THE HEMATOLOGICAL RESPONSE OF AMPHIBIANS TO STRESS AND ITS IMPLICATIONS FOR RESEARCH, MANAGEMENT AND CONSERVATION

By

ANDREW K. DAVIS

(Under the Direction of John C. Maerz)

ABSTRACT

Populations of free-living animals frequently encounter natural and human-driven environmental variation that can affect their physiology and ultimately their long-term viability. In some cases, animals can experience physiological manifestations of such variation long before effects on more traditional population parameters are realized. One component of physiology that is useful for ascertaining how animals cope with such variation is their stress level. Chronically high stress levels can affect the performance of animals by reducing growth rates, long-term survival or increasing disease vulnerability. With this in mind, my dissertation research examines how natural and anthropogenic environmental variation affects stress levels in vertebrates, using amphibians as model subjects. To assess stress levels, investigators traditionally measure the concentration of stress hormones (corticosterone in birds, reptiles and amphibians) in blood plasma of subjects, although this can be logistically difficult because of the rapid response time of hormones and high volume of blood needed for the test. An alternative approach capitalizes on the stress hormone's effect on the host's circulating white blood cells, and in fact this cellular approach has advantages over direct hormone sampling because it is not as time sensitive and requires much smaller blood samples. Although this cellular method is

gaining in popularity among wildlife researchers interested in quantifying stress, it has encountered some resistance in the scientific literature, partly stemming from a poor understanding of the relationship between stress hormones and blood cells. My thesis aimed to address this information gap. Major findings included: 1) a literature review showing how stress causes changes in numbers of circulating white blood cells such that the ratio of neutrophils to lymphocytes (N:L ratio) rises and that this effect is conserved across all vertebrate taxa, 2) experimental administration of stress hormone to salamanders causes a rise in N:L ratios, 3) bringing wild salamanders into captivity causes increases in N:L ratios, 4) rearing salamanders from eggs in crowded conditions causes high N:L ratios in surviving metamorphs, 5) infection with chytridiomycosis can cause changes in blood cell counts that resemble effects of stress, and 6) that amphibians reared in captivity have similar responses to stress as do wild individuals, which justifies the use of captive-rearing initiatives for amphibian conservation. The collective projects in this thesis should not only serve to establish the hematological approach to measuring stress for ecologists, but also to highlight the importance of considering the physiology of the animal when drawing conclusions from research projects or when making decisions for management and conservation of wildlife.

INDEX WORDS: Amphibians, Stress, Corticosterone, White blood cell counts, Hematology, Neutrophil:lymphocyte ratio

THE HEMATOLOGICAL RESPONSE OF AMPHIBIANS TO STRESS AND ITS IMPLICATIONS FOR RESEARCH, MANAGEMENT AND CONSERVATION

by

ANDREW K. DAVIS

B.Sc., Acadia University, 1994

M.Sc. Acadia University, 1999

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2010

THE HEMATOLOGICAL RESPONSE OF AMPHIBIANS TO STRESS AND ITS IMPLICATIONS FOR RESEARCH, MANAGEMENT AND CONSERVATION

by

ANDREW K. DAVIS

Major Professor:	John C. Maerz
Committee:	Steven B. Castleberry Robert J. Cooper M. Kevin Keel

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
July 2010

ACKNOWLEDGMENTS

A wise person once told me, ‘Think of this PhD as a journey, and enjoy the ride.’ Truer words were never spoken. I have indeed enjoyed this ride, and in the past 4 and a half years I have accumulated many memories I will cherish, including wading through sewage treatment ponds, chasing bullfrogs in the dead of night, staring through a microscope for hours, and reading veterinary textbooks from cover to cover – for me, those *are* fond memories. Many people helped me in this journey. I especially thank the students in the Maerz Herpetology Lab for assistance in the field (yes, there was fieldwork!), especially my officemates Joe Milanovich, Sean Sterrett and Andrew Grosse. I will always be indebted to John Maerz, who provided me with a coveted spot in his bustling lab, which allowed me to pursue this degree and to ultimately establish my career in academia. I am also grateful to my colleague, Donna Maney, who helped tremendously with the writing of chapter 2, which set the stage for the entire body of work. Although they are a thousand miles away, I also thank my parents, who nurtured my passion for wildlife at an early age. Finally, and most importantly, I was supported, emotionally and financially(!), by my wife, Sonia Altizer while pursuing this degree. She gave me guidance and suggestions for all of my projects, she was, and is, my source for scientific inspiration, and she constantly pushes me to raise my own bar. I dedicate this thesis to her.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Ecologists and wildlife researchers are becoming increasingly aware of the importance of physiological approaches to assessing animal performance, with the most frequently-used metric being the stress level of the animal (e.g. Groombridge et al. 2004, Romero 2004, Crespi and Denver 2005, Blas et al. 2007) . When animals are stressed, it is a signal to the researcher that the animal perceives a problem or a threat within its current environment, and this information is highly useful for a variety of reasons, such as when gauging the quality of the habitat (Homan et al. 2003) or for predicting future survival of populations (Romero and Wikelski 2001). There are several ways to measure stress in animals. The conventional technique involves assessing the level of stress hormones (usually corticosterone) in blood samples. However, since this hormone rapidly rises when wild animals are captured (within two minutes, Romero and Reed 2005), it can be logistically difficult to obtain blood samples within this time frame, especially if traps are used and animals must be first extracted. Because of this limitation, certain other, indirect metrics have emerged, that are not as time-sensitive. One recent one involves measuring the level of stress hormone metabolites in the feces of animals (Franceschini et al. 2008), which is obviously not influenced by the stress of capture or handling. Similarly, there are new techniques to measure hormone metabolites in feathers of birds (Bortolotti et al. 2009). Another, which is the subject of this thesis, is also an indirect measure of stress hormones, and involves counting numbers of specific white blood cells in blood samples of animals. This approach capitalizes on the effect of stress on neutrophils (or heterophils in birds and reptiles) and lymphocytes, such

that the ratio of the two cells (N:L ratio) is directly related to hormone levels. This thesis was aimed at furthering our understanding of this 'hematological stress index' using amphibians as model subjects, and at establishing the methodology to the scientific community.

