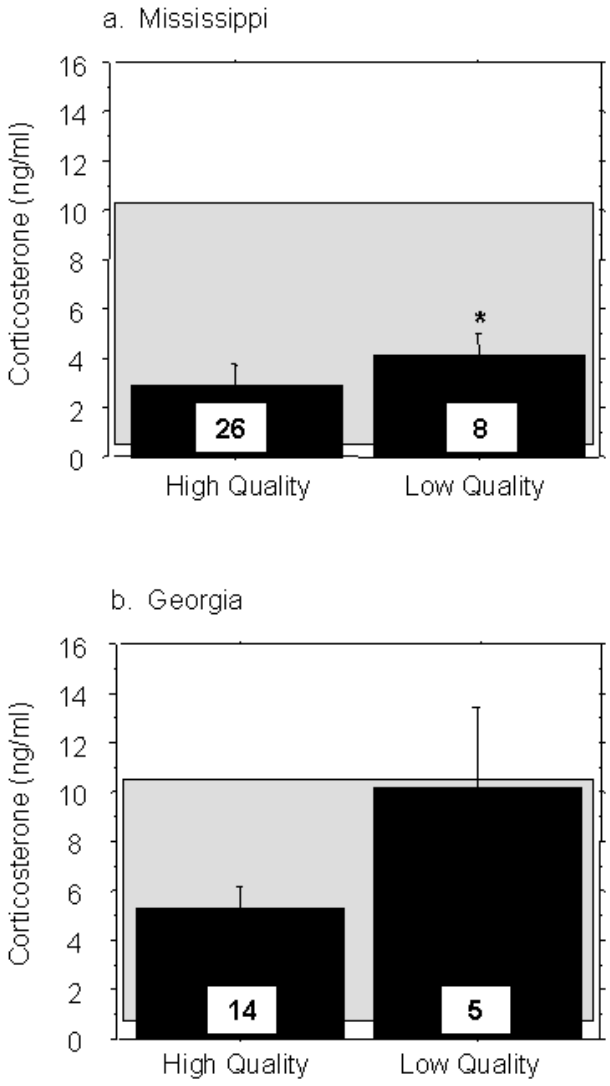


Figure 3. Baseline circulating plasma corticosterone by habitat quality. Corticosterone levels are presented in ng/ml, but analyses were conducted on data that were log transformed to prevent heterogeneity of variance. (a) Tortoises at the low quality site in Mississippi showed significantly higher baseline levels of corticosterone than tortoises at the higher quality sites ($F_{1,32}$=3.95, p=0.05). (b) The corticosterone levels of tortoises in the Mississippi populations were within a normal range of 1 to 10 ng/ml indicated by the gray box, but the corticosterone levels of those in the Georgia population living in low habitat quality exceeded these normal levels.

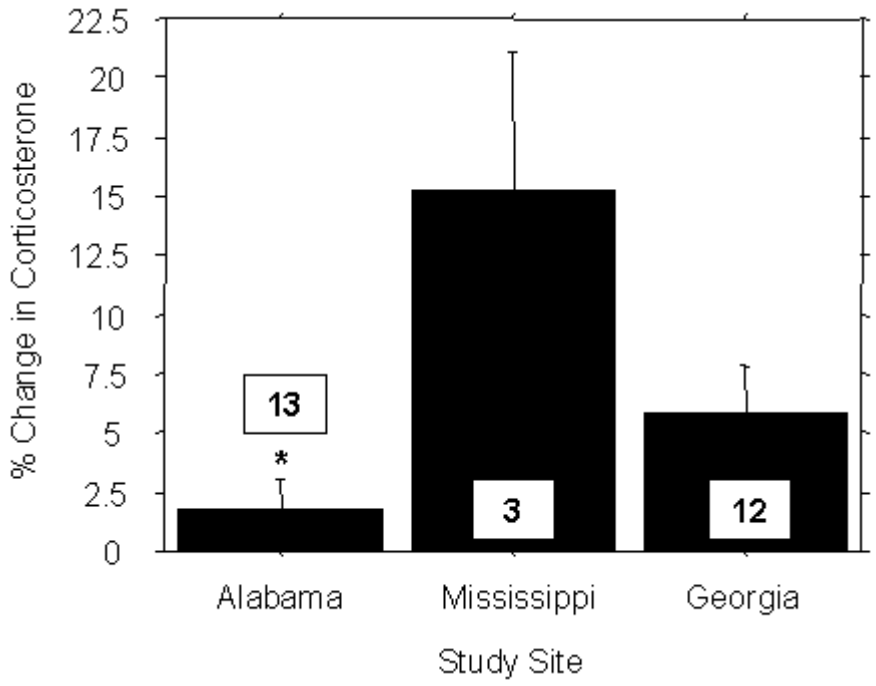


Figure 4. Corticosterone responses to ACTH challenge by geographic location. There was a significant difference in the corticosterone responses to the ACTH challenge among tortoises in the three high quality geographically distant populations ($F_{2,22}$=4.74, p=0.01). Tortoises in the Alabama population had a significantly weaker corticosterone response to the ACTH challenge than tortoises at the Mississippi (p=0.009) and Georgia sites (p=0.02).

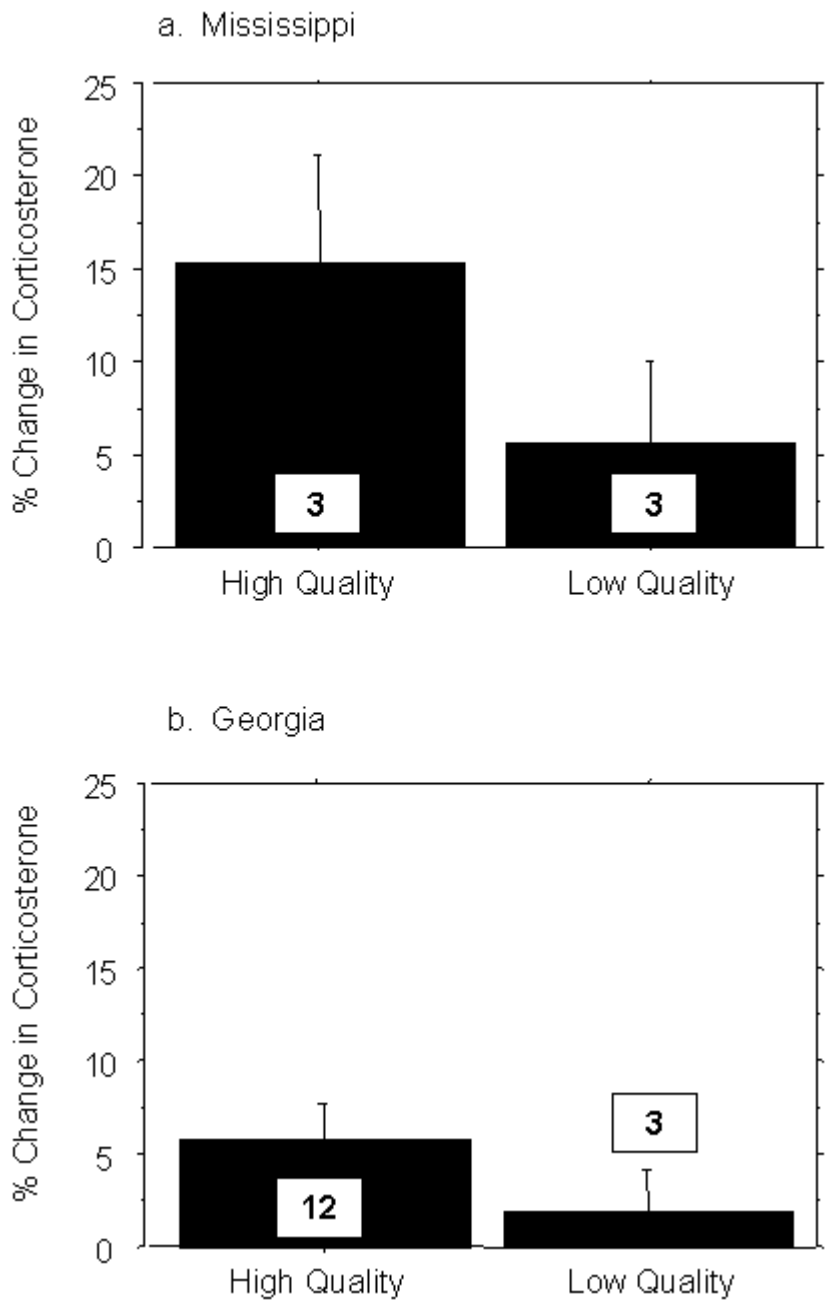


Figure 5. Corticosterone responses to ACTH challenge by habitat quality. There was no significant difference in corticosterone response to the ACTH challenge between tortoises living in high quality versus low quality habitats in either (a) Mississippi ($F_{1,4}$=1.65, p=0.26) or (b) Georgia ($F_{1,13}$=1.88, p=0.19).

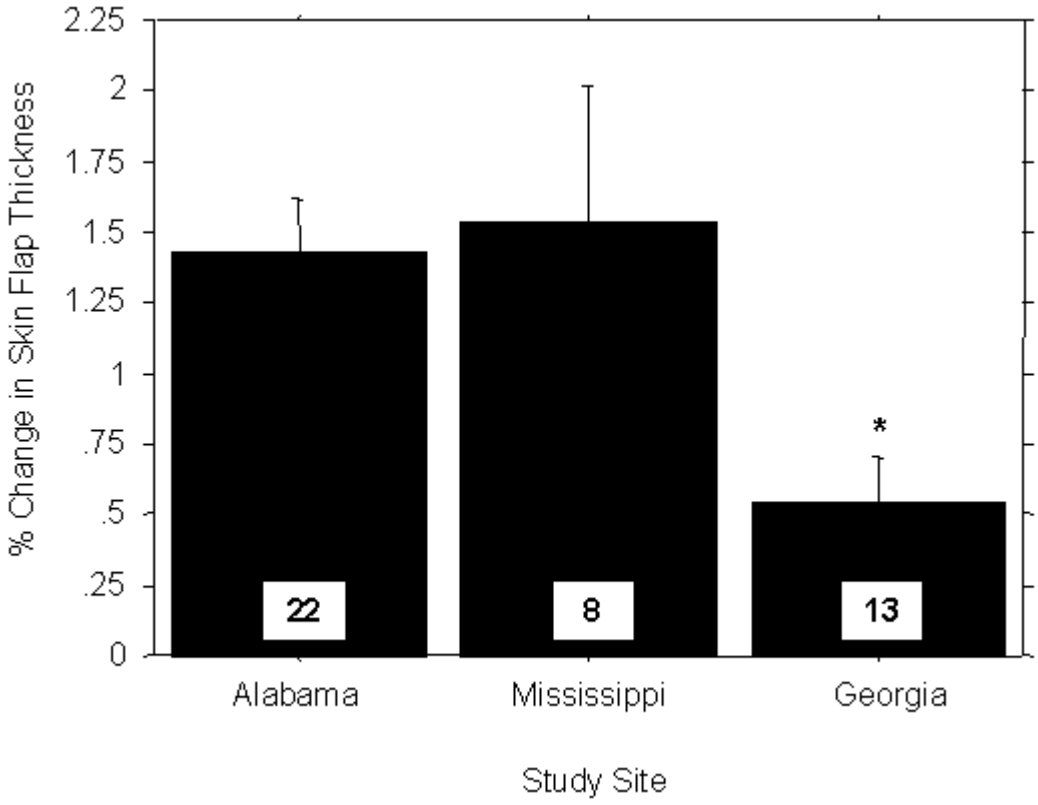


Figure 6. Swelling responses to PHA challenge by geographic location. There was a significant difference in the swelling responses to the PHA challenge among tortoises in the three high quality geographically distant populations ($F_{2,40}$=4.80, p=0.01). Tortoises at the Georgia site had significantly weaker T cell responses to the PHA challenge as indicated by significantly less swelling at the site of injection compared to the Alabama (p=0.006) and Mississippi (p=0.01) sites.

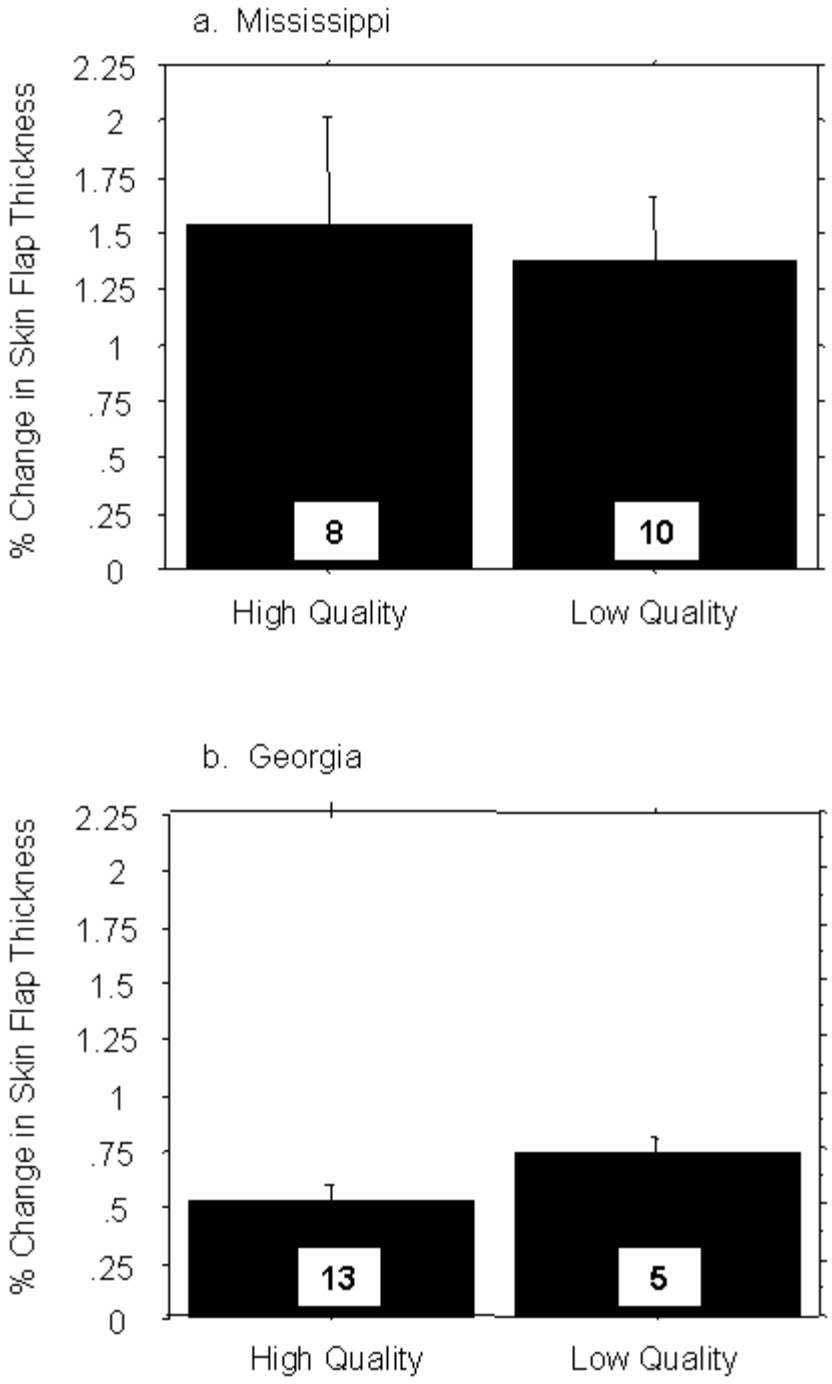


Figure 7. Swelling responses to PHA challenge by habitat quality. There was no significant difference in T cell-related swelling response to the PHA challenge between tortoises living in high quality versus low quality habitats in either (a) Mississippi ($F_{1,16}$=0.09, p=0.76) or (b) Georgia ($F_{1,16}$=0.40, p=0.53).

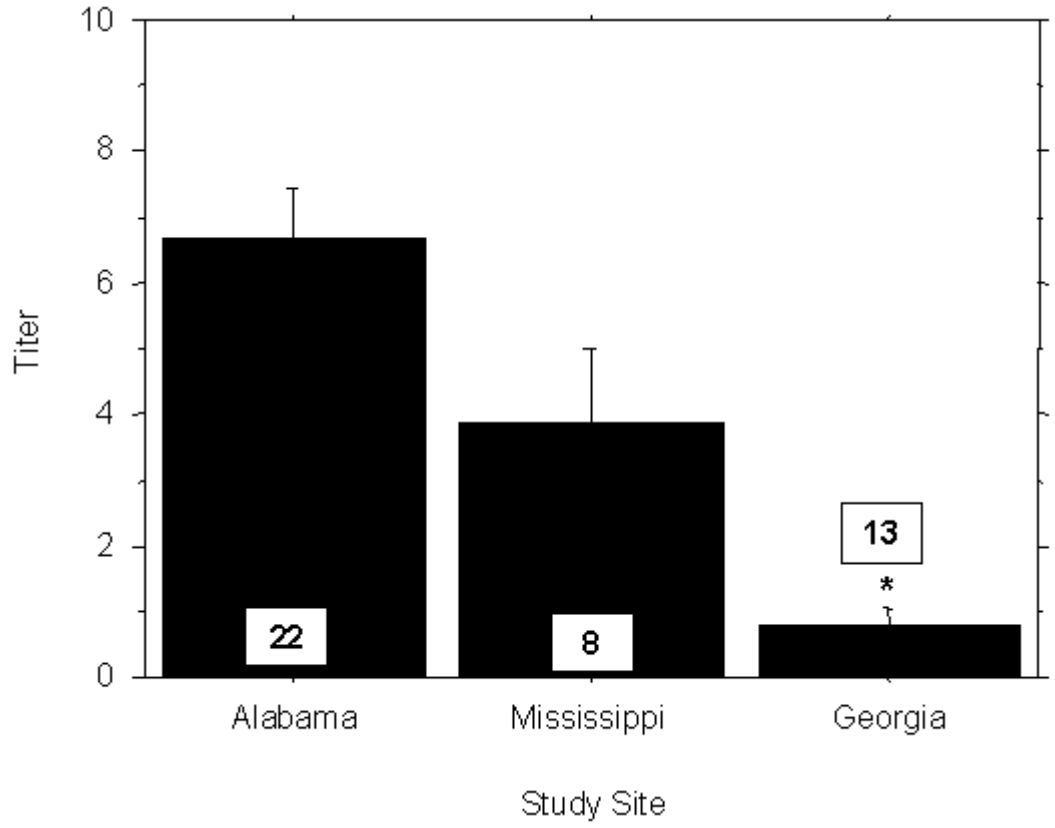


Figure 8. Antibody responses to SRBC challenge by geographic location. There was a significant difference in the antibody titers in response to the SRBC challenge among tortoises at the three high quality geographically distant locations ($F_{2,20}$=6.70, p=0.005). Tortoises in the Georgia population showed a significantly weaker antibody response to the SRBC challenge than tortoises in either Alabama (p=0.0003) or Mississippi (p=0.02).

# CHAPTER TWO

# HANDLING, BLOOD SAMPLING, AND TEMPORARY CAPTIVITY DO NOT AFFECT PLASMA CORTICOSTERONE OR MOVEMENT PATTERNS OF GOPHER TORTOISES (*GOPHERUS POLYPHEMUS*)

## INTRODUCTION

In order to monitor populations of animal species, researchers often capture and physically handle individuals to obtain basic morphometric or physiological data. Handling, though considered fairly innocuous and non-invasive, can initiate a highly conserved stress response in a wide variety of species. For example, in birds it is well established that increased secretion of corticosterone, a glucocorticoid, occurs within minutes of capture and handling (Astheimer et al., 1994; Schwabl, 1995; Romero and Reed, 2005). Other animals that show a similar increase in glucocorticoids as a result of capture and handling include mammals (Widmaier and Kunz, 1993; Morton et al., 1995; Suleman et al., 2004), fish (Fagerlund, 1967; Sumpter et al., 1986), frogs (Coddington and Cree, 1995), turtles (Gregory et al., 1996; Jessop et al., 2004), lepidosaurs (Kreger and Mench, 1993; Tyrrell and Cree, 1998; Jones and Bell, 2004), and alligators (Lance and Elsey, 1999).

In order to avoid the potential effects induced by handling, and to attempt to establish true baseline levels of glucocorticoids, some researchers measure these

hormones in ways that eliminate the acts of capturing and handling. One of the most commonly used non-invasive techniques involves the monitoring of fecal metabolites of glucocorticoids. This technique has been used in studies of a wide range of larger species that are difficult to capture and potentially dangerous to handle (Terio et al., 2004; del Castillo et al., 2005; Rolland et al., 2005). However, monitoring of fecal metabolites is confounded by a multitude of other factors (Millspaugh et al., 2001; Millspaugh and Washburn, 2004). Therefore, comprehensive physiological conservation research, which involves collecting data on stress responsiveness, reproduction, health status, and immunocompetence must rely on the use of blood sampling, innocuous injections, and temporary confinement.

The acute increase in glucocorticoids resulting from capture and handling is generally transient, so in many cases, after animals are returned to their habitats, it is generally assumed that they behave in a fashion that is similar to un-manipulated individuals. As a result, little or no follow-up on behavioral patterns is conducted. However, increases in glucocorticoids have been shown to alter sex steroids and may impact behaviors such as movement patterns and reproductive activities (Greenberg and Wingfield, 1987; Rivier and Rivest, 1991; Pottinger, 1999). Therefore, follow-up is necessary to determine how an animal's subsequent behavior is affected by capture, handling, and manipulation activities.