I initially reviewed the effects of stress hormones on leukocytes across 150 scientific studies from the biomedical, veterinary, ecological and physiological literature [Chapter 2], which showed that stress hormones have a similar effect across all vertebrates, in that they cause neutrophils (heterophils in birds and reptiles) to enter circulation and lymphocytes to leave. This ultimately leads to an increase in the ratio of the two cell types which is directly correlated with stress hormone levels over both short and long timescales. The relationship between stress hormones and N/L ratios was next validated with a controlled laboratory experiment [Chapter 3]. Here, I captured wild salamanders and injected one group with a known dose of corticosterone, and compared the white blood cell response of this group to a group injected with a sham, and to a group that was sampled immediately after capture (control group). Results showed that N:L ratios of the corticosterone group were higher than the control and sham treatment, and that a reduction in eosinophils is associated with increased stress. This was followed by two experiments showing how short-term captivity increases the ratio of neutrophils to lymphocytes in wild, adult salamanders [Chapter 4], and how crowding during larval stages also leads to an increase in N/L ratios of post-metamorphic salamanders [Chapter 5]. Results from these two projects have implications for researchers using herpetofauna as study subjects, since both scenarios (captivity and rearing larvae) were typical of herpetological research. I then examined the effects of a well-known amphibian disease (chytridiomycosis) on the circulating white blood cells of frog larvae [Chapter 6]. These larvae originated from a separate lab experiment in which some individuals unintentionally became infected and others did not. I examined each group and

compared white blood cell counts of infected and non-infected individuals (infection was presumed from mouthpart depigmentation). Results showed how the disease can cause signs resembling the effects of stress, by lowering lymphocyte counts as well as eosinophils counts. Finally, I conducted an experimental assessment of the effect of captive-rearing on amphibian stress levels, which is an example of the utility of this approach in addressing real-world issues of conservation concern [Chapter 7]. This project aimed to validate the use of captive-rearing projects to help bolster declining amphibian populations, and involved comparing the hematological stress levels of captive-reared animals to their wild counterparts. The idea was that since reared amphibians are usually given food *ad libidum* and kept free of predators and parasites, they may not have the ability to respond to real-world stressors when they are released into the wild. Results showed that reared frog and salamander larvae had similar rises in N:L ratios in response to standardized stressors as did wild individuals, which indicates that they in fact, are capable of mounting appropriate stress responses.

I end the thesis with some concluding remarks [Chapter 8] that focus on the main criticisms of this hematological approach. There are indeed several persistent critical comments that come up in manuscript reviews and in discussion with certain colleagues, although these comments are becoming fewer and fewer in recent months, hopefully because of the publication of the chapters in this thesis. If that is the case, then it will have achieved one of my main goals, which was to establish this technique within the scientific community. Moreover, it is my hope that the combined projects in this thesis also serve to highlight the utility of a hematological approach to measuring stress in wildlife management and conservation.

Literature cited

Blas, J., G. R. Bortolotti, J. L. Tella, R. Baos, and T. A. Marchant. 2007. Stress response during development predicts fitness in a wild, long lived vertebrate. Proceedings of the National Academy of Sciences of the United States of America 104: 8880-8884.

Bortolotti, G. R., T. Marchant, J. Blas, and S. Cabezas. 2009. Tracking stress: localisation, deposition and stability of corticosterone in feathers. Journal of Experimental Biology 212: 1477-1482.

Crespi, E. J., and R. J. Denver. 2005. Roles of stress hormones in food intake regulation in anuran amphibians throughout the life cycle. Comparative Biochemistry and Physiology A: 381-390.

Franceschini, M. D., D. I. Rubenstein, B. Low, and L. M. Romero. 2008. Fecal glucocorticoid metabolite analysis as an indicator of stress during translocation and acclimation in an endangered large mammal, the Grevy's zebra. Animal Conservation 11: 263-269.

Groombridge, J. J., J. G. Massey, J. C. Brunch, T. R. Malcolm, C. N. Brosius, M. M. Okada, and B. Sparklin. 2004. Evaluating stress in a Hawaiian honeycreeper, *Paroreomyza montana*, following translocation. Journal of Field Ornithology 75: 183-187.

Homan, R. N., J. V. Regosin, D. M. Rodrigues, J. M. Reed, B. S. Windmiller, and L. M. Romero. 2003. Impact os varying habitat quality on the physiological stress of spotted salamanders (*Ambystoma maculatum*). Animal Conservation 6: 11-18.

Romero, L. M. 2004. Physiological stress in ecology: lessons from biomedical research. Trends in Ecology & Evolution 19: 249-255.

Romero, L. M., and M. Wikelski. 2001. Corticosterone levels predict survival probabilities of Galapagos marine iguanas during El Nino events. Proceedings of the National Academy of Sciences of the United States of America 98: 7366-7370.

Romero, L. M., and J. M. Reed. 2005. Collecting baseline corticosterone samples in the field: is under 3 min good enough? Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology 140: 73-79.

CHAPTER 2

THE USE OF LEUKOCYTE PROFILES TO MEASURE STRESS IN VERTEBRATES: A LITERATURE REVIEW[1]

[1]Davis, A.K., Maney, D.L., and Maerz, J.C. 2008. *Functional Ecology* 22: 760-772. Reprinted here with permission of publisher.

Abstract

A growing number of ecologists are turning to the enumeration of white blood cells from blood smears (leukocyte profiles) to assess stress in animals. There has been some inconsistency and controversy in the ecological literature, however, regarding their interpretation. The inconsistencies may stem partly from a lack of information regarding how stress affects leukocytes in different taxa, and partly from a failure on the part of researchers in one discipline to consult potentially informative literature from another. Here, we seek to address both issues by reviewing the literature on the leukocyte response to stress, spanning the taxa of mammals (including humans), birds, amphibians, reptiles and fish. We show that much of the early literature points to a close link between leukocyte profiles and glucocorticoid levels. Specifically, these hormones act to increase the number and percentage of neutrophils (heterophils in birds and reptiles), while decreasing the number and percentage of lymphocytes. This phenomenon is seen in all five vertebrate taxa in response to either natural stressors or exogenous administration of stress hormones. For the ecologist, therefore, high ratios of heterophils or neutrophils to lymphocytes ('H:L' or 'N:L' ratios) in blood samples reliably indicate high glucocorticoid levels. Furthermore, this close relationship between stress hormones and N:L or H:L ratios needs to be highlighted more prominently in hematological assessments of stress, since it aids the interpretation of results. As with hormone assays, there are challenges to overcome in the use of leukocytes profiles to assess levels of stress; however, there are also advantages to this approach, and we outline each. Given the universal and consistent nature of the hematological response to stress, plus the overwhelming evidence from the veterinary, biomedical and ecological literature reviewed here, we conclude that this method can provide a reliable assessment of stress in all vertebrate taxa.

At one time confined to veterinary practice, methods and techniques that advance the understanding of physiological responses to the environment are now becoming commonplace in ecological studies of wild animals. This development has led to the emergence of a new discipline, coined 'conservation physiology' by Stevenson *et al.* (2005) and explored further by Wikelski & Cooke (2006), who eloquently outlined the questions in conservation that can be addressed using a variety of physiological approaches, especially those that focus on stress. Indeed, stress in animals is clearly an important factor to consider when assessing their welfare in both captive or wild settings. In recent reviews, Wikelski & Cooke (2006) and Romero (2004) outlined a popular method of assessing physiological stress: the measurement of levels of adrenal glucocorticoid hormones, such as corticosterone, in plasma. Measuring these hormones clearly has many applications in ecology and has proven to be an invaluable tool. As with all methods, however, there are drawbacks associated with it. For example, levels of plasma corticosterone rise quickly immediately following capture of wild animals (Romero & Reed 2005), thus making it difficult to obtain baseline measurements in field situations. We review here a complementary method of physiological stress assessment that is becoming popular with ecologists, especially those studying birds: the use of hematological parameters such as relative white blood cell (WBC) counts made from blood smears. This approach may represent an alternate method for measuring corticosterone because, as reviewed below, increases in glucocorticoid hormones cause characteristic changes in the leukocyte component of the vertebrate immune system that can be quantified and related to hormone levels. Moreover, the leukocyte approach offers certain advantages over direct glucocorticoid measurement in that it does not require prohibitively rapid sampling and is relatively inexpensive. Finally, the hematological response to stress is conserved across taxonomic groups, ensuring that this approach to measuring stress can be applied to most

vertebrates, and that results obtained from one taxonomic group should be useful for making predictions in others.

The white blood cell differential in vertebrates

We begin by outlining basic information about white blood cells across vertebrate taxa. Most vertebrates have five types of WBCs: lymphocytes, neutrophils, eosinophils, basophils and monocytes. The morphology of each cell type appears to be conserved across taxa, except in the case of the neutrophil. In birds and reptiles, the neutrophil is replaced with the heterophil, which performs the same immunological function (Hawkey & Dennett 1989, Jain 1993). In amphibians, this cell type is occasionally referred to as a heterophil (e.g. Cabagna *et al.* 2005, Forbes *et al.* 2006); however it appears more similar to a neutrophil (Fig. 2.1) (Thrall 2004) and the majority of authors use that term (e.g. Bennett & Harbottle 1968, Rouf 1969, Wojtaszek & Adamowicz 2003). Reptiles appear to have a sixth cell type, called an azurophil, which most researchers group with monocytes (Hawkey & Dennett 1989, LeBlanc *et al.* 2000).

The immunological function of each of the WBC types has been reviewed extensively elsewhere (Ellis 1977, Jain 1986, Maxwell 1987, Jain 1993, Maxwell & Robertson 1998, Thrall 2004). Briefly, neutrophils/heterophils and lymphocytes make up the majority (i.e. nearly 80% combined) of WBCs in mammals (Jain 1993), birds (Rupley 1997), amphibians (Bennett *et al.* 1972, Cathers *et al.* 1997, Thrall 2004) and reptiles (Eliman 1997, Fisse *et al.* 2004, Werner 2007) (see also Table 2.1). Neutrophils/heterophils are the primary phagocytic leukocyte, and proliferate in circulation in response to infections, inflammation and stress (Jain 1993, Campbell 1995, Rupley 1997, Harmon 1998, Thrall 2004). Lymphocytes are involved in a variety of immunological functions such as immunoglobulin production and modulation of immune

defense (Campbell 1996). The remaining 20% of the leukocytes represent a combination of eosinophils, which play a role in the inflammation process (Jain 1993) and are associated with defense against parasites (Maxwell 1987, Rupley 1997, Kiesecker 2002), monocytes, which are long-lived phagocytic cells associated with defense against infections and bacteria (Campbell 1995, Davis *et al.* 2004) and basophils, the function of which is not clearly understood (Rupley 1997) but is thought to involve inflammation (Campbell 1995).