The impact of mildly invasive research techniques on corticosterone levels and key behaviors is debated by researchers who study Gopher Tortoises (*Gopherus polyphemus*). The Gopher Tortoise is a threatened species that experiences these activities as part of the typical techniques used for conservation actions, such as

relocation. At an extreme, these activities might include trapping, handling, blood sampling, injections with innocuous substances, nasal lavages, and temporary captivity. Unlike many species, trapping of Gopher Tortoises does not cause significant increases in circulating corticosterone levels when they are left in a trap for up to 12 hours (Ott et al., 2000). However, studies have not yet been conducted to determine if more invasive activities than simply trapping the animals may alter corticosterone levels or key behavioral patterns, such as burrow abandonment rates and home range size. Conducting protocols that result in altered movement patterns could be detrimental in the conservation of this species whose habitats and populations have been drastically reduced in size over the last several decades (Auffenberg and Franz, 1982). In this investigation, we examine whether protocols requiring trapping, handling, blood sampling, injections with innocuous substances, nasal lavages, and temporary captivity affect Gopher Tortoises as indicated by changes in their plasma corticosterone levels, movement patterns, burrow usage, or home ranges.

## MATERIALS AND METHODS

*Study Sites*.- Data were collected from adult Gopher Tortoises at the Wade Tract (WT), an 81.5 hectare ecological reserve located on privately-owned land in southwest Georgia (Thomas County) and managed by the Tall Timbers Research Station. The habitat is dominated by nearly pristine, widely spaced, old-growth longleaf pine that provide an open canopy conducive to lush growth of understory plants and the presence of abundant ground level food sources (Johnson, 2004). These qualities, along with well-drained sandy soils and periodic controlled burns to maintain the habitat, create an ideal

environment for Gopher Tortoises (Aresco and Guyer, 1999a; Aresco and Guyer, 1999b).

*Experimental Design*.- A total of 18 randomly-selected adult, reproductively mature Gopher Tortoises at the WT were examined in this study. There were ten males and eight females ranging in weight from 4.0 kg to over 6.0 kg (exceeding the weight allowed by the digital scale). Six of the 18 tortoises served as controls (five males and one female); they were tracked (described below), but they were not trapped and they did not undergo any type of handling, manipulations, or captivity. From June 15 to June 20, 2002, the remaining 12 tortoises (five males and seven females) were trapped using wire live traps (Tomahawk Live Trap Company, custom order) placed at the mouth of their burrows. The floor and foot pedal of the trip mechanism of each trap were partially covered with sand from the burrow apron. The entrance to the burrow and the trap were covered with a 1 $m^2$ piece of burlap that provided shade and prevented trapped tortoises from overheating. The traps were set no later than 08:00 and they were checked twice daily until tortoises were caught. Gopher Tortoises do not show an increase in corticosterone when left in a trap for up to 12 hours (Ott et al., 2000), so no tortoise was left in a trap for longer than 12 hours.

*Handling and Temporary Captivity*.- Upon capture, the tortoise was removed from the trap and a 1 ml blood sample was immediately drawn from the femoral vein using a 1 ml heparinized syringe and 25 gauge needle. Each tortoise was then placed in an individual 37.85 liter Rubbermaid® bin for transport. The cover and sides of the bin were

punctured with numerous 1.5cm air holes, and the bottom of the bin was filled with 5 cm of sand from the burrow apron. The tortoises were transported to the field lab at Tall Timbers Research Station after all traps were checked (within four hours of the first trap check).

After transport, morphological measurements were taken and each tortoise was subjected to simulated adrenal and actual immune challenges following protocols in Kahn et al. (unpublished data). First, tortoises were given a 0.1 ml intraperitoneal (IP) injection of saline. At that time, we also gave them a 0.5 ml subcutaneous injection of 2 mg/ml phytohemagglutinin (PHA) in the ventral webbing of their right medial thigh and a corresponding saline injection in the left thigh. Prior to and 12 hours following the injections, the swelling in each leg was measured to the nearest .001 mm using a digital micrometer at the site of injection. Following the saline and PHA injections, we administered a 5 ml injection of 10% unwashed sheep red blood cells (SRBC). Finally, in order to determine if tortoises were infected with *Mycoplasma agassizii*, the bacterium that causes upper respiratory tract disease (Brown et al., 1999), we flushed 5 ml of 0.9% saline into each naris of the tortoise using a 10 ml syringe with no needle attached. This procedure required that we hold the tortoise's head out of the shell and manually stabilize it behind the occipital bone. At the completion of the manipulations (approximately eight hours after the baseline blood sample was taken), another blood sample was drawn to measure the post-manipulation corticosterone levels. After the 12 hour PHA-induced swelling was measured in the medial thigh, tortoises were returned to their burrows of capture.

As a follow-up, four weeks after undergoing the manipulations protocol, experimental tortoises were trapped again and a blood sample was taken to measure corticosterone. Tortoises were immediately placed back in their burrows of capture. At the completion of the study, all three blood samples from each tortoise were ether-extracted and quantified for corticosterone (ng/ml) using a tritiated steroid radioimmunoassay (Mendonça et al., 1996; Ott et al., 2000) with an intra-assay variability of 10.13%.

*Movement*.- All 18 experimental and control Gopher Tortoises in this study had been tracked in previous years using radiotelemetry so they already had radiotransmitters attached. We conducted radiotelemetry from May 19 through July 14, 2002, which encompasses the timeframe of four weeks before and four weeks following an individual's trap date, or the June 16 control date for control tortoises. Burrow locations were recorded every two to five days with a GPS and recorded on field data sheets. For each tortoise, we calculated total moves made, mean distance per move, number of burrows used, and home range. In addition, we counted the number of days that passed before tortoises made their first, second, and third moves to other burrows after the control date (control tortoises) or after undergoing manipulations and being placed back in their burrow of capture (experimental tortoises).

*Data Analyses*.- We collected a minimum of four radiotelemetry readings on individual tortoises for four weeks prior to and again four weeks following the trap date for experimental tortoises, or June 16, 2002 for control tortoises. We conducted repeated

measures analyses of variance (ANOVAs) to compare pre- and post-manipulation plasma corticosterone levels (experimental tortoises only), movements (total number of moves made from one burrow to another), burrow usage (total number of different burrows used), and home range (95% minimum convex polygon calculated in $m^2$ using CALHOME; Kie et al., 1996). We also conducted a repeated measures ANOVA to analyze the number of days that had passed (only post-manipulation or control date) before the tortoises left their current burrows and moved to the second, third, and fourth burrows. In addition, we compared movement patterns, burrow usage, and home range for tortoises that underwent physiological manipulations versus those that did not, using one-way and repeated measures ANOVAs. All statistical analyses were conducted with StatView for Windows, Version 5.0.1 (SAS Institute Inc.).

## RESULTS

Blood samples were collected successfully from 11 of the 12 experimentally manipulated Gopher Tortoises. There was no significant difference in tortoises' corticosterone levels from pre- to post-manipulation ($F_{1,10}$=0.60, p=0.45, Fig. 9). Immediately after tortoises were removed from the traps, they had a mean of 7.10ng/ml of corticosterone. Approximately eight hours later, after the tortoises experienced handling, manipulations, and temporary captivity, they had a mean of 4.90ng/ml of corticosterone. As a follow-up, ten of the 12 tortoises were trapped and blood sampled again four weeks after they underwent manipulations. There was no significant difference in their corticosterone levels between the baseline measure taken prior to the

manipulations (mean ± SE, 7.10ng/ml ± 1.80) and at the 30 day measure (8.23ng/ml ± 1.50, $F_{1,10}$=0.30, p=0.59).

Overall, the movement measures did not show any significant differences from pre- to post- manipulation (or control date) or between experimental and control groups. Specifically, in the group that underwent manipulations, there was no significant difference in the mean distance that tortoises moved before and after the manipulations were conducted ($F_{1,9}$=0.60, p=0.45). In addition, the control group showed similar patterns (i.e., no significant difference in mean distance moved) before and after the control date ($F_{1,5}$=0.08, p=0.78). There was also no significant difference between the experimental and control groups in the number of days that passed between the first, second, and third moves to other burrows after the manipulation or control date ($F_{1,10}$=0.12, p=0.73), or in the actual number of days that passed between making those moves ($F_{2,10}$=0.81, p=0.45).

The total number of burrows that tortoises used before and after the protocol administration or control date was not significantly different ($F_{1,14}$=2.75, p=0.12), regardless of treatment group ($F_{1,14}$=0.11, p=0.74). Furthermore, tortoises did not show a significant change in home range size from pre- to post-manipulation or control date ($F_{1,14}$=0.73, p=0.41), regardless of treatment ($F_{1,14}$=0.20, p=0.65).

There was no significant difference between the experimental and control groups in the number of movements tortoises made during the 4 weeks before and 4 weeks after the protocol was implemented or control date ($F_{1,14}$=0.03, p=0.86). However, there was an interaction effect for treatment x time ($F_{1,14}$=5.30, p=0.03, Fig. 10). When analyzed separately by treatment, we found that experimental tortoises that were subjected to the

protocol moved significantly more frequently during the four weeks following administration of the protocol as compared to the four weeks prior to implementation of the protocol (post versus pre, mean number of moves ± SE: 4.4 ± 0.65 versus 2.6 ± 0.56, $F_1$=5.87, p=0.04). Control tortoises showed no change from pre- to post-control date in the number of moves they made ($F_{1,9}$=1.07, p=0.35).

## DISCUSSION

Conducting physiological examinations of threatened and endangered species through procedures that are considered by some to be invasive has been shown to increase corticosterone levels in many species and is often thought to potentially disrupt an animal's normal behavior patterns. However, we found that Gopher Tortoises do not experience a change in corticosterone levels and they do not change their behavior in response to trapping, handling, blood sampling, injections with innocuous substances, nasal lavages, and temporary captivity. There are numerous studies indicating that turtle species experience acute handling stress via a significant glucocorticoid response (Gregory et al., 1996; Gregory and Schmid, 2001; Jessop et al., 2004). However, there are also other studies using several reptile models that support our finding, and demonstrate that mildly invasive research activities do not affect study animals in terms of glucocorticoid levels. For example, in a previous study, Kahn et al. (unpublished data) found no significant difference in Gopher Tortoises' corticosterone levels when comparing samples from the initial trap date and one, three, 11, 32, and 52 days in captivity, during which time tortoises also underwent physical manipulations, including those conducted in this study. In addition, the Bearded Dragon (*Pogona barbata*) shows

no significant change in corticosterone levels in captivity at either 3.5 or 24 hours post capture (Cree et al., 2000). Three-Toed Box Turtles (*Terrapene carolina triunguis*) also do not experience a significant increase in fecal glucocorticoid metabolite levels when subjected to capture, handling, attachment of a radiotransmitter, and temporary captivity (Rittenhouse et al., 2005), indicating that this species does not experience a stress response as a result of these activities. Similar findings were documented in mammal species. Koalas do not show an increase in plasma cortisol levels at six hours post capture or at one or seven days in captivity (Hajduk et al., 1992). In addition, African Wild Dogs (*Lycaon pictus*), when compared with control animals, do not show increases in fecal glucocorticoids or increased risk for mortality when they are tranquilized with a dart and radiocollared (Creel et al., 1997). It appears that, at least in the short-term, these mildly invasive manipulations have little or no effect on the glucocorticoid levels of the individuals being studied. The procedures we conducted were both more extensive and more invasive than those conducted in any of these studies, and yet, we also demonstrated no change in Gopher Tortoises' corticosterone levels as a result of our protocols.

None of the key behavioral variables that we studied, except for one, showed any significant differences between experimental and control animals or from pre- to post-manipulation or control date. These findings are similar to results of another recent study that demonstrated no differences in recapture rates or time to recapture between groups of Gopher Tortoises that were previously captured, handled, and marked versus those that were not (Pike et al., 2005). The only significant finding in this study was an interaction effect between treatment and time in the number of movements tortoises

made to other burrows during the four weeks following the manipulation or control date. Despite this finding, tortoises remained within the same home range and continued to use the same burrows. In addition, increased movement during the post-manipulation period, as demonstrated by the experimental group, is also seen in other Gopher Tortoise populations. Specifically, increased movement rates occur later in the active season, usually from July through September as the mating season progresses (McRae et al., 1981; Eubanks et al., 2003). Given these results, it appears that the increase in the experimental group was more typical of Gopher Tortoise behavior than what we observed in our controls.

In general, the movement patterns that we documented are similar to those reported in other studies of Gopher Tortoises. For example, Eubanks et al. (2003) conducted a study of Gopher Tortoises at the Joseph W. Jones Ecological Research Center, a private ecological reserve in southwest Georgia (Baker County) with habitat similar to that of the Wade Tract. They found that female and male tortoises traveled mean distances of 54.0m and 85.2m per move, respectively, from June through October 1997. We found a mean distance of 76.0m per move prior to the manipulations and 52.2m per move after the protocol was implemented, which are in the same general range and the same general time period as the Eubanks et al. (2003) study. Our control tortoises had mean distances traveled of 42.8m per move prior to the control date and 37.6m per move afterwards. Thus, it appears that our experimental tortoises, but not our control tortoises, traveled mean distances that were comparable to the findings of this other study.

The mean number of burrows that tortoises used in the course of our study did not change significantly between pre- and post-manipulation or control date. Experimental tortoises used a mean of 2.4 burrows prior to manipulation and 3.7 burrows post-manipulation, whereas control tortoises used 3.1 and 3.3 burrows pre- and post-control date, respectively. These results are similar to those from other Gopher Tortoise studies. Again, Eubanks et al. (2003) found that female tortoises used an average of about two burrows per month throughout the year, whereas males increased the number of burrows they used from May through September, using a maximum of about five burrows. At another location in Georgia, McRae et al. (1981) documented similar findings to those of the Eubanks et al. (2003) study. They found that tortoises changed burrows infrequently in the early part of the active season, but by summer, tortoises began using two or more burrows per month.

The home ranges that we documented in this study were smaller than those documented in other studies. However, our study was conducted for a period of only eight weeks and most studies report figures for home range over the course of an entire season or year. For example, prior to the manipulation or control date, our tortoises (males and females combined) had a mean home range of 0.05 hectares for the experimental group and 0.17 hectares for the control group. In similar habitat, Eubanks et al. (2003) calculated average annual home ranges for females and males to be 0.4 hectares and 1.1 hectares, respectively, which are very different from our findings. Similarly, in two Florida studies that were conducted over the course of two years, researchers reported annual home ranges for Gopher Tortoises to be 0.6 and 0.31 hectares for females and 1.9 and 0.8 hectares for males (Diemer, 1992; Smith et al.,

1997). The home range data that we report are more comparable to the McRae et al. (1981) study in Georgia where female and male tortoises had home ranges 0.08 hectares and 0.47 hectares, respectively. Perhaps our home ranges are smaller than those reported in most studies due to the abbreviated time frame of our study, spring and early summer months, prior to reported increases in male movement patterns. In fact, we found that in May/June, prior to the manipulations or control date, nine out of 16 tortoises (56%) used only one or two burrows, and a total of only four tortoises (25%) used only one or two burrows post-manipulation or control date (June/July). As a result of these small numbers in burrow usage, the home ranges we report are also small. Ultimately, our data analyses indicate that manipulations had no effect on home range, and we do not think there will be an alteration to the home ranges if examined over the course of an entire season of activity.

In general, few studies have examined direct links between behavior and handling as a stressor. However, studies that did attempt to examine such a link supported our findings that these mildly invasive techniques may not be stressors. Specifically, one study showed that a common territorial lizard, *Anolis sagrei*, does not increase display behaviors in response to investigator handling and temporary confinement, indicating that the research activities are not stressors (McMann and Paterson, 2003). Another study found that despite an increase in corticosterone as a result of four hours of capture stress, male Red-Sided Garter Snakes (*Thamnophis sirtalis parietalis*) do not alter their mating behavior relative to controls (Moore et al., 2000).

The information gained from conducting mildly invasive research techniques may be crucial to the survival and conservation of Gopher Tortoises. The procedures we conducted in our study were both more extensive and more invasive than the procedures conducted in many other studies of this species, and yet, we demonstrated no change in Gopher Tortoises' physiological or key behavioral variables as a result of our protocols. Ultimately, our study provides evidence that these short-term procedures do not significantly affect the corticosterone levels or daily movement patterns of Gopher Tortoises.

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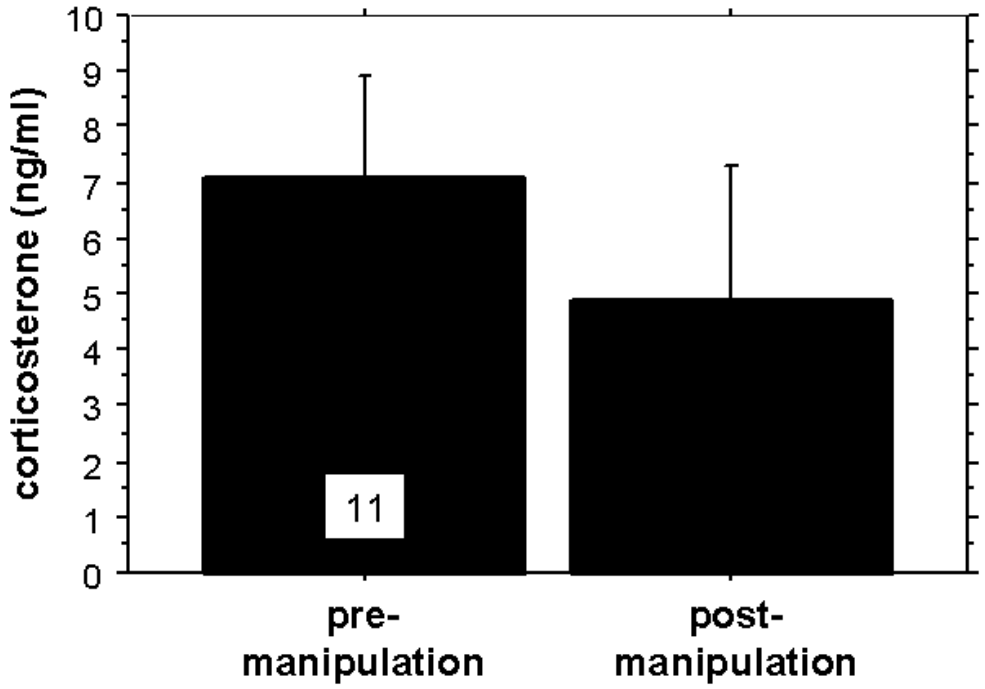


Figure 9. Mean corticosterone levels (ng/ml) ± SE of experimental tortoises at baseline and approximately 8 hours after undergoing trapping, handling, injections, nasal lavage, and temporary captivity ($F_{1,10}$=0.60, p=0.45).

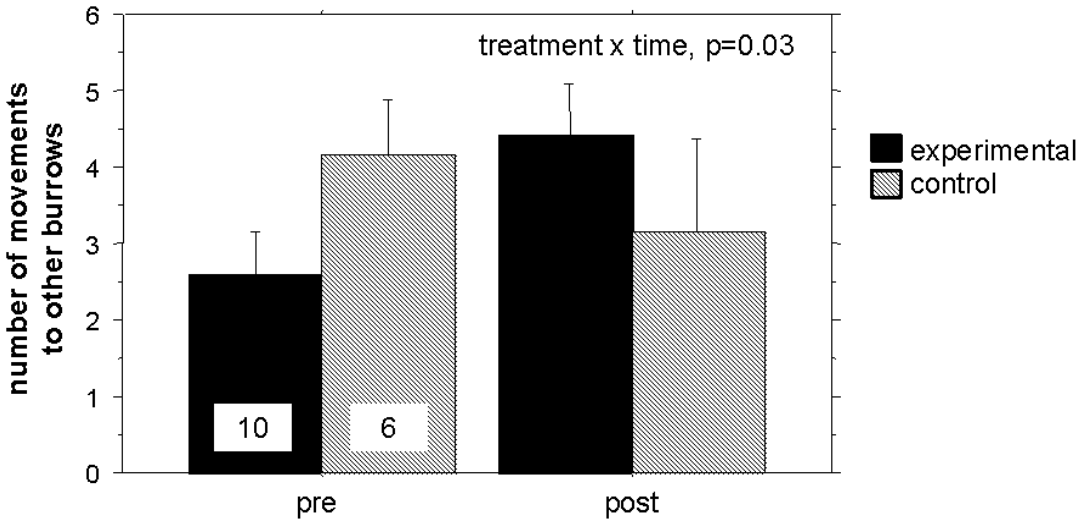


Figure 10. Mean number of movements made ± SE to other burrows by tortoises during the 4 weeks prior to and 4 weeks following implementation of the protocol (experimental group) or control date (control group) (interaction effect of treatment x time, $F_{1,14}$=5.30, p=0.03).

# CHAPTER THREE

# THE EFFECTS OF RELOCATION ON PLASMA CORTICOSTERONE, ADRENAL RESPONSIVENESS AND SEX STEROIDS IN GOPHER TORTOISES (*GOPHERUS POLYPHEMUS*)

## Introduction

Animals living in natural habitats are often faced with a multitude of environmental stressors, including habitat destruction and fragmentation. These stressors have the potential to negatively impact the health and survival of native wildlife species. This can be particularly detrimental to populations of threatened and endangered species. In fact, metapopulation models predict that even dominant species that initially appear to be behaviorally unfazed by moderate habitat destruction are merely experiencing a time-delayed extinction process (Tilman et al. 1994). In order to protect animals whose habitats are becoming fragmented and destroyed, they are sometimes relocated to safer, protected areas that are managed to meet the needs of the animals. In fact, many wildlife species have undergone relocation efforts, including mammals (Chiarello et al. 2004; Goymann et al. 1999; Sigg et al. 2005; Turner et al. 2002), reptiles (Diemer 1989; Edgar et al. 2005; Sullivan et al. 2004), and amphibians (Edgar et al. 2005).