The relative proportions of each WBC type, usually obtained by light microscope examination of 100 leukocytes in a stained blood smear, are what make up the leukocyte 'profile' (also called 'complete blood count', 'leukocyte differential' or 'hemogram') for any animal. The baseline leukocyte profile varies considerably among the vertebrate taxa. In mammals, for example, the most abundant WBC is the neutrophil, whereas in birds, lymphocytes are usually the most common (see Table 2.1). Furthermore, within taxa there is much variation among species.

Stress-induced changes in leukocyte profiles

Leukocyte profiles are particularly useful in the field of conservation physiology because they are altered by stress and can be directly related to stress hormone levels. As Dhabhar *et al.* (1996) pointed out, leukocyte profiles that deviated from normal parameters were routinely used to indicate mammalian hormonal stress responses in the 1940s, before methods were available to directly assess plasma glucocorticoids. Specifically, the changes brought on by stress or glucocorticoid treatment are *increases in numbers of neutrophils (neutrophilia) and decreases in lymphocyte numbers (lymphopenia or lymphocytopenia)*. Moreover, since numbers of neutrophils and lymphocytes are affected by stress in opposite directions, researchers have often

considered the ratio of one to the other, i.e. the relative proportion of neutrophils to lymphocytes (hereafter, 'N:L' ratio) in mammals and amphibians, and heterophils to lymphocytes (hereafter, 'H:L' ratio) in birds and reptiles, as a composite measure of the stress response. This ratio, as read from standard blood smears made before and after a stressful event, is positively related to the magnitude of the stressor and to circulating glucocorticoids (reviewed below). There is also evidence that this ratio is influenced by diseases and infections (or the stress hormones produced as a result of the infection), and we review this evidence later in the paper.

A long history of research on mammals indicates that exogenous treatment with stress hormones (i.e. mimicking a physiological stress response) results in a marked alteration in leukocyte counts within one to two hours (e.g. Dougherty & White 1944, Gordon 1955, Jain 1986, Dhabhar *et al.* 1995). This research, which has been conducted primarily in humans, livestock and mammalian laboratory animals, has involved treatment with cortisol or synthetic glucocorticoids, such as hydrocortisone, dexamethasone or prednisolone. These agents produce neutrophilia or lymphopenia, or both, in cattle (Anderson *et al.* 1999), sows (Kranendonk *et al.* 2005), horses (Burguez *et al.* 1983), bottlenose dolphins (Reidarson & McBain 1999), rats (Cox & Ford 1982), mice (Van Dijk *et al.* 1979), guinea pigs (Fauci 1975) and humans (e.g. Fauci & Dale 1974, Dale *et al.* 1975). Similar research has been conducted using adrenocorticotropic hormone (ACTH), which stimulates release of glucocorticoids from the adrenal glands. Treatment with ACTH increases N:L ratio in boars (Bilandžič *et al.* 2006) and horses (Rossdale *et al.* 1982), probably via its effects on glucocorticoid secretion.

Long-term effects of glucocorticoid hormones on leukocyte populations are seen in humans with chronic medical disorders; for example, Cushing's syndrome is a disorder characterized by chronically elevated levels of plasma cortisol. Not long after the link between

stress hormones and leukocytes was discovered in the early 1940s, medical researchers found that patients with this disorder had chronically elevated neutrophil counts and lower lymphocyte counts compared to healthy individuals (de la Balze *et al.* 1946). Similarly, neutrophils are elevated and lymphocytes depressed in humans with psychological disorders such as depression and schizophrenia, which are characterized by chronically elevated plasma cortisol levels (Kronfol *et al.* 1984). Chronically elevated glucocorticoids may therefore cause long-term elevations in N:L ratios.

The mechanism underlying the effect of stress hormones on leukocyte profiles has been well documented in biomedical studies of mammals (reviewed in Ottaway & Husband 1994, Brenner *et al.* 1998). Stress-induced reductions in circulating lymphocyte numbers are not due to large-scale destruction of cells, but rather to glucocorticoid-induced alterations in the 'trafficking', or redistribution, of lymphocytes from the blood to other body compartments (Dhabhar 2002). In response to glucocorticoids, circulating lymphocytes adhere to the endothelial cells that line the walls of blood vessels, and subsequently undergo transmigration from circulation into other tissues, for example lymph nodes, spleen, bone marrow and skin, where they are sequestered (Cohen 1972, Fauci 1975, Dhabhar 2002). This exodus of lymphocytes from the blood causes a significant reduction in their circulating numbers. In contrast, glucocorticoids also stimulate an influx of neutrophils into the blood from bone marrow and attenuate the egress of neutrophils from the blood to other compartments (Bishop *et al.* 1968). These changes are thought to ensure that the different types of cells are routed to where they are needed during the stress response (Dhabhar *et al.* 1994, Dhabhar *et al.* 1996). For the ecologist seeking a tool to assess stress, they result in an increase in N:L or H:L ratio that is proportional to the level of glucocorticoid release.

Given the very clear effect of stress hormones on leukocyte profiles, it is not surprising that increases in N:L ratios are observed in response to stressors. For example, this phenomenon has been well studied in the context of transport stress in mammals, which has broad ramifications across many fields, including veterinary medicine, biomedical research, agriculture and wildlife management (reviewed in Obernier & Baldwin 2006). Transporting animals from, for example, a vendor to a research laboratory, or from a farm to a slaughter house, causes a hormonal stress response as well as changes in leukocyte profiles. The N:L ratio has been shown to increase after transport in a variety of mammals including horses, goats, swine and cattle (reviewed in Obernier & Baldwin 2006) as well as beagles (Frank *et al.* 2006), dolphins (Noda *et al.* 2007) and rhinos (Kock *et al.* 1999). Other types of stressors also affect hematological parameters of mammals; for example, N:L ratios increase after strenuous exercise in horses (Cardinet *et al.* 1964, Rossdale *et al.* 1982) and humans (reviewed in Brenner *et al.* 1998), after restraint stress in rhesus monkeys (Morrow-Tesch *et al.* 1993) and southern chamois (López-Olvera *et al.* 2007), and after transfer from the wild to captivity in brushtail possums (Baker *et al.* 1998). These examples represent only a fraction of the large literature on the effects of a variety of stressors on leukocyte profiles in a wide range of mammalian species, although we point out that much of this literature has been published in biomedical or veterinary journals and may not readily available to ecologists.

Although the early studies of stress-induced neutrophilia and lymphopenia were conducted in mammals (e.g. Dougherty & White 1944, Gordon 1955), the phenomenon appears to be universal across vertebrates, having been demonstrated not only in mammals but also in birds, amphibians, reptiles and fish. The utility of the avian H:L ratio was first realized by poultry researchers (Gross & Siegel 1983) and is now commonly used to assess the welfare of

chickens under different rearing conditions (Altan *et al.* 2000, Davis *et al.* 2000, Elston *et al.* 2000, Onbasilar & Aksoy 2005, Nicol *et al.* 2006). Much of the early literature on this subject has been reviewed by Maxwell (1993). Throughout this body of work, there are numerous cases where the relationship between leukocytes and stress hormones is highlighted. For example, Davis *et al.* (2000) showed how reduced feed leads to increases in both corticosterone and H:L ratios in domestic chickens (*Gallus gallus*). Importantly, poultry researchers have also discovered that natural variation in the H:L ratios of newly-hatched chicks can be used to assess future susceptibility to diseases, with more susceptible individuals identifiable as chicks with high ratios (Al-Murrani *et al.* 2002, Al-Murrani *et al.* 2006). This research, which has had a measurable impact on the poultry husbandry industry, has laid the groundwork for using leukocyte parameters to infer physiological stress in other avian species.

In recent years, ornithologists have capitalized on the findings in poultry and have increasingly quantified H:L ratios in wild birds across a number of ecological settings. For example, in passerines, H:L ratios increase in response to a wide variety of stressors, including long-distance migration (Owen & Moore 2006), transport (Parga *et al.* 2001, Groombridge *et al.* 2004), parasitic infection (Davis *et al.* 2004, Lobato *et al.* 2005) and radioactive contamination (Camplani *et al.* 1999). H:L ratios have also been associated with other measures of individual health and quality; for example, high ratios (indicating high stress) are associated in pied flycatcher (*Ficedula hypoleuca*) nestlings with reduced growth (Moreno *et al.* 2002), and low ratios are associated with large song repertoires in song sparrows (*Melospiza melodia*) (Pfaff *et al.* 2007). Low ratios in nestling pied flycatchers also positively predict their recruitment into the adult population (Lobato *et al.* 2005). These are but a few examples of a growing body of literature from the ornithological community, and because ornithologists report on leukocyte

parameters more often than researchers in other fields, the body of relevant literature on avian species is growing faster than any other.

A lesser-known, earlier body of literature clearly demonstrates that the stress-induced leukocyte responses are also exhibited by amphibians. In a series of carefully conducted laboratory experiments spanning three decades, Bennett and colleagues showed that the proportion of neutrophils increases while the proportion of lymphocytes decreases in newts and frogs after injection with hydrocortisone (Bennett & Alspaugh 1964, Bennett & Harbottle 1968, Bennett *et al.* 1972), limb amputation (Bennett 1986), osmotic stress (Bennett & Johnson 1973) and exposure to constant light (Bennett & Reap 1978). Increases in neutrophil numbers in amphibians exposed to agricultural pesticides have also recently been recorded (Cabagna *et al.* 2005), while the authors recently found that increased N:L ratios are associated with the onset of reproduction (Davis & Maerz 2008a) and with captivity (Davis & Maerz 2008b) in wild salamanders.

There is some evidence that the hematological response seen in mammals, birds and amphibians also occurs in reptiles. Saad & El Ridi (1988) reported that 3–4 weeks of hydrocortisone treatment caused a pronounced lymphopenia in ocellated skinks (*Chalcides ocellatus*). Similarly, Morici *et al.* (1997) found that long-term treatment with corticosterone elevated H:L ratios in juvenile alligators (*Alligator mississippiensis*). In the same species of alligator, holding juveniles out of water with their jaws held closed caused a rapid rise in plasma corticosterone, followed by a dramatic increase in H:L ratio (Lance & Elsey 1999). In a recent study of box turtles (*Terrapene c. carolina*), H:L ratios were used to determine how individuals responded to captivity in low- versus high-quality housing conditions (Case *et al.* 2005). In this case, individuals housed in high-quality conditions (i.e. with mulched floors, shredded paper and

a hide box) had lower H:L ratios than those housed in low-quality conditions (i.e. only newspaper). Finally, Chen *et al.* (2007) recently showed that increases in rearing density caused increases in H:L ratios in farm-raised soft-shelled turtles (*Pelodiscus sinensis*). These studies suggest that H:L ratios can be used to assess glucocorticoid levels and stress in reptiles; however, we point out that less work has been conducted in this taxon than in others.