The critical endpoints when gauging the success of relocations are that the relocated animals survive to establish stable, self-sustaining populations (Dodd & Seigel 1991; Griffith et al. 1989). However, this determination requires long-term monitoring of relocated populations, and to date, long-term success and failure of relocation projects, particularly for those involving sensitive species like reptiles and amphibians, is still lacking (Dodd & Seigel 1991; Reinert 1991). Adding to the complications involved with collecting long-term data, the parameters for judging the success of relocations are not clearly delineated, despite their wide debate in the literature. This is especially true for reptiles and amphibians that are not considered ideal candidates for relocation (Burke 1991; Seigel & Dodd 2000).

For decades, the long leaf pine forests, sand hill communities, and upland habitats of the southeastern United States have been undergoing extensive real estate development associated with increased urbanization (Diemer 1986). As a result, a variety of animal species living in these habitats have undergone relocation events. One of those is the gopher tortoise (*Gopherus polyphemus*), a keystone species that is threatened in the western portion of its range (western Alabama, Mississippi, and Louisiana). For many years, gopher tortoise populations have been declining at an alarming rate, as much as an 80% decrease in total numbers, as a result of urbanization, agricultural activities, and exploitation as food (Auffenberg & Franz 1982). Thus, they have undergone relocation from their disturbed habitats with the goal of protecting them on managed lands.

Numerous relocation projects have been attempted with gopher tortoises throughout their range, particularly in Florida. Diemer (1989) reviewed many of these

gopher tortoise relocation studies and pointed out the difficulties involved with making comparisons among them and gauging their individual successes. One thing is clear - the key to evaluating relocation success lies in long-term evaluation of survival, reproduction, and health. As a k-selected species, gopher tortoises are a long-lived species that experiences low fecundity. Therefore, for gopher tortoise studies to be considered long-term, researchers would need to follow relocated tortoises for 20 to 30 years to accurately assess these critical factors. Ultimately, these factors lead to three main issues surrounding the assessment of gopher tortoise relocations: 1) It is difficult to obtain genuine long-term (>3 years) data on gopher tortoise relocations; 2) There is no clear consensus on what constitutes relocation success; and 3) Aside from preventing habitat destruction, relocation is the only remaining avenue for protecting gopher tortoises so we need an alternative, immediate means of assessing relocation success. We propose a solution to the third issue: Relocation success can be gauged in the short-term by examining stress, reproduction and overall health via measurable physiological changes in adrenal and gonadal steroids.

It is well established that corticosterone levels increase in response to stressors through the highly conserved mechanisms of the hypothalamo-pituitary-adrenal axis. Acutely, this response can be adaptive in that it allows an animal to respond and adapt to a situation (Chrousos & Gold 1992; Sapolsky et al. 2000). However, chronic stressors can cause a cascade of maladaptive changes, such as impaired adrenal responsiveness (Aguilera 1994; Feek et al. 1983), decreased immunocompetence (Bauer et al. 2001; Chrousos & Gold 1992; Rollins-Smith 2001), and changes in locomotion (Cash & Holberton 1999), foraging behavior (Astheimer et al. 1992; Challet et al. 1995;

Wingfield & Silverin 1986), and reproduction (Greenberg & Wingfield 1987; Pottinger 1999; Rabin et al. 1988; Rivier & Rivest 1991). These effects could be of particular interest when evaluating gopher tortoises that are infected with *Mycoplasma agassizii*, the bacterium that causes Upper Respiratory Tract Disease (URTD) (Brown et al. 1999). Ultimately, if relocation is a stressor for gopher tortoises, then these animals, including those that appear to be healthy, could potentially suffer from chronically elevated corticosterone levels and a variety of other subsequent impairments that could negatively impact the overall success of the relocation in terms of long-term health and survival.

In this study, we investigated gopher tortoise relocation success in the short term by examining physiological markers of stress and reproduction. Specifically, we examined how relocation affects baseline plasma corticosterone levels, adrenal responsiveness, and sex steroids in gopher tortoises at 30 days and 10 months post-relocation. We also determined if habitat quality, either prior to or following the relocation, plays a role. In addition, we conducted relocations during two different seasons in order to characterize potential seasonal differences in the physiological response to relocation.

**Materials and Methods**

*Study Sites*. We conducted a gopher tortoise relocation study at Fort Benning Army Base in Georgia. This location is within the natural range of gopher tortoises, but it is outside the area where they are federally listed as a threatened species. All of the tortoises in this study were scheduled for relocation because their habitats were being developed to accommodate military training activities. Tortoises were relocated to other

areas within the confines of the military installation. We trapped and removed tortoises from areas that ranged in both habitat quality (high quality versus low quality) and military impact (impacted versus not impacted by the military), and it should be noted that these qualities were not necessarily exclusive (i.e., impacted habitat is not necessarily of low quality, etc.).

To classify habitat quality as either high (H) or low (L) quality, we assessed the key characteristics of undisturbed gopher tortoise habitat, looking most specifically for an open canopy and abundant groundcover vegetation (Aresco & Guyer 1999a; Aresco & Guyer 1999b). To determine military impact, we categorized as impacted habitat (I) those sites that experienced daily or weekly military activity, including foot and vehicular traffic, the presence or evidence of tracked and wheeled vehicles conducting maneuvers, and the presence of military unique debris, such as spent ammunition cases. Conversely, those sites that experienced minimal activities, such as occasional foot traffic, with vehicular disturbance limited to transit by wheeled vehicles on established trails, occurring for a total of less than 3 times per year were classified as habitat that was not impacted by the military (N).

We also selected two relocation sites on the army base; 1) a high quality site that was not impacted by the military, and 2) a low quality, military-impacted site. The high quality site was characterized by mixed pine trees, turkey oak, an open canopy, and abundant ground level food sources. The site was managed on a 3 year controlled burn cycle. In addition, the site experienced infrequent foot or vehicular traffic, and training activities were conducted at the site only once or twice a year. Conversely, the low quality habitat had no canopy in some locations and the landscape was predominantly

characterized by desert-like sandy hills. In some areas, the middle story was thick with hardwoods, and the majority of the vegetation at the site was either nonexistent or inedible for tortoises. The site was administratively protected by posting as off-limits to vehicles, but was used extensively on a daily basis for military training exercises. In fact, Guyer et al. (1996) used the site as an impacted habitat in a study of the long-term effects of tracked vehicles on gopher tortoises and reported significant findings in terms of behavior and movement.

During June 2003 and March 2004, prior to the relocations, holding pens were constructed at the two relocation sites to increase site fidelity in gopher tortoises (Lohoefener & Lohmeier 1986; Tuberville et al. 2005). Aluminum flashing (50.8 cm x 15.24 m) was placed 15cm in the ground around the perimeter of each holding area. To provide support for the structure, pieces of 90cm long metal conduit (1.3cm diameter) were placed around the outside of the pen at 1.5 to 3m apart and secured with zipties.

*Relocation*. As described above, tortoises' home habitats were designated as either low (L) or high (H) quality, and as either impacted (I) or not impacted (N) by the military. Tortoises were trapped in their home habitats (see Trapping protocol below) and relocated to one of the two relocation sites (Figure 11). Resident tortoises at the two relocation sites served as controls, and did not undergo relocation. Residents were categorized as HR (high quality habitat residents) or LR (low quality habitat residents), and NR (not impacted residents) or IR (impacted residents).

*Trapping and Blood Collection*. From July to September 2003 (summer 2003 field season) and April to June 2004 (spring 2004 field season), live wire traps (Tomahawk Live Trap Company, custom order) were placed at the mouth of active

gopher tortoise burrows. The floor and foot pedal of the trip mechanism of each trap were partially covered with sand from the burrow apron. The entrance to the burrow and the trap were covered with a $1m^2$ piece of burlap, which made the trap appear to be an extension of the burrow to encourage the tortoise to enter, and it provided a shady area to prevent trapped tortoises from overheating. Tortoises do not show a significant increase in corticosterone levels (the hormone associated with the stress response) when left in a trap for up to 12 hours (Ott et al. 2000). Therefore, to ensure that tortoises were never left in a trap for more than 12 hours, the traps were set no later than 8:00am and they were checked twice daily (from 5am to 8am and from 5pm to 8pm) until tortoises were caught. During 2003, 97.9% of tortoises were trapped and blood sampled after 5pm. However, in 2004, only 84.1% of tortoises were trapped and blood sampled after 5pm while the remaining 15.9% were trapped and sampled prior to 10am. Statistical analysis using a one-way ANOVA showed that baseline corticosterone levels in our 2004 samples did not vary significantly with the time of day that blood was drawn ($F_{1,61}=2.36$, $p=0.13$).

Upon capture, the tortoise was removed from the trap and a 2ml blood sample was immediately drawn from the tortoise's femoral vein using a 3ml heparinized syringe and 22 gauge needle. Blood samples were placed in a cooler with ice for transport. Tortoises were each given an individual identification number written on the carapace in a non-toxic white paint pen. They were then placed in a 37.85 liter Rubbermaid® bin for transport. The cover and sides of the bin were punctured with numerous 1.5 cm air holes, and the bottom of the bin was filled with 5 cm of sand from the burrow apron. The tortoises and blood cooler were transported to the field lab after all traps were

checked (within 4 hours of the first trap check). To undergo the remainder of the protocol, tortoises were held in their individual bins in a climate-controlled environment at a local, temporary holding facility. While in holding, a radiotransmitter (American Wildlife Enterprises, Model AWE-GA) was attached to the carapace of each tortoise using plumber's epoxy. Previous studies have demonstrated that tortoises do not experience an increase in corticosterone levels as a result of transport or holding (Kahn et al. submitted-b).

At the field lab, the sampled blood was centrifuged in a tabletop centrifuge and the plasma was aliquoted for hormone analysis. We later conducted standard tritiated steroid hormone radioimmunoassays (RIA) to determine the amount (ng/ml) of circulating plasma corticosterone (B), 17β-estradiol (E) in females, and testosterone (T) in males (Mendonça et al. 1996; Ott et al. 2000). Briefly, each sample was diethyl ether-extracted and incubated with a 1:10 solution of hormone in order to test extraction efficiency. After the ether evaporated (within 24 hours), samples were resuspended in phosphate buffered saline (PBS) gel. The samples were then aliquoted in duplicate and incubated overnight with the appropriate tritiated hormone and antibody. Finally, samples were centrifuged with charcoal to remove unbound hormone, and the remaining sample was counted and compared with a standard binding curve that was established during each assay. Average extraction efficiency was 86.2% for B, 72.6% for T, and 77.2% for E. Intra-assay variability was 8.64% for B, 4.86% for T, and 3.24% for E, while inter-assay variability was 18.7%, 18.2%, and 11.11%, respectively.

*ACTH Challenge*. While in holding, an ACTH challenge was conducted to determine the gopher tortoises' adrenal responsiveness. They were given an

intraperitoneal (IP) injection of 1mg/ml ACTH (Sigma-Aldrich, adrenocorticotropic hormone from porcine pituitary) in the inguinal region. Dosing was previously established by Kahn et al. at 80μg/kg (submitted-a). Several tortoises received saline instead of ACTH to serve as controls, but previous studies have indicated that tortoises show no adrenal response to saline (Kahn et al. submitted-a). A blood sample was taken 4 hours after the injection to measure the adrenal response. We conducted RIAs to determine the amount (ng/ml) of corticosterone in each sample for both the baseline blood sample taken in the field and for the 4 hour post-ACTH injection blood sample.

At the completion of the protocol, control tortoises (resident tortoises living in the relocation habitats that did not experience a relocation) were returned to their burrows of capture, while experimental tortoises were relocated to the pen at either the disturbed or undisturbed relocation site where they were released at the burrow apron of inactive or abandoned burrows. Thirty days later, tortoises were trapped again and the protocol was repeated in order to compare B, T, E, and stress responsiveness between Day 0 (pre-relocation/control date) and Day 30 (30 days post-relocation/control date). The tortoises that were examined in summer 2003 were trapped again 10 months later on Day 0 2004 to compare their steroid hormone levels and stress responsiveness in the longer-term.

*Data analysis*. All corticosterone, 17β-estradiol, and testosterone measures were log transformed and goodness of fit tests were conducted using Kolmogorov's D Test to ensure normality and homogeneity of variance. Outliers for each data set were calculated using Grubb's Test Statistic, and were removed from the analyses.

Differences in corticosterone levels were examined from pre- to post-ACTH injection using percent change calculated using the following equation:

$$\frac{[\text{B (ng/ml) at 4hrs post - ACTH injection}] - [\text{B (ng/ml) at baseline}]}{[\text{B (ng/ml) at baseline}]} \times 100$$

Negative percent values were made positive by adding 101 to all values, which were then arcsin transformed for normality.

Two-way repeated measures analyses of variance (ANOVA) were used to determine changes in steroid hormone levels (B, T, and E) from pre-relocation/control date (Day 0) to 30 days post-relocation/control date (Day 30) by relocator/resident status and sex. Further one-way ANOVA analyses were conducted to assess differences in steroid levels by habitat quality and military impact for relocators according to changes in their habitat quality and military impact. However, sample sizes were too small to conduct analyses by sex and habitat, so sex was not used as a variable in the habitat analyses.

A two-way repeated measures ANOVA analyzing the effects of relocator/resident status and sex was used to determine changes in hormone levels from 2003 pre-relocation/control date (Day 0 2003) to approximately 10 months post-relocation/control date (Day 0 2004) in tortoises followed longitudinally through the two seasons. Finally, body condition index (BCI) was obtained by taking the residuals of a simple regression plotting straight carapace length (cm) against mass (kg). We found that body condition index did not vary significantly by season ($F_{3,151}$=1.68, p=0.10), but

it did vary significantly by sex ($F_{3,151}$=5.82, p<0.0001). Therefore, body condition index was accounted for in all statistical analyses conducted by sex. The statistical software program JMP version 6.0.0 (SAS Institute Inc.) was used to conduct all statistical analyses. Analyses were considered significant when p<0.05.

**Results**

A total of 155 tortoises were monitored in this study. In summer 2003, there were 70 tortoises in the study, including 36 relocators and 34 control residents. Forty-five of these tortoises (32 relocators and 13 control residents) were followed into spring 2004 in order to examine the physiological effects of relocation 10 months later. In order to conduct the seasonal comparison, data collected during summer 2003 were compared to data collected for 85 new tortoises added to the study in spring 2004, which included 44 relocators and 41 control residents.

*Baseline Corticosterone*. A one-way ANOVA indicated that there was no significant difference in baseline corticosterone levels in initial blood samples taken during June, July, and August 2003 ($F_{2,67}$=1.09, p=0.34) or in samples taken during April and May 2004 ($F_{1,83}$=2.61, p=0.11). Therefore, we combined data for June through August into a single season (summer 2003), and we combined data for April and May into another season (spring 2004). A two-way ANOVA found baseline corticosterone levels to be significantly higher for all tortoises in spring than in summer ($F_{1,147}$=11.96, p=0.0007), and females had significantly higher baseline corticosterone levels than males during both seasons ($F_{1,147}$=5.70, p=0.02). However, there was no interaction effect between season and sex ($F_{3,147}$=1.37,p=0.24, Figure 12). As a result of these

findings, all further corticosterone data analyses were conducted by sex within each season.

During summer 2003, there was no overall significant change in corticosterone levels from Day 0 (pre-relocation) to Day 30 (post-relocation) of the study ($F_{1,47}$= 0.14, p=0.71). However, when we examined differences in corticosterone levels from Day 0 to Day 30 between relocator/resident status and sex, we found a significant relationship ($F_{1,47}$= 4.89, p=0.03, Figure 13a). Specifically, during summer 2003, female relocators showed a decrease in plasma corticosterone levels from pre- to post-relocation, while the male relocators showed an increase in corticosterone levels during that same time period (male relocators versus female relocators, $F_{1,26}$=14.14, p=0.0009). However, there was no overall significant difference between relocators and residents ($F_{1,47}$= 1.99, p=0.16).

To assess the longitudinal effects of relocation, tortoises from the summer 2003 study were blood sampled again ten months later. A repeated measures ANOVA comparing Day 0 2003 with Day 0 2004 indicated that there was no significant difference in circulating plasma corticosterone levels by relocator/resident status or by sex ($F_{1,39}$= 2.43, p=0.13).

In a separate relocation study conducted with new individuals during spring 2004, there was no significant difference in corticosterone levels from Day 0 to Day 30 of the study between relocators and residents or by sex ($F_{1,55}$= 0.25, p=0.61, Figure 13b). However, there was an overall significant decrease in corticosterone levels from Day 0 to Day 30 of the study ($F_{1,55}$= 14.64, p=0.0003).

Although we found no overall significant difference in corticosterone levels from Day 0 to Day 30 between relocators and residents during either season or longitudinally,

we conducted further examination of the corticosterone levels of only the relocators (i.e., excluding control resident tortoises) according to habitat quality (high versus low quality) and military impact on the habitat (impacted versus not impacted). Sex was not included as a variable in these analyses due to small sample sizes. We found that changes in relocated tortoises' corticosterone levels from pre- to post-relocation during each field season were not related to habitat quality (summer $F_{2,25}$= 1.34, p=0.28; spring $F_{3,37}$= 0.17, p=0.91) or military impact on the habitat (summer $F_{2,25}$= 1.34, p=0.28; spring $F_{3,37}$= 0.49, p=0.69). Similarly, there was no effect of habitat quality or military impact on the corticosterone levels of relocated tortoises that were followed longitudinally from summer 2003 into spring 2004 ($F_{2,29}$= 1.47, p=0.25). It should be noted that in the longitudinal study, low quality habitat was the same as impacted habitat and high quality habitat was the same as not impacted habitat.

*ACTH Challenge*. Overall, tortoises that received ACTH showed a significantly stronger adrenal response than those that received saline during both summer 2003 ($F_{1,62}$=31.38, p<0.0001) and spring 2004 ($F_{1,78}$=16.43, p<0.0001). For those tortoises that received ACTH (i.e., excluding those that received saline), there was no significant difference in adrenal response between Day 0 and Day 30 of the study, regardless of relocator/resident status or sex during either summer 2003 ($F_{1,20}$=1.20, p=0.29, Figure 14a) or spring 2004 ($F_{1,45}$=1.71, p=0.20, Figure 14b).

For tortoises that were followed longitudinally from summer 2003 into spring 2004, there was also no significant difference in adrenal response when examined by relocator/resident status and sex ($F_{1,15}$=2.10, p=0.16). However, when grouping the relocated and resident tortoises that were followed longitudinally into a single group, the

response to ACTH was significantly stronger in summer than in spring ($F_{1,15}$=5.93, p=0.02), and male tortoises showed significantly stronger adrenal responses than females during both seasons ($F_{1,15}$=5.36, p=0.03, Figure 15). Sample sizes were too small to analyze longitudinal data according to habitat quality.

*Sex Steroids*. Baseline levels of circulating plasma testosterone (T) in male tortoises were significantly higher during summer 2003 than spring 2004 ($F_{1,58}$=5.36, p=0.0003). Specifically, there were significantly lower T values in May as compared with values in July and August ($F_{3,58}$=7.20, p=0.0003) so further analyses were conducted by season.

During summer 2003, there was a significant decrease in T levels from Day 0 to Day 30 ($F_{1,15}$=5.24, p=0.04), but the decrease was not related to relocator/resident status ($F_{1,15}$=0.57, p=0.46, Figure 16a). It was also not related to habitat quality or military impact ($F_{2,9}$= 1.19, p=0.35). When these male tortoises were followed longitudinally into spring 2004, they experienced a significant decrease in T ($F_{1,20}$=34.24, p<0.0001). However, the decrease again was not related to relocator/resident status ($F_{1,20}$=1.09, p=0.31) or relocators' habitat quality or military impact on habitat ($F_{2,12}$=0.38, p=0.70).

During spring 2004, there was no significant change in T levels from Day 0 to Day 30 ($F_{1,32}$=1.55, p=0.22), and there was no significant difference between relocators and residents ($F_{1,32}$=3.54, p=0.07, Figure 16b). In addition, there was no significant difference in T levels by habitat quality ($F_{2,15}$= 2.01, p=0.17) or military impact ($F_{3,14}$= 1.35, p=0.30).

In female tortoises, there was no significant difference in Day 0 baseline circulating plasma levels of 17β-estradiol (E) between summer 2003 and spring 2004

($F_{1,46}$=1.41, p=0.24). However, there was a significant difference in E levels by month, specifically between May and August ($F_{3,45}$=2.69, p=0.05) so analyses were again conducted by season.

There was an overall significant decrease in E from Day 0 to Day 30 in both summer 2003 ($F_{1,10}$=5.13, p=0.05) and spring 2004 ($F_{1,22}$=36.00, p<0.0001). In summer 2003, there was no effect of relocator/resident status ($F_{1,10}$=0.03, p=0.87, Figure 17a), while in spring 2004 there was a significant relationship between E and relocator/resident status ($F_{1,22}$=14.91, p=0.0008, Figure 17b). Specifically, female relocators in spring 2004 did not demonstrate as great a decrease in E from Day 0 to Day 30 as did the female residents ($F_{1,22}$=14.91, p=0.0008). Furthermore, changes in E were not related to habitat quality (summer 2003 $F_{2,5}$= 0.22, p=0.81; spring $F_{2,14}$= 0.66, p=0.53) or military impact (summer 2003 $F_{2,5}$= 0.22, p=0.81; spring $F_{3,13}$= 0.61, p=0.62).

Finally, female tortoises that were followed longitudinally from summer 2003 into spring 2004 did not experience a significant change in E between seasons ($F_{1,9}$=4.46, p=0.06). Longitudinal analyses by habitat quality and military impact on habitat could not be conducted on E levels of females due to the small sample sizes in each group.

**Discussion**

To date, no data are available regarding the physiological consequences of relocation on gopher tortoises. We predicted that placing relocated gopher tortoises into a new habitat would cause an increased, potentially chronic activation of individuals' HPA axes leading to numerous subsequent maladaptive effects, including changes in

adrenal and gonadal steroids. However, given our sampling timeline, from 30 days to 10 months, our findings indicate that within this short time period following the relocation/control date, relocation does not increase corticosterone levels or cause a deterioration of the adrenal response, and it does not affect sex steroids. Of course, we may have missed a peak in corticosterone by waiting to measure the response until 30 days post-relocation. However, it is clear that even if there was a peak in the corticosterone response at 3 days, 7 days, or even 14 days, our data indicate that corticosterone levels return to baseline and are the same as resident controls by 30 days post-relocation.