The leukocyte response to stress has been well studied in fish despite some confusion in the early literature over cell identification and nomenclature (Ellis 1977). Furthermore, thrombocytes (the equivalent to mammalian platelets) have sometimes been included in early investigations of 'leukocyte differentials' of fish (Saunders 1968), which makes comparison with modern counts difficult because most authors do not routinely report these non-leukocytic cells in differential counts (Thrall 2004). This trend has apparently continued in the ichthyology community, with authors of recent hematological studies also reporting thrombocytes in differential counts (e.g. Silveira-Coffigny *et al.* 2004). Nevertheless, neutrophils and lymphocytes appear to be readily quantifiable in fish, and the same leukocyte responses to stress and to exogenous glucocorticoid treatment (neutrophilia and lymphopenia) can be measured. Ellsaesser & Clem (1986), Bly *et al.* (1990) and Harris & Bird (2000) provide excellent reviews on these responses. In general, acute stress induces both neutrophilia and lymphopenia in fish (e.g. Pulsford *et al.* 1994), although sometimes only lymphopenia is reported (Larsson *et al.* 1980), and these stress-induced changes have been shown repeatedly to be related to elevated glucocorticoids. Neutrophilia, lymphopenia and increased N:L ratios are apparent after treatment with either cortisol or hydrocortisone (Ellsaesser & Clem 1986, Wojtaszek *et al.* 2002). Treatment with ACTH also induces neutrophilia and lymphopenia in fish (McLeay 1973). These responses in fish are therefore identical to those seen in other vertebrate taxa.

Many ichthyologists have capitalized on the leukocyte stress response in fish as a tool for understanding the physiological effects of exposure to heavy metals and other contaminants in a wide range of species (e.g. Mishra & Srivastava 1979, Dick & Dixon 1985, Murad & Houston 1988, Dethloff *et al.* 1999, Witeska 2005). In fact, ichthyologists consider changes in the differential leukocyte count to be one of the most sensitive indicators of acute stress in fish (Wedemeyer *et al.* 1990). Thus, the size of the literature set on leukocyte responses to environmental contaminants in fish is indeed large, and although it is too extensive to review adequately here, we do highlight a selected subset of examples. A number of laboratory studies have exposed fish to heavy metals such as lead (Witeska 2005), zinc (Mishra & Srivastava 1979), copper (Dick & Dixon 1985, Dethloff *et al.* 1999) or cadmium (Murad & Houston 1988), and in each of these studies lymphopenia was reported, with neutrophilia reported in most (neutrophils were not always counted). The exception is a study of Mozambique tilapia (*Oreochromis mossambicus*) where exposure to copper resulted in the opposite pattern (Nussey *et al.* 1995). Experimental exposure to polluted water (i.e. industrial effluent) resulted in lymphopenia in flounders (*Platichthys flesus*) (Larsson *et al.* 1980). Finally, although not necessarily related to environmental contaminants, fish have also been exposed other stressors, including immersion in near-freezing water (Bennett & Gaudio Neville 1975), 24 hour light (Valenzuela *et al.* 2008) and transport (Ellsaesser & Clem 1986). In all cases, lymphopenia and neutrophilia ensued.

Advantages of leukocyte stress indicators

The researcher using plasma glucocorticoid levels to assess stress is generally interested in two different measures: the baseline level prior to capture and handling, and the peak level during the

stress response. Both of these measures provide information relevant to the stress physiology of the animal. Whereas obtaining a blood sample during the peak phase of the stress response is usually not a problem, quantification of baseline levels is more difficult because it requires the collection of a blood sample within a few minutes of capture. The exact timing of the rise is not known precisely in most species; however, the existing literature demonstrates that it typically occurs within 5 min and can begin in as little as 2 min after capture in birds (Romero & Romero 2002, Romero & Reed 2005). Examples of two responses are compared in Fig. 2.2, which shows the time course of the adrenal response to stress in skinks (taken from Langkilde & Shine 2006) and in rats (taken from Muir & Pfister 1987). This rapid hormonal response means that a field investigator wishing to take a baseline hormone sample from most any vertebrate needs to monitor traps closely and must be very skilled at rapid blood collection. In fact, a recent study of house sparrows (*Passer domesticus*) emphasizes this point (Lynn & Porter 2008). In this project, the corticosterone stress response was found to begin as soon as individuals entered traps, even though they did not exhibit prolonged escape behavior. For the researcher interested in glucocorticoid hormones, this study indicates that traps cannot be left unattended, even for brief periods.

Unlike the hormonal response to stress, the initial *leukocyte* response begins over a time span of hours to days, depending on the taxon. To demonstrate this point for mammals, we have reproduced two figures from an early study by Cardinet *et al.* (1964), who examined the short-term effect of strenuous exercise on leukocytes of the horse (Fig. 2.3). In this simple but effective study, a horse was taken across a 32-mile course over 8 hours and its blood was sampled every two hours for leukocyte examination. The resulting counts were compared to those made during a day with no exercise. Figure 2.3 shows how lymphocytes (A) remained

lower than normal throughout the exercise, and then took 14 hours after the end of the exercise to return to normal levels. Meanwhile, neutrophil numbers (B) increased throughout the exercise, then gradually declined upon termination, but had not yet returned to the baseline level 14 hours later. It is important here to point out that neutrophil counts were not appreciably elevated until 4 hours after the start of the exercise period. Similarly, Burguez *et al.* (1983) showed that injections of cortisol did not significantly elevate N:L ratios until 2 hours after injection in foals and 4 hours later in adult horses. In contrast to these studies on domestic mammals, more recent work shows that the leukocyte response may occur on a more rapid time scale in wild mammals. In a study of wild ungulates, neutrophil numbers were found to double, and lymphocytes reduced by half, within one hour of capture (López-Olvera *et al.* 2007). Even this time course, however, is not so rapid that sampling becomes problematic.