Despite our findings that indicate no change in corticosterone as a result of relocation, we did measure seasonal variation in corticosterone levels, which contradicts findings from a previous study (Ott et al. 2000). Specifically, we found that baseline levels of plasma corticosterone were significantly lower in summer than in spring, regardless of sex or habitat. While both our study and the Ott et al. study reported values that were very similar and all values were within the normal range, we may have found significant seasonal differences because our overall sample sizes were larger than those in the Ott et al. study, and we analyzed the data using a repeated measures ANOVA, while they used a two-way general linear model (Kahn et al. submitted-a; Ott et al. 2000).

Currently, there are no published relocation studies with which we can compare our physiological findings. Therefore, we can only compare our baseline corticosterone levels at capture and how the levels vary seasonally. In a closely related species, the desert tortoise (*Gopherus agassizii*), B levels have also been reported to vary

significantly throughout the season, but within a smaller range of B values, from 0.2ng/ml up to 6.45ng/ml, and by sex (Lance et al. 2001). Female desert tortoises show the same seasonal pattern of corticosterone as the gopher tortoises in our study (higher in spring than in summer), but the opposite was found in male desert tortoises (Lance et al. 2001). This difference in seasonal patterns of corticosterone between male gopher and desert tortoises could be the result of different physiology in the two species since they are not direct sister taxa, and they are restricted by different biological constraints as a result of inhabiting different environments. Like the male desert tortoise, the soft-shelled turtle *Lissemys punctata punctata* also shows seasonal variation in corticosterone with significantly higher levels in summer than in spring (Ray et al. 2003), so this pattern is not uncommon.

Our findings also demonstrated sex differences in circulating corticosterone values. Despite the low (but normal) corticosterone levels in both sexes during both summer and spring, females had significantly higher levels than males during both seasons. Studies of other herpetofauna have demonstrated similar findings in that females generally exhibited higher corticosterone levels than males (Licht et al. 1983). This is directly opposite to the findings of Lance et al (2001) and Lane and Rostal (2002a) who found that male desert tortoises consistently had significantly higher mean corticosterone values than females throughout the season. As mentioned above, this could be related to differences in the physiology of these closely related species.

Corticosterone values for gopher tortoises subjected to an ACTH challenge were only recently documented to determine if low baseline corticosterone values in gopher tortoises are indicative of adrenal health (normal adrenal secretion) or adrenal

impairment (adrenal exhaustion which limits secretion). In gopher tortoises, corticosterone peaks in response to an ACTH challenge at 4 hours post-injection (Kahn et al. submitted-a). Desert tortoises, however, when tested for a corticosterone response to ACTH at 1, 2, 4, 8, and 24 hours, show a peak at only one hour post-injection (Lance & Rostal 2002b). In this study, we found that gopher tortoises showed a stronger adrenal response to the ACTH challenge during summer than spring. We also found that over a period of 10 months, tortoises that were relocated from one low quality habitat to another low quality habitat showed a stronger adrenal response than tortoises moved from any type of habitat to a high quality habitat. It appears that gopher tortoises that are associated with low quality habitats prior to and following relocation may be physiologically primed to respond more vigorously to a stressor. A similar pattern was found in nestling tree swallows (*Tachycineta bicolor*); those exposed to contaminants in their environments showed no significant difference in baseline corticosterone levels when compared with controls, but they demonstrated a higher level of corticosterone secretion than controls in response to an ACTH challenge (Mayne et al. 2004).

Relocation did not significantly impact the seasonal sex steroid cycle of gopher tortoises. We found that T levels in male gopher tortoises are not affected by relocation, but they do vary seasonally in that they are significantly higher in summer than spring. In many turtle and tortoise species, high T levels in summer coincide with a peak in spermatogenesis and the mating season (Rostal et al. 1998), while in spring, during the period following hibernation, low T occurs while seminiferous tubules are regressed and leydig cells are hypertrophied (Rostal et al. 1994). These T data coincide with those of the Ott et al. study (2000) in which they showed that gopher tortoise T levels increase

significantly in July compared to the spring months and remain elevated throughout the remainder of the active season. Desert tortoises show the same seasonal pattern of T levels (Lance et al. 2001; Lance & Rostal 2002a; Rostal et al. 1994), as do painted turtles (*Chrysemys picta*), although painted turtles show an additional transient peak in late spring (Licht et al. 1985). We also found that although T levels do not change significantly in gopher tortoises in spring, there is a significant decrease in T from early to late in the summer as the season progresses toward overwintering. In desert tortoises, the late active/pre-hibernation season is again associated with hypertrophied leydig cells and maximum-sized seminiferous tubule diameters (Rostal et al. 1994). Our T values are similar to findings of desert tortoise studies; our male tortoises showed a peak in T at just over 250ng/ml in summer, while male desert tortoises showed a peak in T at 244ng/ml in one study and just over 300ng/ml in others (Lance & Rostal 2002a; Rostal et al. 1998). However, our peak in T values for gopher tortoises is much different from peaks reported in other turtle species, including Galápagos tortoises at 6ng/ml (Rostal et al. 1998), painted turtles at 20ng/ml (Licht et al. 1985), and the common snapping turtle ay 50ng/ml (Mahmoud & Licht 1997).

Female gopher tortoises did not exhibit a significant seasonal difference in E, but when we examined the E data by month, we found that E is significantly higher in August than in May. This is similar to many other turtle species that exhibit significantly higher levels of E in summer (early vitellogenesis) than in spring (pre-nesting) (Mahmoud & Licht 1997; Rostal et al. 1998). Similarly, Ott et al. found that female gopher tortoises had basal levels of E in spring (1-2ng/ml), but they experienced a significant increase in E during August (peak of 7ng/ml) which lasts through October

(Ott et al. 2000). However, our study indicated that E levels decreased significantly both from early to late summer and early to late spring, and these decreases were not related to habitat quality or military impact on habitat. Female desert tortoises show a similar significant decrease in E from early to late spring, and like female gopher tortoises, the spring E values were also significantly lower than summer E values which peaked in wild caught desert tortoises at approximately 500pg/ml (Lance & Rostal 2002a). Female leatherback sea turtles also show a significant decrease in E from early to late in the nesting season (Rostal et al. 2001). Summer values in our study peaked at approximately 2.5ng/ml while spring values peaked at approximately 1.7ng/ml. These values are similar to the summer peak documented in female gopher tortoises in the Ott et al. study. However, Galápagos tortoises (*Geochelone nigra*) exhibit a mean peak in E values of 75.5pg/ml ± 11.9 SE (Rostal et al. 1998) slightly less than leatherback sea turtles (*Dermochelys coriacea*) with a mean value of 190.95pg/ml ± 16.8 SE at the start of nesting. These variations between and among species may be expected as differences in plasma E values can vary up to an order of magnitude (Licht 1995).

In this study, we did not demonstrate significant changes in the adrenal and sex steroids of gopher tortoises following a relocation event. However, gopher tortoises are a long-lived species so we may not realize the full effects of relocation for many years to come. In the meantime, integrative research that documents current relocation efforts is critical and should include monitoring physiological parameters, as well as examining behavioral patterns, reproductive success, disease, and mortality. These combined data will give us a more complete picture of relocation success and population stability in the

short-term, and may provide us with much needed insight into the physiology and conservation management needs of gopher tortoises over the long-term.

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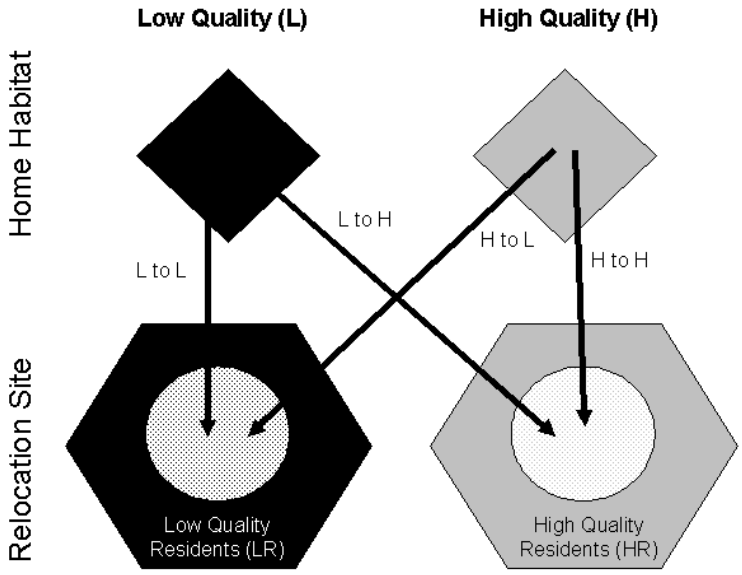
**a. Habitat Quality**

| Relocation Category | Summer 2003 | Spring 2004 |
|---|---|---|
| L to L | 12 | 2 |
| L to H | 10 | 1 |
| H to L | 0 | 25 |
| H to H | 14 | 16 |
| LR | 5 | 5 |
| HR | 29 | 36 |




**b. Military Impact**

| Relocation Category | Summer 2003 | Spring 2004 |
|---|---|---|
| I to I | 12 | 22 |
| I to N | 10 | 14 |
| N to I | 0 | 5 |
| N to N | 14 | 3 |
| IR | 19 | 27 |
| NR | 15 | 14 |

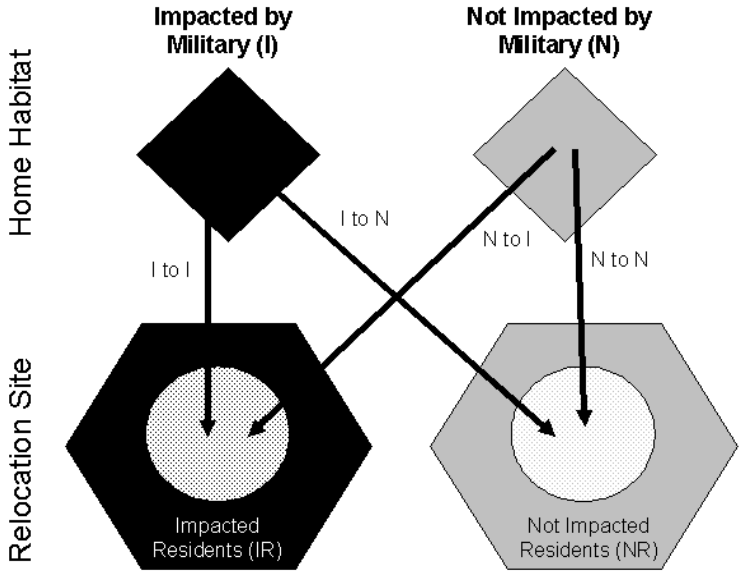



Figure 11. Relocation plan. Tortoises' home habitats were designated as either low (L) or high (H) quality, and as either impacted (I) or not impacted (N) by the military. Tortoises were trapped in their home habitats and relocated to one of the two relocation

sites, also designated as either high or low quality and either impacted or not impacted by the military. This resulted in the formation of four relocation categories according to habitat quality (L to L, L to H, H to L, and H to H) and four other relocation categories according to military impact (I to I, I to N, N to I, and N to N). Resident tortoises at the two relocation sites served as controls, and did not undergo relocation: HR - high quality habitat residents, LR - low quality habitat residents, NR - not impacted residents, and IR - impacted residents.

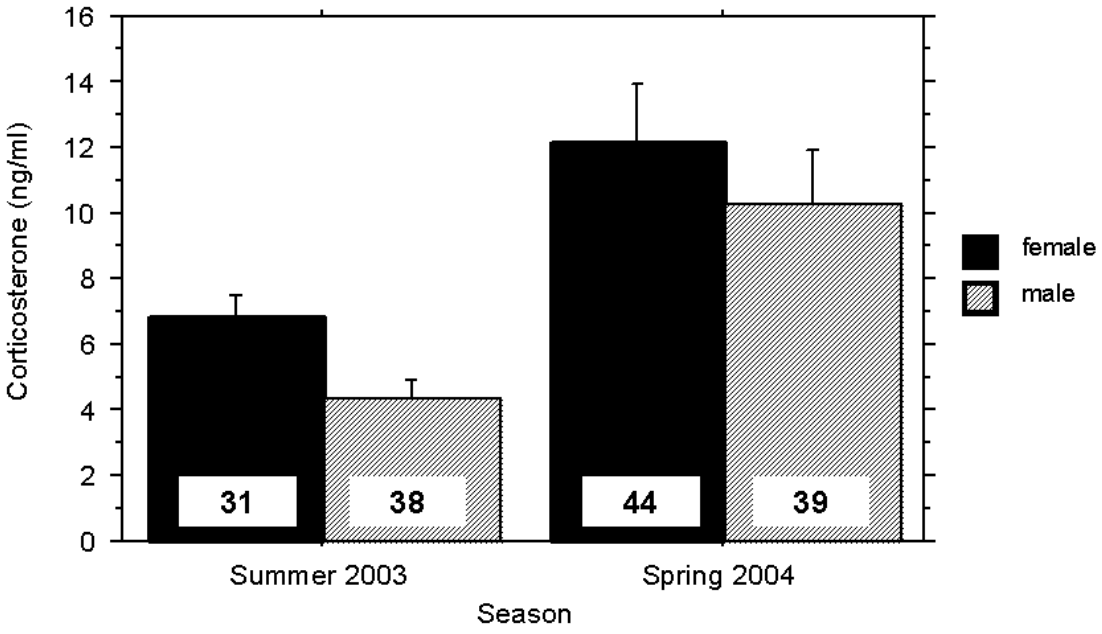



Figure 12. Baseline corticosterone by season and sex. Baseline corticosterone levels were significantly higher for all tortoises in spring than in summer ($F_{1,147}$=11.96, p=0.0007), and females had significantly higher baseline corticosterone levels than males during both seasons ($F_{1,147}$=5.70, p=0.02).

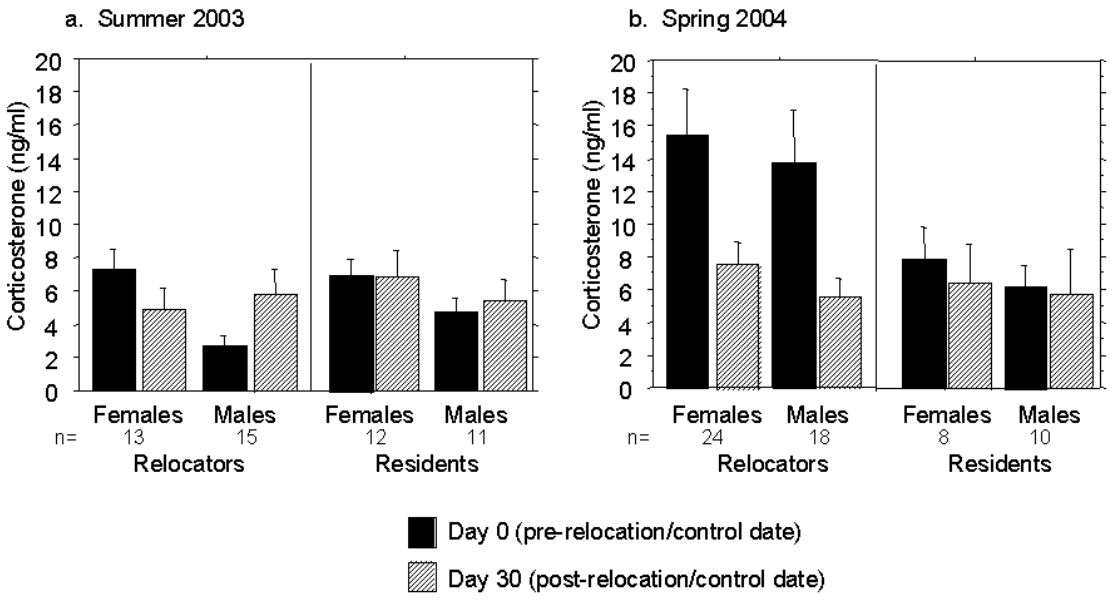



Figure 13. Change in corticosterone from pre- to post-relocation or control date by relocator/resident status and sex. (a) During summer 2003, there was no significant change in corticosterone levels from Day 0 (pre-relocation) to Day 30 (post-relocation) of the study ($F_{1,47}$= 0.14, p=0.71) and no significant difference between relocators and residents ($F_{1,47}$= 1.99, p=0.16). However, female relocators showed a decrease in plasma corticosterone levels from pre- to post-relocation, while the male relocators showed an increase in corticosterone levels during that same time period (male relocators versus female relocators, $F_{1,26}$=14.14, p=0.0009). (b) During spring 2004, there was no significant difference in corticosterone levels from Day 0 to Day 30 between relocators and residents or by sex ($F_{1,55}$= 0.25, p=0.61). However, there was an overall significant decrease in corticosterone levels from Day 0 to Day 30 of the study ($F_{1,55}$= 14.64, p=0.0003).

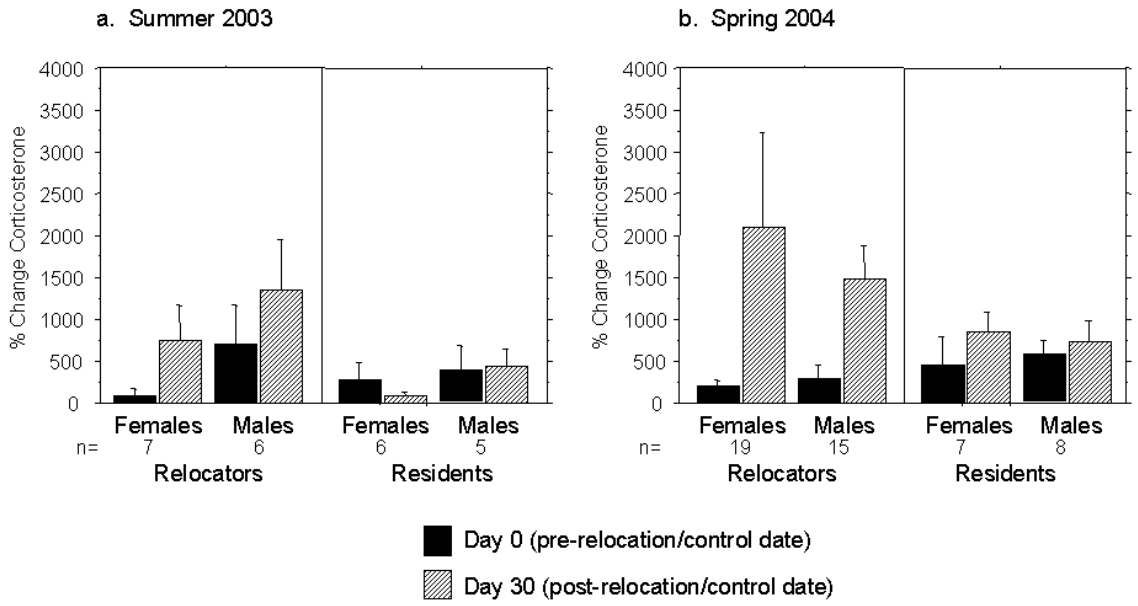



Figure 14. Percent change in corticosterone post-ACTH injection (i.e., excluding saline controls) from pre- to 30 days post-relocation or control date by relocator/resident status and sex. There was no significant difference in adrenal response between Day 0 and Day 30, regardless of relocator/resident status or sex during either (a) summer 2003 ($F_{1,20}$=1.20, p=0.29) or (b) spring 2004 ($F_{1,45}$=1.71, p=0.20).

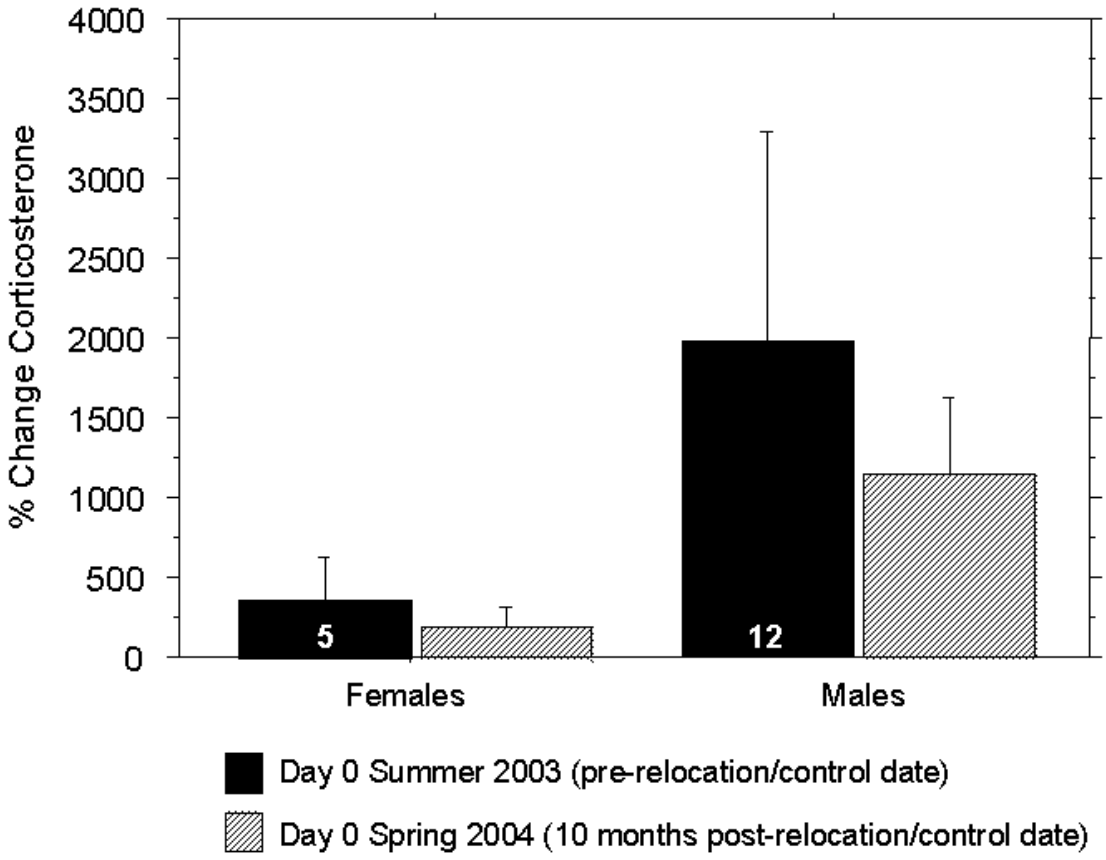



Figure 15. Percent change in corticosterone post-ACTH injection from pre- to 10 months post-relocation or control date by sex. There was no significant difference in adrenal response when examined by relocator/resident status and sex ($F_{1,15}$=2.10, p=0.16). However, with relocators and residents combined, the response to ACTH was significantly stronger in summer than in spring ($F_{1,15}$=5.93, p=0.02), and male tortoises showed significantly stronger adrenal responses than females during both seasons ($F_{1,15}$=5.36, p=0.03).