Research with birds also demonstrates a relatively slow leukocyte response time that makes obtaining baseline samples convenient. Davis (2005) found that H:L ratios do not increase significantly within one hour of capture in house finches (*Carpodacus mexicanus*). To demonstrate this idea graphically, we have reproduced a figure from that study as well as one from another study (Lindström *et al.* 2005) in which corticosterone was measured after capture in house finches (Fig. 2.4). In both studies, individuals were captured and sampled three times over a period of one hour. Lindström *et al.* measured plasma corticosterone (Fig. 2.4A), whereas Davis quantified H:L ratios using blood smears (Fig. 2.4B). Plasma corticosterone increased dramatically within 30 min (Lindström *et al.* 2005), whereas H:L ratios did not increase significantly throughout the hour (Davis 2005). In a separate set of individuals held for one hour and sampled only once at 60 min, H:L ratios did not increase at all, indicating that the slight

trend of increasing H:L ratios seen in Fig. 2.4B may have been caused by repeated sampling, not the handling time *per se*.

The time lag associated with the leukocyte response to stress may be the longest in ectothermic animals, perhaps owing to their temperature-dependent metabolism, which is slow at low temperatures (Pough 1980). Bennett *et al.* (1972) found that experimental injections with hydrocortisone did not alter leukocyte differentials in newts (i.e. no neutrophilia or lymphopenia was observed) until 3 days later, whereas similar experiments with frogs showed that some individuals responded within 12 hours, but others took 144 hours (Bennett & Harbottle 1968). If this slow response is due to a slow metabolism, we might expect equally slow leukocyte responses to stress in reptiles since their metabolism is also slow at low temperatures (Pough 1980). Similarly, the time course of the leukocyte response in fish is lengthy. The effects of transport stress on peripheral blood neutrophil counts in channel catfish (*Ictalurus punctatus*) were not detectable until 12 hours post-stress and peaked at 24 hours (Bly *et al.* 1990). Thus, among ectotherms, the leukocyte response time appears to be much slower than that of endothermic birds and mammals.

Few researchers have directly quantified the endogenous hormonal and leukocyte responses with the intent of comparing their relative utility, with the exception of scientists studying poultry. MacFarlane *et al.* (1989) measured the leukocyte (H:L ratio) and adrenal hormone responses in chickens that were chronically exposed to a variety of stressors, including beak trimming, air fouling, coccidiosis, heat stress, continuous noise and intermittent electric shocks. These treatments initially caused the typical rises in both plasma corticosterone and H:L ratios. After seven days, however, corticosterone was no longer elevated, suggesting acclimation to the stressors (see Romero 2004). Interestingly, H:L ratios remained elevated, and this led the

investigators to conclude that the leukocyte response to stress was more enduring and perhaps a more reliable indicator of long-term stress than corticosterone sampling. Whether acclimation to stressors occurs in two dissociable phases, one characterized by a reduction in the adrenal response followed by another involving the leukocyte response, is a question that should be explored by future research.

Besides allowing more time for researchers to obtain baseline samples, the use of leukocyte profiles for assessing stress in vertebrates offers additional advantages. First, the technique is relatively inexpensive, requiring only microscope slides, stain and a microscope. Second, the blood sample required for a blood smear is very small. Corticosterone assays require 30—60 μL of blood, which is not easily taken (if to be done so non-destructively, at least) from animals less than 15 g in weight (Washburn *et al.* 2002). In contrast, leukocyte profiles can be obtained from blood samples as small as 5–10 μL (A. K. Davis,personal observation), which in theory means that smaller animals with lower blood volumes can be studied.

Predictive value of leukocyte profiles

One of the reasons for assessing stress in ecological settings is to detect early warning signals of potential problems in vertebrate populations. For example, corticosterone levels have been used to predict survival in populations of Galápagos marine iguanas (*Amblyyrhynchus cristatus*) (Romero & Wikelski 2001). Leukocyte profiles also have predictive power, and some of the best examples of this power come from human medical studies in which patients can be followed for years after routine blood smears are made. Multiple studies report that low lymphocyte counts (which the authors of these studies attribute to elevated glucocorticoids) reliably predict the risk of mortality associated with a range of ailments, such as coronary artery disease (Ommen *et al.*

1997), haemodialysis (Reddan *et al.* 2003), heart failure or myocardial infarction (Thomson *et al.* 1995, Ommen *et al.* 1998, Acanfora *et al.* 2001) and surgical implantation of defibrillators (Ommen *et al.* 2002). The N:L ratio was found to be a good predictor of cardiovascular risk, more so than high neutrophil counts or low lymphocyte counts (Horne *et al.* 2005). Other examples of the predictive capacity of leukocyte stress parameters come from avian research. High H:L ratios have been associated in birds with susceptibility to infection (Al-Murrani *et al.* 2002), slow growth rates (Moreno *et al.* 2002) and survival to the next breeding season (Lobato *et al.* 2005, Kilgas *et al.* 2006). These associations make H:L ratios valuable for predicting future problems in both populations and in individuals.