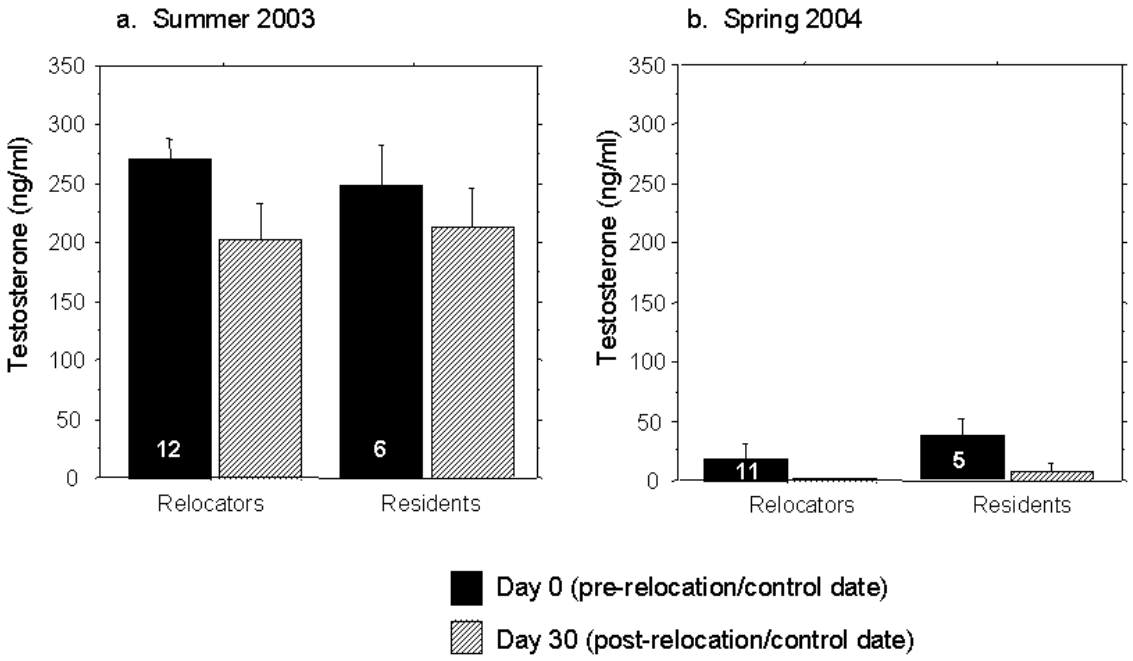



Figure 16. Change in testosterone from pre- to post-relocation or control date by relocator/resident status. (a) During summer 2003, there was a significant decrease in T levels from Day 0 to Day 30 ($F_{1,15}$=5.24, p=0.04), but the decrease was not related to relocator/resident status ($F_{1,15}$=0.57, p=0.46). (b) During spring 2004, there was no significant change in T levels from Day 0 to Day 30 ($F_{1,32}$=1.55, p=0.22), and there was no significant difference between relocators and residents ($F_{1,32}$=3.54, p=0.07).

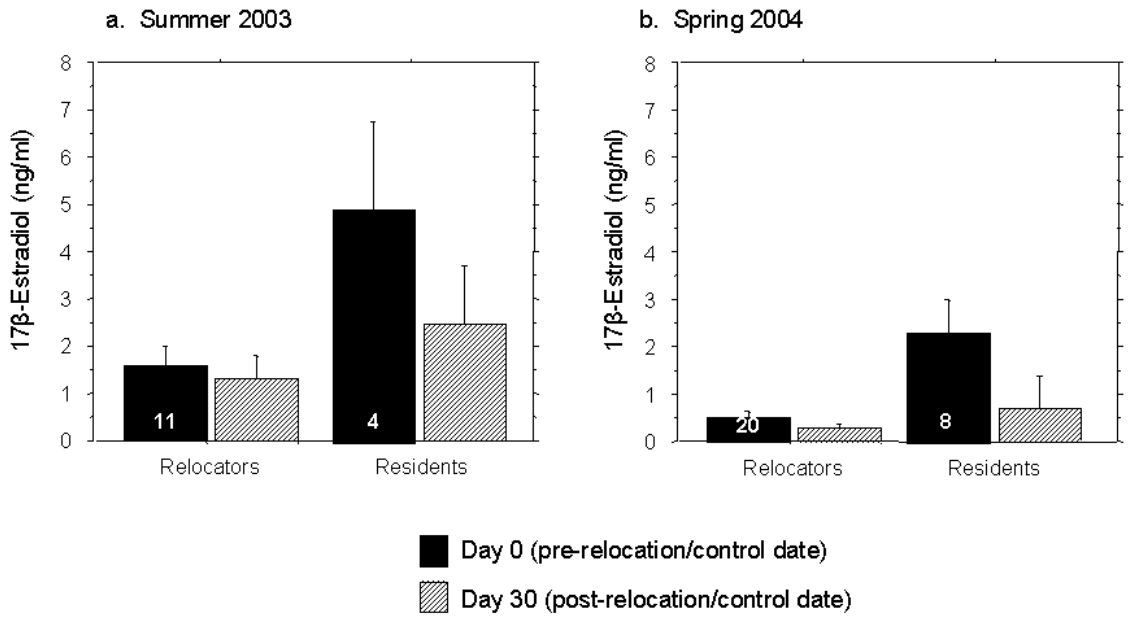



Figure 17. Change in 17β-estradiol from pre- to post-relocation or control date by relocator/resident status. There was an overall significant decrease in E from Day 0 to Day 30 in both (a) summer 2003 ($F_{1,10}$=5.13, p=0.05) and (b) spring 2004 ($F_{1,22}$=36.00, p<0.0001). In summer 2003, there was no effect of relocator/resident status ($F_{1,10}$=0.03, p=0.87), while in spring 2004 there was a significant relationship between E and relocator/resident status ($F_{1,22}$=14.91, p=0.0008).

# CHAPTER FOUR

# THE PHA CHALLENGE AS A MEASURE OF LYMPHOID RESPONSE IN GOPHER TORTOISES (*GOPHERUS POLYPHEMUS*)

## Introduction

The increased interest in infection and disease in wild animal populations has prompted many researchers to include measures of immune function in conservation-based studies. These measures of immune function can then be related to metabolism (Martin et al., 2003; Ots et al., 2001), social behavior (Hawley et al., In press), mating success and reproduction (Rolff, 2002), and ultimately, energetic trade-offs that affect fitness and survival (Lochmiller & Deerenberg, 2000; Møller & Saino, 2004; Norris & Evans, 2000).

Avian literature has provided the most extensive presentation of field-based techniques for measuring immune response. For example, Matson et al. (2005) developed a hemolysis-hemagglutination assay that requires minimal handling and blood sampling to examine avian species differences in natural antibody-mediated complement, an innate humoral immune response. Still others have used sheep red blood cells (SRBC) in order to examine antibody response (Boa-Amponsem et al., 2000; Deerenberg et al., 1997; Hawley et al., In press). Field-based immune measures are also used with other taxa, including both mammals (Heise & Van Acker, 2000; Wronski et

al., 2003) and reptiles (Berger et al., 2005; Kahn et al., submitted; Merchant & Britton, 2006).

The cell-mediated immune response, directed by T cells, is of particular interest to many researchers because of its critical role in the acquired immune response. T cells not only interact directly with pathogens to eliminate them, but they release cytokines that influence the innate response and they are obligatory prerequisites for the full activation of the humoral immune response. T cells mature in the thymus, a primary lymphoid organ that responds in size, and likely in function, to changes in the environment (Martin, 1976). Therefore, measures of cell-mediated immune function, and T cell response specifically, may provide information regarding the physiological condition of an animal as it relates to the environment.

The PHA (phytohemagglutinin) challenge has been used in ecological studies for many years as a relatively simple measure of adaptive, cell-mediated immune function. PHA is derived from red kidney bean (*Phaseolus vulgaris*) extract and is a potent T cell mitogen and leukoagglutinator (Geppert, 1998). Since T cells are sensitive to environmental factors (El Ridi, 1998), the PHA challenge is often used to determine the responsiveness of an animal's cell-mediated immune system to environmental changes (Smits et al., 1999). For example, Northern leopard frogs (*Rana pipiens*) demonstrate a decrease in *in-vitro* T cell proliferation in response to an injection of PHA when they are exposed to low temperatures for extended periods of time (Maniero & Carey, 1997). Similarly, *Mauremys caspica*, a turtle that hibernates in cold winter months, has shown seasonal variation in *in-vitro* T cell responses to PHA (Muñoz et al., 2000). In addition, nestling bluebirds (*Sialia mexicana*) exposed to high levels of lead shot show a

significantly lower *in-vivo* T cell response to PHA than birds in low exposure and lead-free treatment groups (Fair & Myers, 2002). Finally, lymphocyte proliferation, as determined by the *in-vitro* response to PHA, is enhanced in loggerhead sea turtles (*Caretta caretta*) that are exposed to organochlorines in their environment (Keller et al., 2006). Therefore, the PHA challenge can be an effective tool for studying the relationship between cell-mediated immune response and environmental factors across many taxa.

It is well established that turtles and tortoises have thymus-derived splenic lymphocytes, often referred to as T-like cells (El Ridi, 1998), and these cells are able to be cultured *in-vitro* (Ulsh et al., 2001). Like the T cells of other animals, turtle T cells are sensitive to environmental factors, and can be depleted in adverse conditions (El Ridi, 1998). Therefore, challenging turtles and tortoises *in-vivo* or *in-vitro* with a T cell mitogen, like PHA, has the potential to provide information about the physiological condition of the animals, which may be related to habitat, disease, and other conservation factors. For example, green turtles (*Chelonia mydas*) diagnosed with symptomatic fibropapillomatosis have demonstrated impaired *in-vitro* immune responses to whole blood stimulation with PHA, as compared with healthy controls (Cray et al., 2001; Work et al., 2001). The PHA challenge is an effective measure of immune response in turtles, and with further study, it may be used as one of several indicators of immune status and health.

One animal that could benefit from immune assessment using PHA is the gopher tortoise (*Gopherus polyphemus*), a threatened species whose native habitats, the longleaf pine forest and upland sandhills of the southeastern United States, have become

increasingly fragmented. In addition to being subjected to extensive habitat disturbance, these tortoises are susceptible to a bacterial illness, Upper Respiratory Tract Disease (URTD). Therefore, the general cell-mediated immune response may be an important variable to monitor in these animals as it may provide insight through correlational analysis into the development and progression of URTD. Ultimately, evaluating gopher tortoises' responses to a PHA challenge may allow us to better manage populations with disease.

To that end, we conducted a preliminary *in-vivo* PHA study on gopher tortoises and found that these animals exhibit a maximum swelling response to a PHA challenge at 12 hours post-injection (Kahn et al., submitted). However, the 12 hour time frame is consistent with an innate, but not necessarily an adaptive immune response (Munford, 2004). In addition, other non-reptilian species that have been challenged with PHA show substantially different response times than those established for gopher tortoises. For example, in human blood stimulated *in-vitro* with PHA, lymphocytes increase RNA synthesis and become enlarged in 24 to 48 hours post-exposure, with DNA synthesis at approximately 72 hours post-exposure (Sasaki & Norman, 1966). In addition, many bird species, including zebra finches (Smits & Williams, 1999), American goldfinches (Navara & Hill, 2003), tropical house sparrows (Martin et al., 2005), and western bluebirds (Fair & Myers, 2002), among others, respond to an *in-vivo* PHA challenge in 24 hours. Therefore, we question whether PHA stimulates a T cell response in tortoises in 12 hours, or if it instead initiates an innate immune response characterized by the presence of edema and macrophages in the injected tissue. We conducted a histological assessment of the swelling response at 12 hours and 24 hours following an *in-vivo* PHA

challenge to determine if it caused a lymphoid, cell-mediated immune response in gopher tortoises, as it does in other animals. We also compared the lymphoid response between biopsied tissue samples of PHA- and saline-injected tortoises at those time points.

**Materials and Methods**

*Study Site and Trapping*. A total of 58 gopher tortoises were trapped at Fort Benning in Georgia from April through June 2004. These tortoises were part of a larger group being studied in a concurrent research project. Live wire traps (Tomahawk Live Trap Company, custom order) were placed at the mouth of active gopher tortoise burrows. The floor and foot pedal of the trip mechanism of each trap were partially covered with sand from the burrow apron. The entrance to the burrow and the trap were covered with a $1m^2$ piece of burlap to make the trap appear to be an extension of the burrow to encourage the tortoise to enter, and to provide a shady area to prevent trapped tortoises from overheating. The traps were set no later than 08:00 and they were checked twice daily until tortoises were caught. Tortoises were never left in a trap for more than 12 hours because gopher tortoises do not show a significant increase in corticosterone (the hormone associated with the stress response) if left in a trap for up to 12 hours (Ott et al., 2000).

Upon capture, tortoises were each placed in an individual 37.85 liter Rubbermaid® bin for transport. The cover and sides of the bin were punctured with numerous 1.5 cm air holes, and the bottom of the bin was filled with 5 cm of sand from

the burrow apron. The tortoises were transported to the field lab after all traps were checked (within 4 hours of the first trap check).

*PHA Challenge*. We conducted the PHA challenge *in-vivo* as opposed to *in-vitro* to ensure that the immune responses included the body's physiological regulatory influences (Pechhold et al., 2002). At the field lab, each tortoise was placed with its carapace face down on the research bench and a rear leg was stabilized manually out of the shell. A non-toxic permanent marker was used to make a small marking on the ventral surface of the skin flap that connects the medial aspect of the gopher tortoise's thigh to the abdominal region. A measurement of skin flap thickness was taken at the marking using a digital micrometer (W.W. Grainger, Mitsuoyo, 0-25mm) to the nearest thousandth of a millimeter. The measurement was taken three times and an average of the three measurements was recorded. Then a 0.5ml solution of 2mg/ml PHA-P (Sigma-Aldrich PHA-P, lectin from *Phaseolus vulgaris*) mixed with phosphate buffered saline was given to the tortoise subcutaneously at the marking where the measurement was taken. Dosing and timing of measurements were previously established by Kahn et al (submitted). As a control, ten tortoises received the same protocol in the ventral skin flap on the other medial thigh with a 0.5ml saline injection. At 12 hours post-injection, the swelling resulting from the PHA and saline injections was measured three times using a digital micrometer and the average recorded. Half of the tortoises were randomly selected to receive a biopsy of the swelling at 12 hour post-injection using a 3mm sterile dermal biopsy punch (Miltex). The biopsied tissue was excised using a scalpel and placed individually in a 1.5ml eppendorf with 10% formalin. After 72 hours,

the tissue was transferred to another 1.5ml eppendorf containing 70% ethanol until histological processing. In event of bleeding that occurred as a result of the biopsies, direct pressure was applied to the biopsied location and styptic powder was used when necessary. At 24 hours post-injection, the injected skin flaps of the remaining tortoises were again measured three times using a digital micrometer and the average was recorded. These tortoises then underwent the same biopsy procedure as the tortoises at 12 hours post-injection. The same researcher conducted all measurements and biopsies (PFK). All tortoises were returned to their burrows of capture after the 24 hour biopsies were conducted. Tortoises were trapped again 30 days post-injection as part of a concurrently running study, and only four of the 108 tortoises showed minor infections (red skin, generalized swelling, evidence of discharge) in the area of the injection.

The tissue samples taken at 12 and 24 hours post-injection were embedded in paraffin and sectioned at 10μm. All sections of each tissue sample were collected and placed on slides that were then stained with hematoxylin and eosin (H&E). Each set of slides from an individual tortoise was reviewed using an Axiovert light microscope. For each tortoise, a single tissue section was chosen from that individual's set of slides for further examination. The particular section that was selected for each tortoise had a clear, single layer of cells which accurately represented the cellular composition of the other tissue sections. This section was then examined more closely and systematically at magnifications of 2.5x, 10x, and 20x, and cellular responses were classified and recorded (details below).

We originally intended to characterize the cellular response to PHA by counting lymphocytes, heterophils, macrophages, eosinophils, and basophils, similar to the

methods used by Martin et al. (2006). However, with careful review, we found lymphocytes to be the primary cell type found in the responses, with few other types of immune cells present. As a result, we used an alternative two-step categorization process. First we categorized the immune responses based on the size of the focal accumulations of the lymphoid cells using the categories presented in Table 2: negligible accumulations (3-10 cells), small (11-30 cells), medium (31-50 cells), large (51-100 cells), and extra large (101+ cells). Using those categories, we then scored the overall responses from 1 to 4 according to the classifications in Table 3. A minimum score of 1 indicated a negligible response containing 1 or more negligible lymphoid accumulations (of 3 to 10 cells each). A maximum score of 4 indicated a marked response characterized by at least one extra large accumulation (101+ lymphoid cells), as well as other size accumulations. All scoring was conducted by three trained reviewers. The scoring was done blindly, without the reviewers being aware of which slides were experimental and which were controls. In addition, each reviewer provided a score without having seen the scores provided by the other reviewers and without consulting one another.

*Data analysis*. Pre- and post-swelling measures were compared using a repeated measures analysis of variance (ANOVA). In addition, percent change in swelling was calculated using the following equation:

$$\frac{(\text{post - injection swelling}) - (\text{pre - injection swelling})}{(\text{pre - injection swelling})} \times 100$$

The percentages had to be ArcSin transformed to ensure normality, but this requires all percentages to be greater than 0. Therefore, a constant (34) was added to the percentages prior to the transformation.

A coefficient of variance was calculated for the three scores given for each slide using the following equation:

$$\frac{\text{standard deviation} * 100}{\bar{x}}$$

The average coefficient of variance was 19.09%. Since this percentage indicates relatively low variance among reviewers' assessments, we averaged the individual scores to conduct further statistical analyses. Scores for tissue slides were compared by treatment and time using two-way ANOVAs. Data were considered statistically significant when $p<0.05$. The statistical software program JMP version 6.0.0 (SAS Institute Inc.) was used to conduct all statistical analyses.

**Results**

Using a repeated measures ANOVA on the measures of skin flap thickness, we found that the PHA injection caused a significantly greater swelling response in tortoises than that caused by the saline injection at both 12 and 24 hours post-injection ($F_{2,51}=10.54$, $p<0.0001$, Figure 18). The PHA injection (i.e., excluding the saline injection) caused a significant increase in swelling at the site of injection at both 12 hours and 24 hours post-injection ($F_{2,41}=42.75$, $p<0.0001$), though there was no significant difference in swelling between those two time points ($p=0.54$). The saline

injection caused no increase in skin flap thickness at 12 or 24 hours post-injection ($F_{2,10}$=0.45, p=0.65).

A total of 58 biopsies were conducted on tortoises that received PHA; 27 tortoises at the 12-hour measure and 31 tortoises at the 24-hour measure. In addition we conducted biopsies on 10 tortoises that received saline as a control; 4 tortoises at the 12-hour measure and 6 tortoises at the 24-hour measure. All biopsies were conducted identically and we assumed that all tortoises had equal vascularization. Overall, we found that the PHA injection caused a lymphoid response that was not present in biopsies from control samples (Figure 19). In fact, lymphoid cells were the most predominant cell type in biopsies taken from PHA-injected tortoises. In general, the response to the PHA injection was primarily characterized by angiocentric inflammation and focal accumulations of lymphoid cells around venules, capillaries, and lymphatic vessels. Most samples also had a small number of macrophages and heterophils present, and nine samples (none of which were controls) were infused with heterophils, indicating infection at the injection site (Figure 20).

According to the lymphoid response scoring system, tortoises that were injected with PHA showed a significantly stronger lymphoid response than tortoises that received saline ($F_{1,64}$=10.47 p=0.002), regardless if the sample was taken at 12 or 24 hours ($F_{1,64}$=0.004, p=0.95, Figure 21). At the twelve hour measure, tortoises that were injected with PHA (i.e., not saline) received a mean score of 2.5 indicating that they had at least one, but likely more than one large lymphoid accumulation and several small and medium-sized accumulations in their biopsies. Tortoises that received the saline injection had a mean biopsy score of only 1.5 at the 12-hour measure and 1.3 at the 24-

hour measure indicating that they showed primarily negligible, if any, lymphoid accumulations at both time points ($F_{1,8}$=1.10 p=0.33).

**Discussion**

A previous study found that gopher tortoises mount a maximum swelling response to an *in vivo* PHA challenge at 12 hours post-injection (Kahn et al., unpublished data). In this study we found that gopher tortoises mount a maximum lymphoid response to PHA that lasts from 12 to 24 hours post-injection. Despite our expectations that tortoises would exhibit an innate response at 12 hours post-injection, we identified perivascular lymphocyte accumulations at that time point that were similar to those described in biopsies of chickens tested *in-vivo* with PHA at 24 hours (Goto et al., 1978). Although we did not use immunocytochemistry to characterize the lymphocytes as T cells or T-like cells as opposed to other cell types, we are confident that lymphocytes were accurately identified in our samples for several reasons. First, the lymphocyte accumulations at both 12 hours and 24 hours post-injection were not likely B cells since it takes substantially longer to mount a B cell response. Secondly, we do not believe that the cells we identified as lymphocytes were thrombocytes, despite their morphological similarities in reptiles (Alleman et al., 1992) because we did not document any cells in our tissue samples that resembled thrombocytes according to the description provided by Mader (1996). Third, there were few cell types present in the gopher tortoises' responses to PHA, so we were clearly able to differentiate the presence of macrophages from lymphocytes in the tissues. It is not surprising that we found macrophages because it has been previously established that monocytes (the precursors

to macrophages) and the cytokines they release (IL-6 specifically) are required for the successful proliferation of PHA-stimulated T cells (Ceuppens et al., 1988). In addition, macrophages have also been positively identified in biopsies of chickens injected with PHA (Goto et al., 1978).

It was clear from this study that the saline control caused no significant change in the extent of swelling or lymphoid response. We encountered similar findings in a previous study in which we examined only the swelling response (Kahn et al., submitted). If future studies continue to find that saline controls result in no swelling response in gopher tortoises, we suggest that experiments using PHA on gopher tortoises eliminate the use of saline controls, as has been suggested for birds (Smits et al., 1999).

It is critical that we study the immune status of threatened species like the gopher tortoise as just one of several integrated factors that may assist in our attempt to conserve their populations. Our findings indicate that using the PHA challenge as a measure of cell-mediated immune response has the potential to elucidate one aspect of gopher tortoises' immunocompetence. However, Martin et al. (2006) caution against equating PHA-related swelling specifically with cell-mediated immunocompetence due to the complex nature of immune responses. For that reason, we propose that establishing a normal range of responses to this challenge, and using the results in coordination with other immune and physiological measures, will allow us to better evaluate the health and immune status of gopher tortoises.

**Acknowledgements**

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| Classification of Lymphocyte Accumulations | Number of Cells per Accumulation |
|---|---|
| Negligible | 3-10 |
| Small | 11-30 |
| Medium | 31-50 |
| Large | 51-100 |
| Extra large | 101+ |

Table 2. Classification of lymphocyte cell accumulations in response to the PHA challenge. In order to score the tissue slides from the biopsies taken during the PHA challenge, lymphocyte accumulations were first classified according to the size (number of cells) per accumulation.

| Score | Response/ Accumulations | Negligible | Small | Medium | Large | Extra Large |
|---|---|---|---|---|---|---|
| 1 | None | 1+ | 0 | 0 | 0 | 0 |
| 2 | Mild | 0+ | 1-10 | 1-10 | 1 | 0 |
| 3 | Moderate | 0+ | 0+ | 11-20 | 2+ | 0 |
| 4 | Marked | 0+ | 0+ | 0+ | 2+ | 1+ |

Table 3. Overall lymphocyte response to the PHA challenge. Tissue slides from the biopsies taken during the PHA challenge were given a final score of 1 to 4, depending on the number and size of the lymphocyte accumulations. A score of 1 indicates no response to the PHA/saline injection and means that there were either no lymphocyte accumulations in the tissue or that the accumulations that existed were no larger than a negligible size (3-10 cells). A score of 2 indicates a mild response to the PHA/saline injection; the tortoises showed up to 10 small lymphocyte accumulations, up to 10 medium sized accumulations, and/or a single large lymphocyte accumulation. A moderate response with a score of 3 indicates that the tortoises showed at least 11 medium sized lymphocyte accumulations and/or 2 large accumulations. Finally, a tortoises was given a score of 4 indicating a marked response if they responded to the PHA/saline injection with at least 2 large lymphocyte accumulations and/or one extra large accumulation.

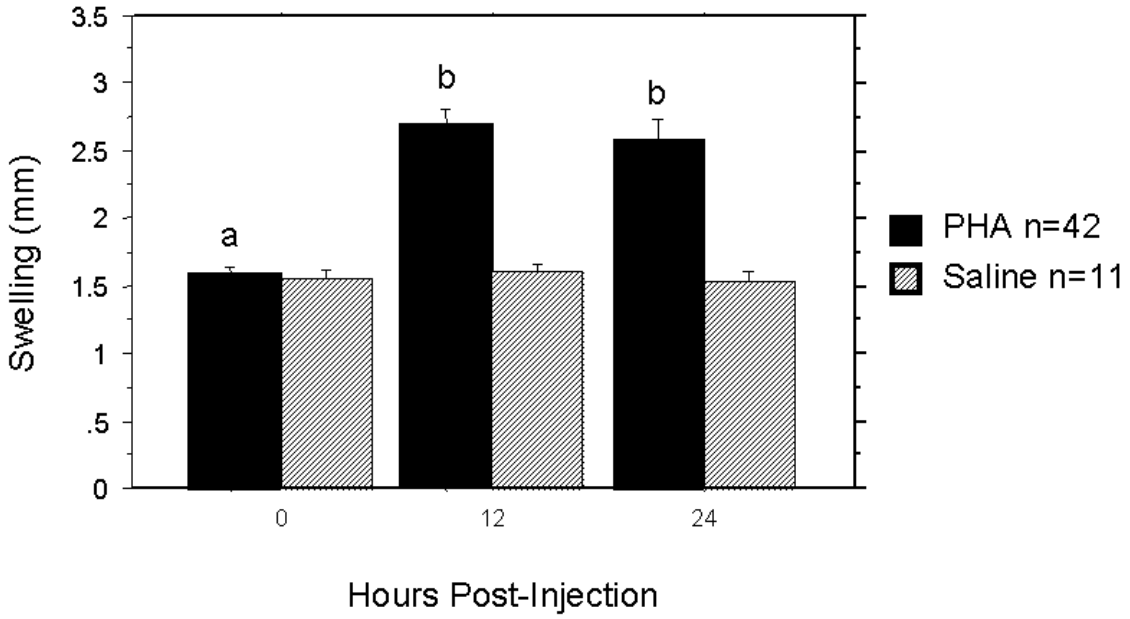


Figure 18. Swelling response to PHA Challenge at 12 hours and 24 hours post-injection ($F_{2,51}$=10.54, p<0.0001). The PHA injection (i.e., excluding the saline injection) caused a significant increase in swelling at the site of injection at both 12 hours and 24 hours post-injection ($F_{2,41}$=42.75, p<0.0001), though there was no significant difference in swelling between those two time points (p=0.54). The saline injection caused no increase in swelling at 12 or 24 hours post-injection ($F_{2,10}$=0.45, p=0.65).

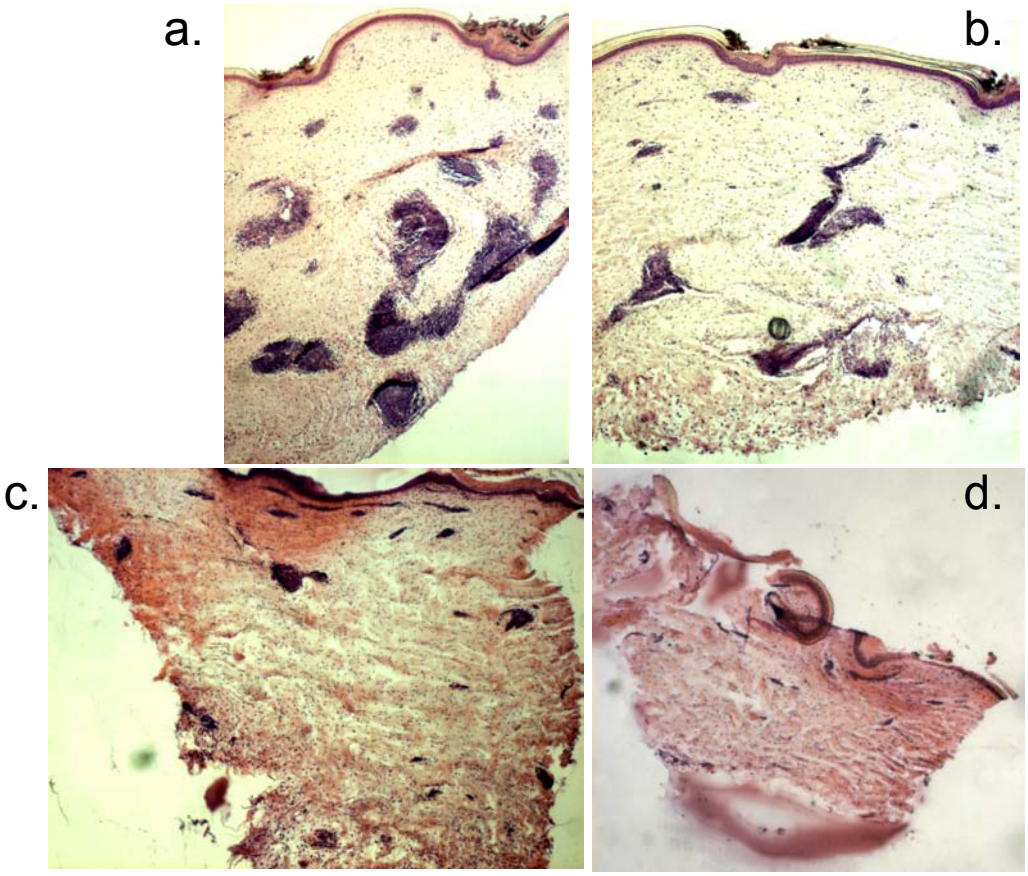


Figure 19. Photographs of lymphoid responses to PHA using Axiovert light microscope (2.5x). (a) Marked lymphoid response (score of 4) - lymphoid cells were the most predominant cell type in biopsies taken from PHA injected tortoises. In general, the response to the PHA injection was primarily characterized by angiocentric inflammation and focal accumulations of lymphoid cells around venules, capillaries, and lymphatic vessels; (b) Medium lymphoid response; (c) Mild lymphoid response; (d) Negligible lymphoid response, primarily documented in saline-injected, not PHA-injected tortoises.

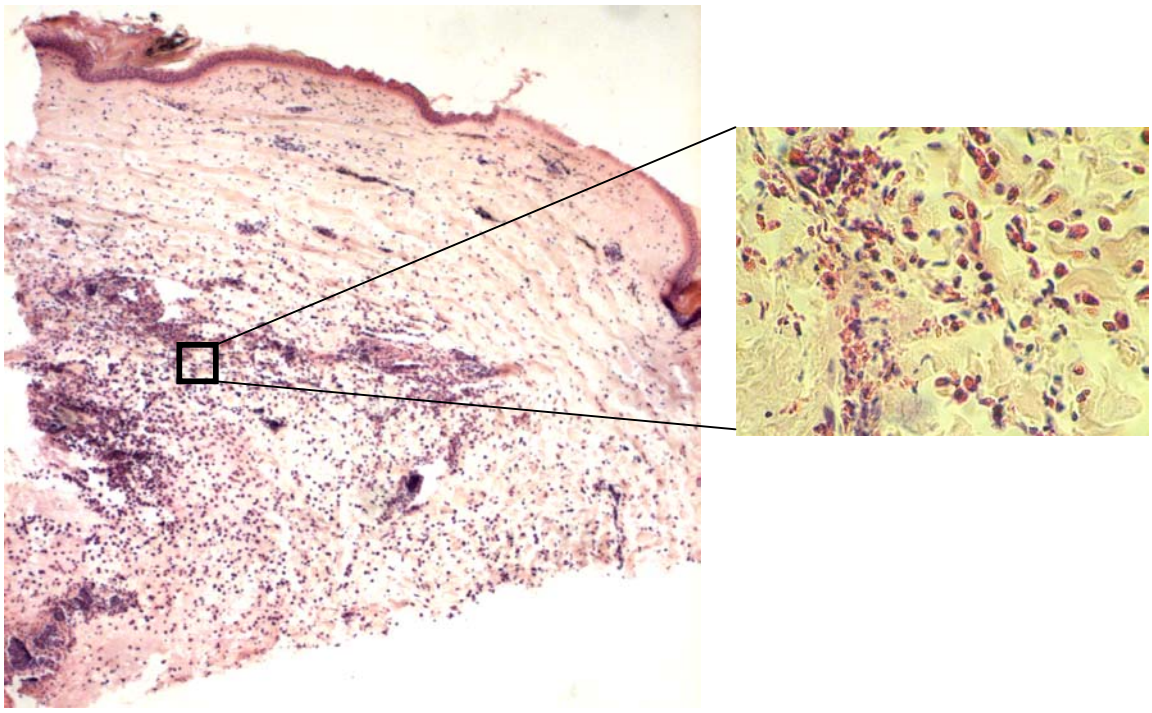

Figure 20. Photographs of tissue sample infused with heterophils (granular cells) at the site of injection 12 hours post-injection: 2.5x (left) and 20x (right).

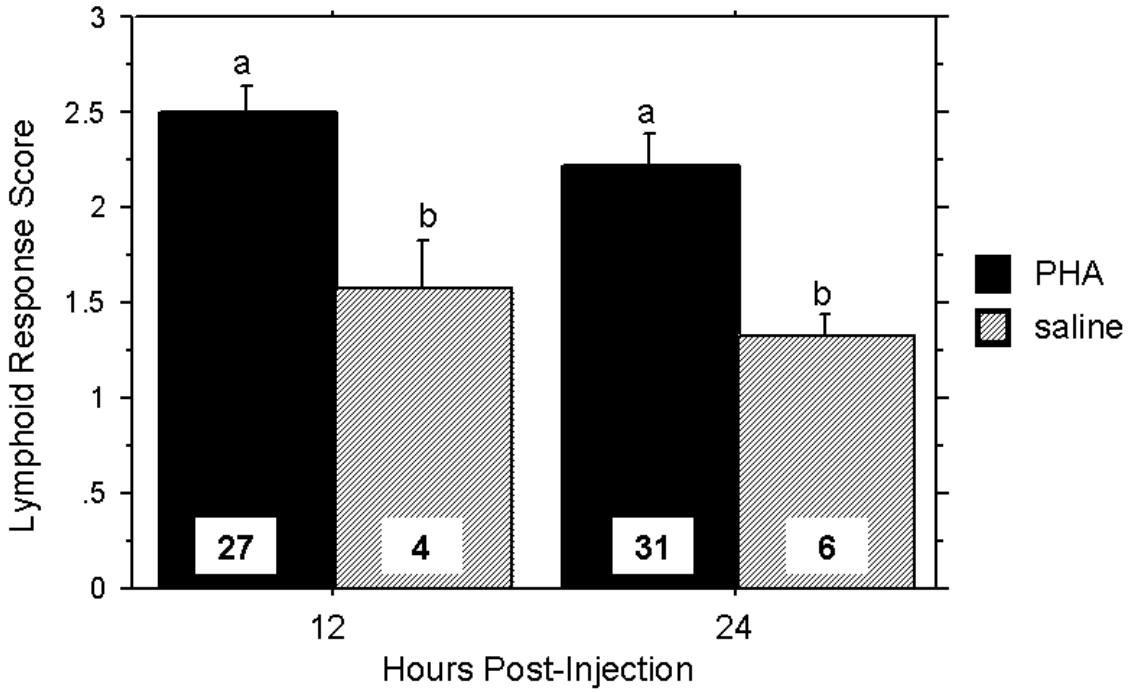


Figure 21. Lymphoid response to PHA and saline at 12 hours and 24 hours post-injection. According to the lymphoid response scoring system, tortoises that were injected with PHA showed a significantly stronger lymphoid response than tortoises that received saline ($F_{1,64}$=10.47 p=0.002), regardless if the sample was taken at 12 or 24 hours ($F_{1,64}$=0.004, p=0.95).

# CHAPTER FIVE

# THE EFFECTS OF RELOCATION ON UPPER RESPIRATORY TRACT DISEASE AND IMMUNE STATUS IN GOPHER TORTOISES (*GOPHERUS POLYPHEMUS*)

## Introduction

A critical aspect in the conservation of threatened wildlife species involves monitoring of health status and immune function (Meffe 1999; Scott 1988). Global changes, often caused by anthropogenic activities, can result in environmental stressors, including habitat loss, climate change, and environmental contamination (Daszak et al. 1999). One potential impact of these environmental stressors is that they can lead wildlife populations to become more susceptible to disease outbreaks, either through existing or novel pathogens, which can result in changes in population densities (Daszak et al. 2000; Deem et al. 2001; Scott 1988). Therefore, worldwide population declines in wildlife species have prompted studies that explore the idea that infectious agents may be major contributors to our biodiversity crisis (Deem et al. 2001).

Translocation is used extensively in wildlife management practice to protect threatened species in their natural habitats. However, translocation can cause changes in population density, habitat, social structure, and food selection, and as a result, may serve as a stressor to both the relocated animals and the resident populations (Deem et al.

2001). Animals experience stress through the highly conserved response of the hypothalamo-pituitary-adrenal axis, which results in the release of corticosterone. It is well established that the neuroendocrine and immune systems are highly integrated, and that chronic increases in corticosterone can results in changes, usually decreases, in immunocompetence (Apanius 1998; Chrousos & Gold 1992; Maule & VanderKooi 1999). Therefore, if translocation is a stressor, then the process of relocating animals may also decrease immunocompetence, thereby negating the use of this technique for conservation purposes.

The gopher tortoise (*Gopherus polyphemus*) is a species native to the southeastern United States that frequently undergoes relocation as a method of conservation. Due to extensive habitat loss, the gopher tortoise is federally listed as a threatened species in the western portion of its range (southwestern Alabama, Mississippi, and Louisiana), and it is considered a species of special concern in most of the eastern portion (Georgia and Florida). Despite the increased use of relocation as a means to protect this species, no data are available to document the relationship between relocation and changes in immune function. This relationship is particularly important for gopher tortoises because they are susceptible to Upper Respiratory Tract Disease (URTD), a bacterial infection caused by *Mycoplasma agassizii* (Brown et al. 2001; Brown et al. 1999b). Clinical symptoms of this strain of mycoplasmosis include ocular and palpebral edema, conjunctivitis, purulent ocular discharge, serous, mucoid, or purulent discharge from the nares, difficulties in breathing, and lethargy, and the combination of these symptoms can lead to dehydration, emaciation, and ultimately death as a result of cachexia (Jacobson et al. 1991; Schumacher et al. 1997). It has been

suggested that mycoplasma infection may be chronic and/or cyclic in gopher tortoise populations, and that anthropogenic or environmental stressors, including relocation, may induce symptomatic disease (Diemer Berish et al. 2000; McLaughlin et al. 2000).

Upper Respiratory Tract Disease was first identified in a population of desert tortoises (*Gopherus agassizii*) in the western Mojave Desert of California in 1988 (Jacobson et al. 1991). Only a year later, in 1989, URTD was documented for the first time in a population of gopher tortoises located on Sanibel Island off the coast of Florida (McLaughlin et al. 2000). Since that time, studies have documented dramatic population declines in both species, and cited URTD as one of the main contributing factors (Berry 1996; Gates et al. 2002; Rabatsky & Blihovde 2000; Seigel et al. 2003). As a result, most studies of these species now use an enzyme-linked immunosorbent assay (ELISA) to determine if the animals have ever been exposed to *M. agassizii* (Schumacher et al. 1993). Use of this technique allows researchers to document individuals and populations affected by *M. agassizii*.

While the actual presence of infection and disease is not easily identifiable in many wild animal populations (Scott 1988), it is possible to determine mycoplasmal infection in gopher tortoises by conducting polymerase chain reaction (PCR) on the 16S rRNA gene sequence of DNA samples (Brown et al. 1995). Identifying the presence of *M. agassizii* is essential because the clinical signs of mycoplasmosis often overlap with those of other infectious agents, such as herpesvirus and iridovirus (Jacobson 1993; Origgi & Jacobson 2000; Westhouse et al. 1996).

Although measuring actual infection and antibody status are important, conducting general immune measures of cell-mediated and humoral immune response

may be beneficial in determining overall health status. Numerous studies have challenged animals with innocuous agents, such as phytohemagglutinin (PHA) and sheep red blood cells (SRBC) to elicit general T cell and antibody immune responses, respectively. Results from these challenges have the potential to provide us with a well-rounded picture of an animal's health and have been used successfully as biomarkers of health status. For example, green sea turtles (*Chelonia mydas*) afflicted with fibropapillomatosis show significantly lower T cell responses to PHA than healthy turtles (Cray et al. 2001; Work et al. 2001). Therefore, if an animal that appears to be healthy and asymptomatic for disease responds weakly to a PHA challenge, it could indicate the presence of a disease process which would warrant further investigation.

Relocation has become a common conservation protocol for protecting gopher tortoises, but it has the potential to be a stressor for the animals, which could suppress immunocompetence and prevent populations from becoming established and stable in their new habitats. Therefore, it is important to examine changes in gopher tortoise health to determine if relocation is a successful conservation technique for this species. Although ELISA and PCR have become the standards for assessing disease status in gopher tortoises, they do not provide adequate information on overall health, particularly for those with sub-clinical or asymptomatic infection. Therefore, this study examines the results of these specific tests, and documents the immune responses of gopher tortoises to general immune challenges to determine how relocation affects gopher tortoise immune status and overall health. Prior to relocation and again 30 days post-relocation, we examined tortoises for the presence of URTD by measuring changes in antibody status and changes in the presence of the disease-causing bacterium,

*Mycoplasma agassizii*. We also examined changes in tortoises' general cell-mediated and humoral immune responses using PHA and SRBC challenges, respectively. We conducted these examinations during summer 2003 and again in spring 2004 in order to make seasonal comparisons.

## Materials and Methods

### *Relocation*

This study took place at Ft. Benning Army Base in Georgia. All relocations took place within the confines of the installation among several training compartments. During summer 2003 (June, July, and August) and spring 2004 (April and May), tortoises were relocated from their home habitats to one of two relocation sites, depending on their antibody status to *Mycoplasma agassizii* (positive or negative for antibodies, see ELISA antibody testing below). Home habitats and relocation sites were located 2 to 4 km apart. Relocated tortoises were placed inside a 3 hectare pen at the relocation site, both to increase site fidelity and to prevent the possible transmission of disease between relocators and residents. As controls, we also examined the resident tortoises living in the relocation habitats that did not experience a relocation. To further prevent disease transmission during the course of the study, all field and lab methods were sterile. All equipment that came into contact with a tortoise, including traps, transport bins, and measuring tools were thoroughly cleaned with hospital-grade disinfectant after each use.

After trapping tortoises in their home habitats on Day 0 (see trapping protocol below), a radiotransmitter (American Wildlife Enterprises, Model AWE-GA) was attached to the carapace of each relocated and resident tortoise using plumber's epoxy.

Then tortoises were transported in individual 37.85 liter Rubbermaid® bins to a climate controlled environment at a local, temporary holding facility. Upon receiving the ELISA antibody results (within 7 days), experimental tortoises were relocated to the 3 hectare holding pen at the relocation site where they were released at the burrow apron of inactive or abandoned burrows. Control tortoises were returned to their burrows of capture. However, if the burrow of capture was located within the confines of the 3 hectare holding pen, then the tortoises were placed at the closest available neighboring inactive or abandoned burrow outside the penned area (in all cases less than 6 meters away from the burrow of capture, and well within the normal home range). All of the following procedures were conducted prior to the relocation or control date (Day 0), and again 30 days post-relocation or control date (Day 30).

*Trapping and Blood Collection*

On Day 0 of the study, wire live traps (Tomahawk Live Trap Company, custom order) were set at the mouth of active gopher tortoise burrows no later than 08:00 and they were checked twice daily until tortoises were caught. Tortoises were never left in a trap for more than 12 hours because tortoises do not show a significant increase in corticosterone (the hormone associated with the stress response) if left in a trap for up to 12 hours (Ott et al. 2000).

Upon capture, the tortoise was removed from the trap and a 2ml blood sample was drawn from the tortoise's femoral vein using a 3ml heparinized syringe and 22 gauge needle. Blood samples were placed in a cooler with ice for transport. Tortoises were each given an individual identification number written on the carapace in a non-

toxic white paint pen. They were then placed in their individual bin for transport. The cover and sides of the bin were punctured with numerous 1.5 cm air holes, and the bottom of the bin was filled with 5 cm of sand from the burrow apron. The tortoises and blood cooler were transported to the field lab after all traps were checked (within 4 hours of the first trap check).

*Presence of Disease – URTD Antibody Testing*

At the field lab, the blood was centrifuged in a tabletop centrifuge, and the plasma was aliquoted for ELISA testing at the University of Florida's Mycoplasma Testing Laboratory to determine tortoises' antibody status to *Mycoplasma agassizii*, the bacterium that causes Upper Respiratory Tract Disease. Antibody status was provided categorically as negative, suspect, or positive, based on a titer system. A titer of 0 was considered antibody negative, and indicated that the tortoise had not been exposed to *M. agassizii*. Titers of 64, 128, and 256 indicated that the tortoise was positive for antibodies and had been previously exposed to *M. agassizii*. A titer of 32 was considered suspect by the University of Florida and they advise that these tortoises be retested in 6 weeks to properly determine their status. However, due to time constraints imposed by military training activities, we needed to relocate the tortoises immediately so for our purposes, tortoises that tested with a titer of 32 (n=5) were relocated along with the antibody positive tortoises in order to prevent the possible transmission of disease to the antibody negative population during relocation.

*Presence of Disease – PCR*

While at the field lab on Day 0, nasal swabs were conducted on each tortoise to determine if the animals were infected with the bacterium *M. agassizii*. The tortoise's head was held out of the shell and manually stabilized behind the occipital bone by one of the field technicians. A second field technician swabbed each naris using a single sterile mini-swab dipped in sterile saline. The swab was immediately placed back in the sterile collection tube with SP4 media and frozen at -20C until further processing.

All swabs were DNA extracted using the boil method. Briefly, swabs were placed in v-bottom screw cap tubes with 100µl sterile water and boiled at 100C for 10 minutes. Tubes were immediately placed in the freezer at -20C for another 10 minutes. Samples were then centrifuged at 13,000rpm for 5 minutes, and tested for extraction efficiency. Twelve samples showed no DNA after the boil method extraction so they were re-extracted using a High Pure PCR Template Preparation Kit (Roche), which yielded DNA in all but 3 cases.

PCR was conducted on all of the successfully extracted samples using the methods and primers described in Brown et al. (1995) and Brown et al. (1999a). Briefly, 5µl of each extracted sample was placed in a tube with 20.5µl dd$H_2O$, 5µl buffer, 4µl $MgCl_2$, 5µl dnTPs, 0.5µl Taq, and 5µl of each primer (Invitrogen, 16S-471F, specific for gram-positive bacteria; 16S-1055R, specific for the Genus *Mycoplasma*). Samples were placed in the thermocycler for a six-step process that took approximately 3 hours to complete. Gels for each sample, as well as positive and negative controls and a 100 step ladder, were run for approximately 12 minutes. Tortoises were assigned a status of PCR

positive if the band was present at 567bp, indicating the presence of DNA for *M. agassizii*, and they were assigned a status of PCR negative if no band was present.

*Cell-Mediated Immune Response - PHA Challenge*

During summer 2003, a non-toxic permanent marker was used to make a small marking on the ventral surface of the skin flap that connects the medial aspect of the gopher tortoise's thigh to the abdominal region. A measurement was taken at the marking using a digital micrometer (W.W. Grainger, Mitsuoyo, 0-25mm) to the nearest 0.001 mm. The measurement was taken three times and an average of the three measurements was recorded. Then 0.5ml of 2mg/ml PHA/phosphate buffered saline (Sigma-Aldrich PHA-P, lectin from *Phaseolus vulgaris*) was injected into the tortoise subcutaneously at the marking where the measurement was taken. Dosing and timing of measurements were previously established by Kahn et al. (unpublished data). As a control, some tortoises received the same protocol in the ventral skin flap on the other medial thigh with a 0.5ml saline injection. At 12 hours post-injection, the swelling resulting from the PHA and saline injections was again measured three times using a digital micrometer and averaged.

Several modifications were made to the PHA challenge during 2004. First, few tortoises were administered saline as a control for PHA during 2004 since tortoises in the 2003 study, as well as tortoises in a previous study (Kahn et al., unpublished data), established that the saline injection does not cause a swelling response. Secondly, an additional measurement of the swelling response was taken at 24 hours in a subset of tortoises to ensure that the previously established timing of the response was correct in

this particular population. Finally, after the post-injection measurements were recorded at 12 and 24 hours, a biopsy was conducted of the swelling using a 3mm sterile dermal biopsy punch (Miltex). The tissue samples were processed as described in Kahn and Mendonça (unpublished data). Briefly, we categorized the immune responses of gopher tortoises to PHA and saline injections based on the size of focal accumulations of lymphoid cells. We ultimately scored each response from 1 to 4, with 1 being a negligible response and 4 being a marked response.

*Humoral Immune Response - SRBC Challenge*

During summer 2003, while receiving the PHA injection on Day 0 at the field lab, 38 tortoises (28 relocators and 10 resident controls) also received a 5ml intraperitoneal injection of 10% unwashed SRBC (sheep red blood cells from defibrinated sheep blood, Colorado Serum Company, Denver, CO) mixed with phosphate buffered saline (PBS). Dosage was previously established by Kahn et al. (unpublished data). A 1ml blood sample was taken 30 days post-relocation to measure tortoises' antibody responses to SRBC using a hemagglutination titration assay (Hay et al. 2002; Wegmann & Smithies 1966). Briefly, in 96-well v-bottom plates, 20µl of plasma was serially diluted across 10 wells, each containing 20µl PBS, resulting in dilutions ranging from 1:2 to 1:1024. Well number 11, the negative control, contained only 20µl PBS (no plasma), and well number 12, the positive control, contained only 20µl anti-sheep hemolysin (Colorado Serum Company, Denver, CO). A total of 20µl of 2% washed SRBC/PBS suspension was added to each well, and the plates were incubated at room temperature for 24 hours. Wells with samples were compared with controls, and antibody titers were expressed as

the $\log_2$ of the highest dilution of plasma showing hemagglutination, which was equal to the well number. Titers ranged from 0 through 5, with 0 being no response and 5 being a maximum response. The SRBC challenge was not repeated in spring 2004.

*Data analyses*

Chi-square tests were used to compare nominal variables, such as URTD antibody status (positive or negative for antibodies) and PCR results (positive or negative for the presence of *M. agassizii*) by season, relocator/resident status, and sex. In some cases, sample sizes were less than five, so results should be viewed with caution. Chi-square tests via Wilcoxon/Kruskal Wallis tests of rank sums (for ordinal data) were used to analyze differences between relocators and residents in SRBC titers in summer 2003 and in cellular lymphoid response to the PHA challenge at 12 and 24 hours in spring 2004. Lymphoid response was also analyzed by sex using a chi square, but sample sizes for the SRBC challenge were too small to analyze the responses by sex.

A repeated measures analysis of variance (ANOVA) on Day 0 was used to compare the swelling responses of tortoises injected with PHA in one leg, and saline in the other. Due to the small samples size of tortoises that received saline during spring 2004, data for the two seasons were combined for the overall PHA versus saline analysis. When examining the Day 0 (pre-relocation or control date) results of only the tortoise skin flaps that were injected with PHA (i.e., excluding the ones that received saline), we found that the swelling responses varied, not by season, but by month ($F_{3,141}$=5.81, p=0.0009). Samples taken during the month of June were excluded due to small sample size (n=5). Therefore, all remaining analyses of PHA data were conducted

by month, instead of by season. Two-way and repeated measures ANOVAs were used to analyze PHA swelling by month and relocator/resident status, but sample sizes were too small to include sex as a variable.

The statistical software program JMP version 6.0.0 (SAS Institute Inc.) was used to conduct all statistical analyses. Data were considered significant when $p<0.05$.

**Results**

*Presence of Disease - Antibody Status to M. agassizii*

Prior to relocation, the percentage of tortoises that showed a positive antibody status to *M. agassizii* was significantly higher in spring than in summer, 43.5% versus 27.1%, respectively ($\chi^2$=4.52, df=1,153, p=0.03). However, there was no significant difference in antibody status by sex during either season (summer $\chi^2$=1.74, df=1,68, p=0.18; spring $\chi^2$=0.48, df=1,83, p=0.48). Thirty days after the relocation or control date, there was no significant difference in antibody status between relocators and residents during either season (summer $\chi^2$=1.76, df=1,36, p=0.18; spring $\chi^2$=0.02, df=1,60, p=0.88).

We also analyzed the antibody data from pre- to post-relocation or control date according to *change* in antibody status; we compared tortoises that changed their antibody status (i.e., from positive to negative or from negative to positive) with those that maintained their antibody status (i.e., remained positive or negative throughout the study). From pre- to post-relocation or control date, we found there was a significant difference in the number of tortoises that changed their antibody status versus those that maintained their antibody status, according to both season and relocator/resident status (season, $\chi^2$=13.05, df=3,94, p=0.004; relocator/resident status, $\chi^2$=12.30, df=3,94,

p=0.006, Figure 22). Specifically, there was no significant difference between relocators and residents during summer 2003 ($\chi^2$=3.81, df=2,34, p=0.14); none of the residents and only 2 relocators (7.1%) changed their antibody status during summer 2003, both from antibody negative to antibody positive. However, in spring 2004, 2 resident tortoises (10.5%) changed their status from antibody positive to antibody negative, while 16 relocators (37.2%) changed their status ($\chi^2$=9.94, df=3,56, p=0.01). Nine of the relocators (20.9%) in spring 2004 changed their antibody status from negative to positive and 7 relocators (16.3%) changed their status from positive to negative.

*Presence of Disease – PCR for M. agassizii*

Nasal swabs obtained prior to relocation in spring 2004 from 38 tortoises were analyzed using PCR to determine if the tortoises were infected with *M. agassizii*; 81.6% tested negative and the remaining 18.4% tested positive for the presence of the bacteria. While 80.7% of those that tested negative for the bacteria also tested negative for antibodies, 19.4% tested positive for antibodies, indicating that they had been exposed to the bacteria at some point in their lives.

Of those that tested positive for the bacteria on Day 0 (n=7), almost half (3 out of 7) did not test positive for exposure according to the ELISA test, indicating that they had not yet mounted an immune response to the presence of the bacteria. There was no significant sex difference in the presence or absence of *M. agassizii* ($\chi^2$=0.01, df=1,36, p=0.91).

Thirty days post-relocation or control date, there was no significant difference between relocators and residents for the presence of *M. agassizii* ($\chi^2$=0.64, df=1,27,

p=0.42). In addition, only 2 of the 12 tortoises that tested bacteria negative on Day 30 tested positive for antibodies (16.7%), while almost half of tortoises that tested positive for the bacteria tested negative for antibodies (47.1%). There was still no difference by sex ($\chi^2$=0.35, df=1,27, p=0.54).

We obtained PCR results for a total of 16 tortoises (11 relocators and 5 residents) both pre- and post-relocation or control date (Figure 23). Eight of those tortoises (5 relocators and 3 residents) maintained their infection status: 5 remained negative (4 relocators and 1 resident) and 3 remained positive (1 relocator and 2 residents). However, 6 relocators changed their status: 5 tested negative for the presence of the bacteria on Day 0, but tested positive on Day 30, while one relocator tested positive on Day 0 and negative on Day 30. Only two residents changed status, from negative to positive.

*Cell-Mediated Immune Response - PHA Challenge Swelling Response*

A one-way repeated measures ANOVA indicated that prior to relocation (Day 0), tortoises that received PHA showed a significantly greater swelling response (as determined by percent change in skin flap thickness at the site of injection) than those that received saline at both 12 hours and 24 hours post-injection ($F_{1,44}$=12.14, p=0.001), but there was no significant difference between the two time points ($F_{1,44}$=0.30, p=0.58). We focused further data analysis on the 12 hour measure.

When examining the Day 0 PHA results at 12 hours (i.e., excluding saline results), we found that there was a significant difference in swelling response by month ($F_{3,141}$=5.13, p=0.002); swelling responses obtained in August were significantly larger

than those obtained in April. Therefore, we combined April and May into a single field season (spring), and July and August into a separate field season (summer).

Thirty days after the relocation or control date, tortoises still exhibited a significantly greater swelling response to PHA than saline at both 12 and 24 hours post-relocation or control date ($F_{1,41}$=18.21, p=0.0001). When examining the results of only the PHA injections (i.e., excluding saline) at 12 hours on Day 0 and Day 30 using a repeated measures two-way ANOVA, we found that there was no significant difference between relocators and residents or field season ($F_{1,95}$=1.25, p=0.26). However, there was an overall significantly stronger response to the PHA challenge 30 days after the relocation or control date than on Day 0 ($F_{1,95}$=4.53, p=0.03, Figure 24).

*Cell-Mediated Immune Response - PHA Challenge Lymphoid Response*

A chi square test based on histological findings indicated that prior to the relocation or control date, tortoises that received PHA showed a significantly greater lymphoid response than those that received saline ($\chi^2$=7.34, df=1,47, p=0.006). There was also a significant difference by sex: males showed a significantly stronger response to the PHA challenge (i.e., excluding saline) than females ($\chi^2$=7.24, df=1,41, p=0.007). However, there was no significant difference in lymphoid response whether the response was examined at 12 or 24 hours ($\chi^2$=0.34, df=1,41, p=0.55). Furthermore, there was no significant difference in lymphoid response based on the month that samples were taken ($\chi^2$=2.30, df=1,41, p=0.12).

Thirty days post-relocation, tortoises that were injected with PHA again showed a significantly greater lymphoid response than those that received saline ($\chi^2$=11.98,

df=1,36, p=0.0005), and there was no significant difference in the responses between the 12 and 24 hour measures ($\chi^2$=1.81, df=1,29, p=0.17). In terms of relocation, there was no significant difference in the 12 hour PHA response (i.e., excluding the saline response) between relocators and residents 30 days post-relocation or control date ($\chi^2$=1.14, df=1,29, p=0.28, Figure 25). In addition, males and females showed no significant difference in their lymphoid response to the PHA challenge on Day 30 ($\chi^2$=2.40, df=1,29, p=0.12).

*Humoral Immune Response - SRBC Challenge*

In summer 2003, a total of 38 tortoises were administered the SRBC challenge (28 relocators and 10 resident controls). Titers indicating antibody response ranged from 0 (no response) through 5 (the strongest response). After the relocation or control date, 33 out of the 38 tortoises (86.8%) had a titer of 0, 1 or 2, while the remaining 5 tortoises ranged in titer from 2.5 to 5. According to a chi-square test, there was no significant difference in SRBC titer between relocators and residents ($\chi^2$=0.04, df=1,36, p=0.82, Figure 26), and no significant difference by sex ($\chi^2$=3.41, df=1,36, p=0.06). However, due to the small sample size (n<5 for each titer in the resident category), the results of the chi square test may be unreliable. The SRBC Challenge was not repeated in spring 2004.

**Discussion**

Conservation methods like relocation have the potential to detrimentally affect the health of the animals they are designed to protect. Overall, it does not appear from this study

that the act of relocation causes short-term changes in the disease status or immunocompetence of gopher tortoises. Overall, 30 days after the relocation or control date, we did not find significant differences between relocators and residents in their URTD antibody status (positive versus negative for antibodies to *Mycoplasma agassizii*), URTD PCR status (positive versus negative for the presence of *Mycoplasma agassizii*), cell-mediated immune responses, or humoral immune responses. However, we did find that significantly more relocated tortoises showed changes in their antibody status than residents. In addition, this study lasted only 30 days during each season, and we did not examine all of the factors that may be associated with the development or progression of disease, including innate measures of the blood's bacterial killing properties, natural antibodies and antibody-mediated complement (Matson et al. 2005; Tieleman et al. 2005). Therefore, it is possible that there may be longer term effects and other aspects of the immune system that were affected that we did not document.

While we identified only one difference between relocators and residents in their URTD status, we did find seasonal differences in both URTD and general immune variables. Specifically, we found a significantly greater percentage of antibody positive tortoises in spring than in summer, and tortoises showed a greater T cell response to PHA in August than in April. It is not surprising that we documented seasonal changes because immune response, including response to bacterial infection, is known to vary seasonally in many animals (Nelson & Demas 1996; Nelson et al. 2002 and references therein). In fact, studies have indicated seasonal variation in the immune responses of the full range of species, including birds (Møller et al. 2003), reptiles (Muñoz & De la Fuente 2001a), amphibians (Rollins-Smith 2001 and references therein), mammals

(Lochmiller et al. 1994) and fish (Zapata et al. 1992). While other studies of turtle species have indicated stronger responses to PHA in spring than in summer (Muñoz & De la Fuente 2001b), our study found the opposite to be true, a stronger response in summer than in spring. However, our findings could be related to the increased prevalence of antibody positive tortoises during spring because a previous study documenting the pathology of URTD in gopher tortoises found that tortoises that tested positive for antibodies to *M. agassizii* had smaller thymus glands (the location of T cell maturation) than controls (McLaughlin et al. 2000).

Aside from seasonal variation, we found that URTD antibody status (positive versus negative, according to titer) changed rapidly within the 30 days of the study. Little is known about reptile antibodies, so we do not know with certainty if antibody status can actually change within 30 days or if there is an issue with the ELISA test that quantifies antibody status. With any test, including the ELISA, there is a chance of false positive or false negative results. Therefore, the changes in antibody status that we documented may be the result of false test results or they could indicate genuine rapid changes in immune status. Other studies of gopher tortoises have also found that URTD antibody results vary greatly both within and between populations, and that results often change substantially when populations are re-tested (Smith et al. 1998). This puts conservation managers in a difficult position because gopher tortoises are regularly relocated based on a single ELISA test of antibody status. If antibody status can change in such a short time period, then the URTD testing protocol associated with relocation may not be a reliable source of data on which to depend when making critical conservation decisions.

In addition to changes in antibody status, we found that tortoises also changed their status in terms of infection with the bacteria. PCR results indicated that 9 out of 15 tortoises that tested negative for infection with *M. agassizii* prior to the relocation or control date tested positive 30 days later. It is highly unlikely that any of these tortoises contracted the bacteria as a result of handling during this study because all field and lab methods were sterile. All equipment that came into contact with a tortoise, including traps, Pesola, calipers, worktable, and transport/holding bins, was cleaned with the hospital-grade disinfectant Nolvasan prior to use with another tortoise. In addition, field technicians sprayed their hands with Nolvasan after contact with each tortoise.

Instead of contracting the bacteria during the course of the relocation study, we suggest two scenarios. First, these tortoises could have already been infected with the bacteria prior to the relocation study when they had originally tested negative for infection. The acts of trapping, handling, and relocating these animals (even if back to their home habitats) may have prompted the onset of an immune response in conjunction with a possible acute stress response which rendered them more vulnerable to the effects of the bacteria. Although a previous study that we conducted indicated that relocation is not a stressor for tortoises at 30 days post-relocation (Kahn and Mendonça, unpublished data), there may have been an acute stress response in the days following relocation that could have initiated an immune response resulting in the development of antibodies to a current, but latent infection with the bacteria.

A second scenario that explains changes in infection status involves the techniques used to collect DNA samples. The nasal swabs that we obtained from the gopher tortoises in this study were most likely not sufficient to collect adequate samples

to test for *M. agassizii*. Gopher tortoises' external nares open into a ventro-lateral depression in the skull. This depression is continuous with a large dorsal nasal cavity where *M. agassizii* can exist out of the reach of a mini-tip swab (McLaughlin et al. 2000). A preferred method for collecting samples to determine the presence of *M. agassizii* involves conducting a nasal lavage with sterile SP4 media that is flushed throughout the nasal cavities and collected in a sterile cup. This method is more likely to come into contact with bacteria deep in the nasal cavity where it can be flushed out, successfully collected and analyzed using PCR. Therefore, it is possible that many of our tortoises that tested negative for infection with *M. agassizii* on Day 0 actually were infected with the bacteria but we were not able to collect an adequate sample. This scenario could also explain why almost 20% of tortoises that tested negative for *M. agassizii* infection tested positive for antibodies to the bacteria.

Furthermore, half of the tortoises that tested positive for *M. agassizii* infection simultaneously tested negative for antibodies to the bacteria. In other words, half of the bacteria positive tortoises did not have a detectable immune response to the bacteria at the time that they were ELISA tested. This could happen in cases where tortoises were recently infected and did not have time to mount a humoral immune response to the pathogen. However, we suggest, as others have, that the bacteria can exist in the tortoise as a chronic, periodically latent infection (Brown et al. 1999a; Diemer Berish et al. 2000; McLaughlin et al. 2000). Conversely, we could also hypothesize that a tortoise may be infected with the bacteria making them so ill that they can no longer successfully mount an immune response. Longer term studies examining the relationships among antibody

response, presence of bacteria, and display of clinical symptoms may provide further insight into these hypotheses.

Tortoises showed low titers indicating a minimal humoral immune response when subjected to an experimental antigen challenge with sheep red blood cells. The overwhelming majority of immune responses (86.8%) ranged in titer from 0 to 2 on the hemagglutination titration assay. Two possible reasons exist. First, if the immune response is seasonal, as we have shown, then perhaps summer is a time when humoral immune response is low. A second possibility is that the tortoises were only given a single immunization, and humoral immune response sometimes requires multiple immunizations in order to achieve a maximum response. Third, tortoises may not mount a substantial response to sheep red blood cells if the antigen does not constitute an imminent threat to the health of gopher tortoises. Since the responses were so low in 2003 and presented little variation, we did not repeat the SRBC immune challenge in 2004.

Overall, relocation does not appear to affect URTD-specific or general immune status in gopher tortoises in the short-term (30 days). However, our study indicates that perhaps testing animals for disease using strictly cellular and molecular techniques is insufficient. While these methods do provide answers as to whether animals have been exposed to disease causing agents, or if they are currently infected, they do not elucidate the actual disease status when what we are genuinely asking is if these animals are *sick*. For example, Jacobson (1991) documented the extensive presentation of symptomatic disease in desert tortoises that all tested with the highest possible titer for exposure to *M. agassizii*. Clearly, we would consider these animals to be sick. In fact, one of the

tortoises was found dead several months after being tested. Conversely, in 1998, Smith et al. documented a population of 15 gopher tortoises in Florida that tested antibody positive for exposure to *M. agassizii*. However, none of these animals presented any symptoms of disease. Do we also consider these animals sick and a threat if exposed to antibody negative populations? Long-term monitoring of gopher tortoise populations using the current techniques will be necessary to determine the immunological effects of relocation, but we should expand our health-related data collection to incorporate the documentation of clinical symptoms. It will undoubtedly be beneficial to correlate the results with other physiological measures, such as adrenal and reproductive hormones. Ultimately, an integrative approach to studying disease status may allow us to better understand disease processes and lead us to utilize more successful conservation techniques to protect threatened and endangered species.

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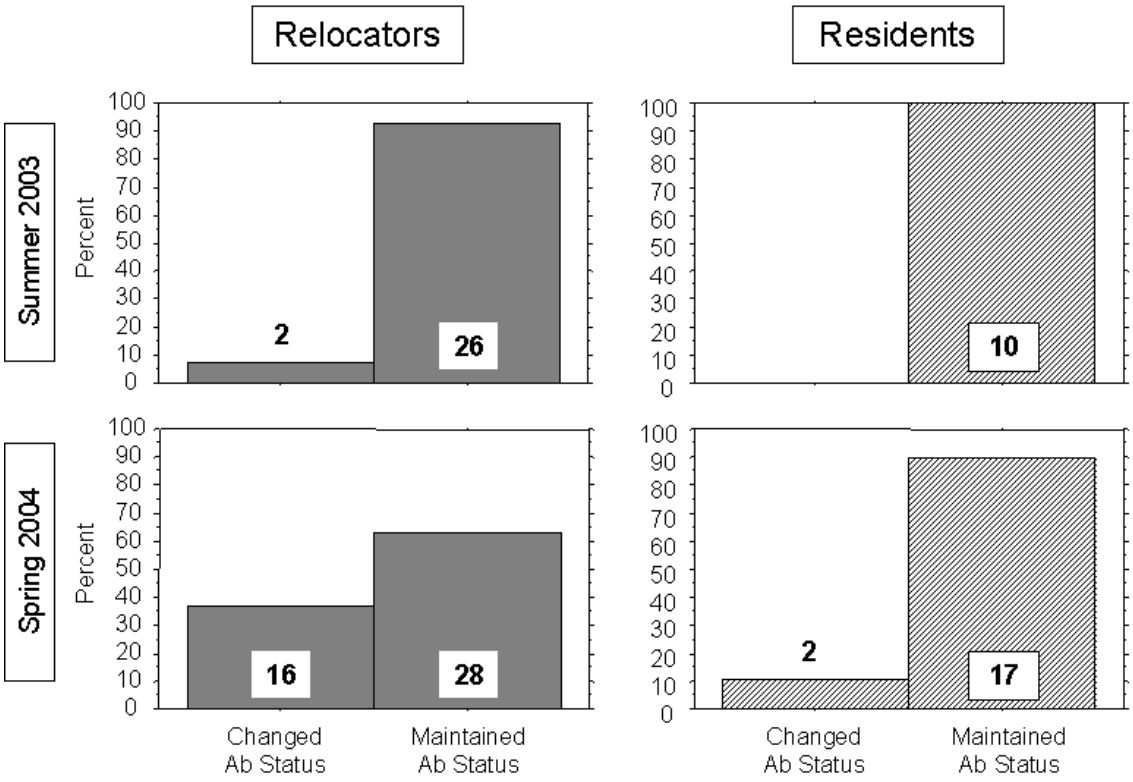


Figure 22. Change in antibody (Ab) status from pre- to post-relocation or control date. From pre- to post-relocation or control date, we found there was a significant difference in the number of tortoises that changed their antibody status versus those that maintained their antibody status, according to both season and relocator/resident status (season, $\chi^2$=13.05, df=3,94, p=0.004; relocator/resident status, $\chi^2$=12.30, df=3,94, p=0.006). There was no significant difference between relocators and residents during summer 2003 ($\chi^2$=3.81, df=2,34, p=0.14). However, in spring 2004, 2 resident tortoises (10.53%) changed their status from antibody positive to antibody negative, while 16 relocators (37.21%) changed their status ($\chi^2$=9.94, df=3,56, p=0.01).

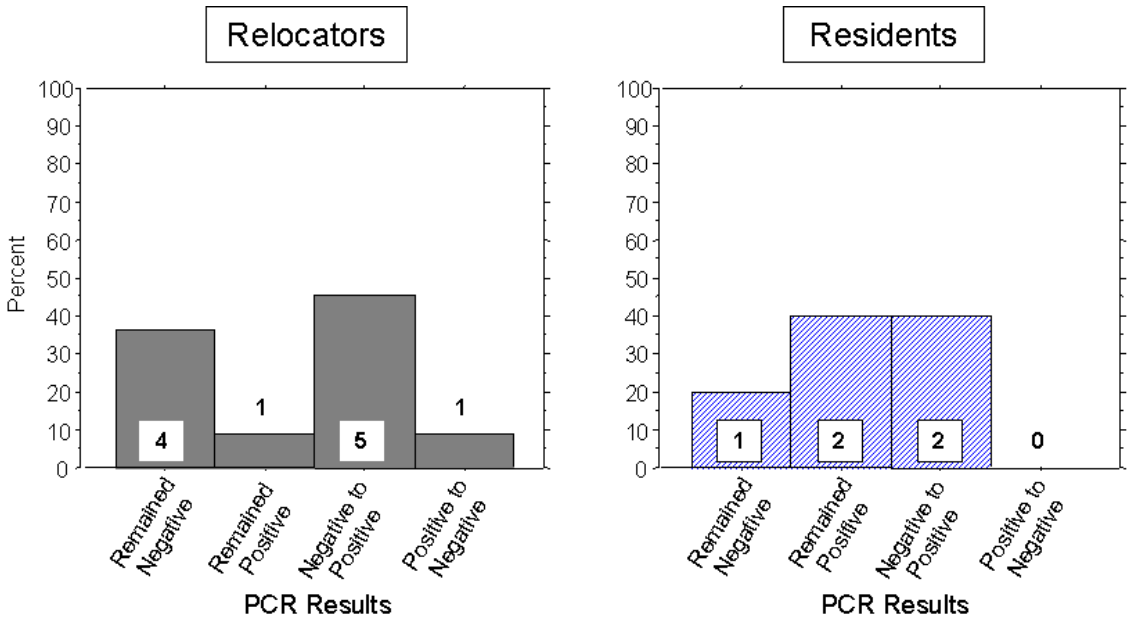


Figure 23. Change in PCR status from pre- to post-relocation or control date. Eight tortoises with PCR results both pre- and post-relocation or control date maintained their infection status: 5 remained negative and 3 remained positive. However, 6 relocators changed their status: 5 tested negative for the presence of the bacteria on Day 0, but tested positive on Day 30, while one relocator tested positive on Day 0 and negative on Day 30. Only two residents changed status, both from negative to positive.

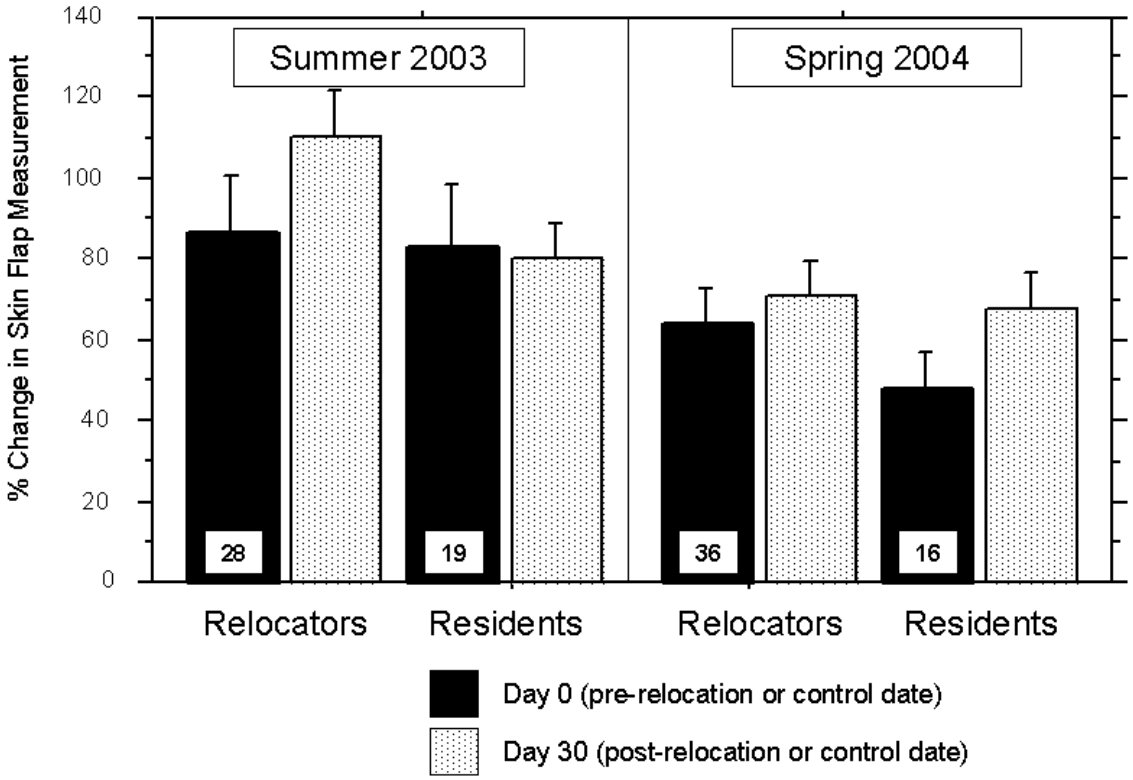


Figure 24. Response to PHA challenge: Percent change in skin flap thickness from pre- to post-relocation or control date. There was no significant relationship among relocator/resident status, field season, and PHA response in tortoises that received PHA (i.e., not saline) on both Day 0 and Day 30 with measurements taken at 12 hours post-injection ($F_{1,95}$=1.25, p=0.26). However, there was an overall significantly stronger response to the PHA challenge 30 days after the relocation or control date than on Day 0 ($F_{1,95}$=4.53, p=0.03).

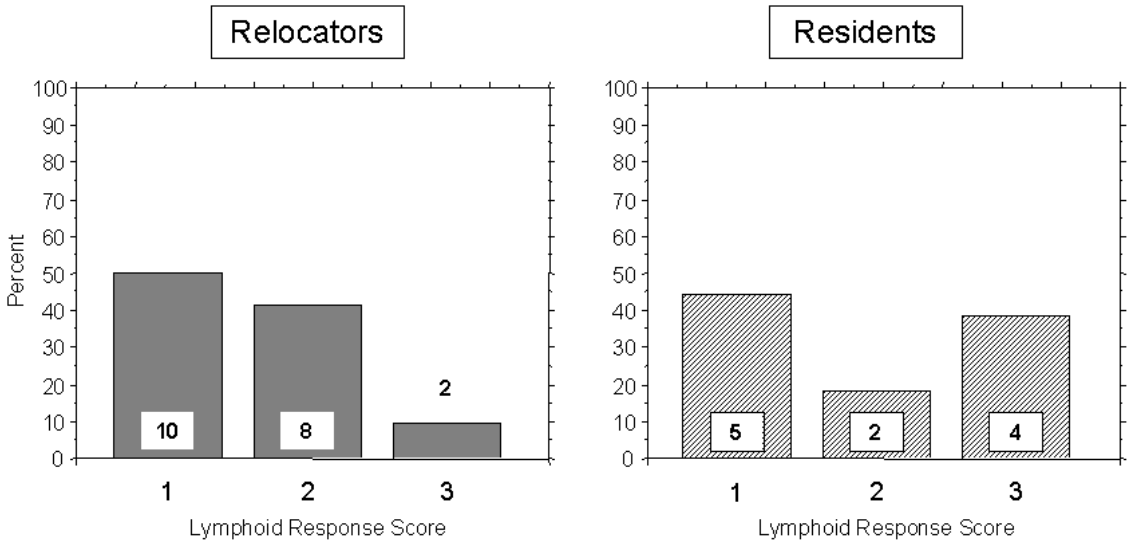


Figure 25. Post-relocation or control date lymphoid response to the PHA challenge. There was no significant difference in the 12 hour PHA response (i.e., excluding the saline response) between relocators and residents 30 days post-relocation or control date ($\chi^2$=1.14, df=1,29, p=0.28).

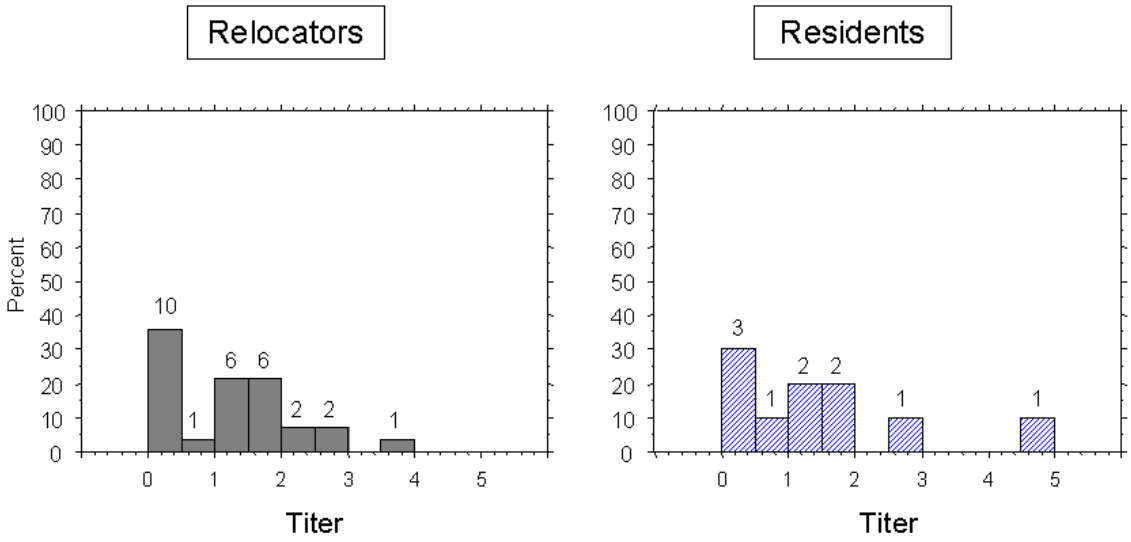


Figure 26. Post-relocation or control date SRBC titer. There appears to be no significant difference in SRBC titer between relocators and residents on Day 30 ($\chi^2$=0.04, df=1,36, p=0.82). However, the small sample size makes the results of the chi square test unreliable.

# CONCLUSION TO DISSERTATION

Habitat destruction plagues the southeastern United States and threatens the existence of gopher tortoise populations. As a result, relocation is the only remaining option to protect this species from extinction. My studies indicate that relocation success can be assessed with the same standard physiological measures and challenges that have been used with a wide variety of vertebrate taxa. I successfully assessed adrenal competence, reproductive health, and immune status prior to and following the relocation of gopher tortoises at Ft. Benning, Georgia during two seasons, spring and summer, and to and from different quality habitats.

Determining the variation in physiological responses of gopher tortoises across their natural geographic range and in different quality habitats was a critical first step in assessing the utility of measuring physiological parameters to determine relocation success. To that end, I established appropriate dose-response curves for adrenal and immune function in this species and found that gopher tortoises do not have the same response times as many other previously studied animals. I used the newly developed dose-response curves that are specific to gopher tortoises in order to implement protocols in the field. I determined that tortoises' baseline levels of corticosterone and their responses to the adrenal and immune challenges varied significantly according to geographic location, habitat quality, and population.

In the relocation study, I predicted that placing relocated gopher tortoises in a new location would cause an increased, potentially chronic activation of individuals' HPA axes leading to numerous subsequent maladaptive effects, including changes in adrenal and gonadal steroids. However, given our sampling timeline, from thirty days to ten months, my findings indicate that within this short time period following the relocation/control date, relocation does not increase corticosterone levels or cause a deterioration of the adrenal response. It also does not significantly affect sex steroids. There is the possibility that I may have missed a change in these parameters by waiting to measure the responses until 30 days post-relocation. However, it is clear that even if there were changes in these variables at three days, seven days, or even fourteen days, my data indicate that all physiological parameters returned to baseline and were the same as resident controls by 30 days post-relocation and remained that way even ten months post-relocation.

Despite my findings indicating no change in corticosterone as a result of relocation, I did document seasonal variation in corticosterone levels, which contradicts findings from a previous study (Ott et al. 2000). Specifically, I found that baseline levels of plasma corticosterone were significantly lower in summer than in spring, regardless of sex or habitat. I also documented similar findings for sex steroids. Specifically, testosterone levels in male gopher tortoises were not affected by relocation, but they did vary seasonally in that they were significantly higher in summer than spring. In many turtle and tortoise species, high testosterone levels in summer coincide with a peak in spermatogenesis and the mating season (Rostal et al. 1998), while in spring, during the period following hibernation, low testosterone occurs while seminiferous

tubules are regressed and leydig cells are hypertrophied (Rostal et al. 1994). While female gopher tortoises did not exhibit a significant seasonal difference in 17β-etsradiol, I found that levels were significantly higher in August than in May. This is similar to many other turtle species that exhibit significantly higher levels of 17β-etsradiol in summer (early vitellogenesis) than in spring (pre-nesting) (Mahmoud and Licht 1997; Rostal et al. 1998).

Conservation methods like relocation have the potential to detrimentally affect the health of the animals they are designed to protect so I also predicted that relocation would be detrimental to the immune status of gopher tortoises. However, it does not appear from my study that the act of relocation causes short-term changes in the disease status or immunocompetence of gopher tortoises. In fact, 30 days after the relocation or control date, I did not find significant differences between relocators and residents in their URTD antibody status (positive versus negative for antibodies to *Mycoplasma agassizii*), URTD PCR status (positive versus negative for the presence of *Mycoplasma agassizii*), cell-mediated immune responses, or humoral immune responses. However, I did find that a greater percentage of relocated tortoises showed changes in their antibody status than residents. This puts conservation managers in a difficult position because gopher tortoises are regularly relocated based on a single ELISA test of antibody status. If antibody status can change in such a short time period, then the URTD testing protocol associated with relocation may not be a reliable source of data on which to depend when making critical conservation decisions.

Gopher tortoises are a long-lived species so we may not realize the full effects of relocation for many years to come. In the meantime, integrative research that documents

current relocation efforts is critical and should include monitoring the physiological parameters that I measured in my studies, as well as examining behavioral patterns, actual reproductive success, and the development and progression of disease. These combined data will give us a more complete picture of relocation success and population stability in the short-term, and may provide us with much needed insight into the physiology and conservation management needs of gopher tortoises over the long-term. However, even if research ultimately determines that relocation is not physiological detrimental to gopher tortoises, removing this keystone species from its native habitats may result in dire consequences for many of the gopher tortoises' 360 other commensal and obligate species, and will ultimately contribute to the Earth's biodiversity crisis.

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