Interpreting changes in leukocyte profiles

Controversy surrounding the use of leukocyte profiles in ecology seems to center around the perception that data collected on leukocyte parameters are difficult to interpret. As with any tool, it is important to understand what leukocyte profiles can and cannot tell us. Although they can give information about whether an individual may be subjected to more or less stress relative to other individuals, the information obtained from one blood smear tells us little if anything about the *ability* of that individual to mount an immune response. Researchers who attempt to tie leukocyte profiles to immune function or fitness are faced with a dilemma: does heterophilia indicate stress and illness (and therefore low fitness), or does it indicate a superior ability to respond to infection (high fitness)? Does lymphopenia indicate an active stress response, or a lack of parasites? Or does it indicate immunosuppression? This troubling paradox is illustrated in the literature on plumage brightness and coloration in passerine birds. Saks *et al.* (2003) reported a negative correlation between the brightness of the yellow feathers of greenfinches (*Carduelis*

chloris) and heterophil count, and interpreted this result to mean that the brighter birds were in better health because their heterophil counts were low. In contrast, Dufva & Allander (1995) found the opposite relationship between yellow coloration and H:L ratios in great tits (*Parus major*), and argued that heterophilia (high heterophil count), which in this case was associated with bright coloration, indicated an "efficient immune response" and "superior immunity to parasites." Similarly, Figuerola *et al.* (1999) reported that yellow coloration correlated positively to H:L ratio in cirl buntings (*Emberiza cirlus*), and suggested that the high ratios indicated an "absence of parasites and infectious diseases and correspondingly better overall health." Although Dufva & Allander (1995) and Figuerola *et al.* (1999) considered alternative explanations, they concluded that brighter coloration signals better health. If, however, H:L ratios are related to stress, as we have reviewed here, the brighter birds in their studies were actually experiencing more stress, and possibly greater rates of infection, than the duller ones. We recently found that in northern cardinals (*Cardinalis cardinalis*), the more deeply saturated, redder birds had higher H:L ratios than less colorful birds (Maney *et al.* 2008). Our result is consistent with Dufva & Allander (1995) and Figuerola *et al.* (1999); however, we believe that the saturated coloration is associated with greater stress, perhaps due to greater energy expenditure on mate seeking and/or territory defense. This result is consistent with that reported by (Mazerolle & Hobson 2002), who found that birds defending high quality territories (and presumed to be most fit) had higher H:L ratios than those in lower quality territories. Note that none of these H:L data give any information regarding 'immunocompetence' or 'immunosuppression' because the immune systems of these birds were not systematically challenged by the researchers in the course of these studies. Leukocyte profiles inform only on the relative proportions of WBC types that are currently circulating in blood. They do not

indicate the numbers of heterophils or lymphocytes that are available in reserve in other body compartments, or (without doing another smear after a stress paradigm) how many would be released or redistributed in response to a stressor or infectious agent. Assessing leukocyte profiles as described here is therefore not the same as measuring immune responses *per se*, which can be more directly quantified using other methods.

A second important challenge in the interpretation of leukocyte profiles is that the 'normal' parameters for many species of wildlife are not known. Thus, ecologists are often faced with the problem of trying to determine if the leukocyte differentials they obtained from blood smears (i.e. the proportions of lymphocytes, neutrophils, etc.) differ from what normally occurs in their study species. In the field of wildlife medicine, investigators attempt to address this issue by reporting 'reference' hematological parameters (i.e. means and minimum and maximums) for species that have not yet been examined hematologically, and these can include leukocyte profiles, plasma chemistry values (Eliman 1997, Hrubec *et al.* 2000, Werner 2007) and sometimes even red and white blood cell morphology (Martinez-Silvestre *et al.* 2005, Sacchi *et al.* 2007). If the ecologist is fortunate enough to study an animal that has had leukocyte reference values established, their task of interpreting the leukocyte profiles at hand is certainly made easier. However, we point out that any set of hematological 'reference' values must be viewed with one caveat in mind, which is that the animals examined in such studies usually come from captive collections, or if wild-caught, from a single population, and as such their hematological parameters may not necessarily be representative of the species as a whole. Therefore they should be treated more as a starting place for future work than a set of absolutes. Furthermore, reference values can vary among investigators because of differences in handling procedures prior to sampling, or captive housing conditions (see Case *et al.* 2005), leading to cases where

two or more groups of investigators report vastly different 'reference' hematological parameters for the same species. In one such case, Cathers *et al.* (1997) reported leukocyte profiles of American bullfrogs (*Rana catesbeiana*) with lymphocyte and neutrophil proportions of 63% and 22%, respectively. Meanwhile, Coppo *et al.* (2005) reported values of 27% and 61% for lymphocytes and neutrophils, respectively, for the same species. This example highlights the inherent difficulty in making comparisons of hematological parameters between investigators; however, we point out that this problem can be overcome, at least in experimental scenarios, by comparing leukocyte parameters from experimental groups of animals with those from non-manipulated controls (e.g. Bennett & Johnson 1973, Davis & Maerz 2008b, Valenzuela *et al.* 2008).

A third challenge in the interpretation of leukocyte profiles is distinguishing stress responses from those of inflammation or disease. Given that leukocytes make up the primary line of defense in the innate immune system of vertebrates, it is not surprising that infection and diseases cause alterations in their numbers. In fact, the effect of disease on leukocyte profiles is similar to that of stress in that neutrophilia/heterophilia and lymphopenia are commonly observed in all taxa. Indeed, it is well established that neutrophils/heterophils, being phagocytic, proliferate in circulation to combat infections (Jain 1986, Latimer *et al.* 1988, Campbell 1996), and the increase in this cell type alone can cause increases in N:L or H:L ratios during infections (e.g. Davis *et al.* 2004). Therefore, interpreting changes in leukocyte profiles (i.e. attributing them to stress vs. infection) can be problematic, especially if infection status is not known. Indeed, the two factors are closely related: stress is known to lead to susceptibility to diseases (e.g. Al-Murrani *et al.* 2002), and infections or diseases can cause increases in stress (e.g. Lindström *et al.* 2005). The two may be dissociated, however, by looking at other hematological

parameters. In addition to causing relative neutrophilia and lymphopenia, infections commonly cause general increases in monocytes, which also phagocytize foreign particles and infections (e.g. Jain 1986, Campbell 1996, Davis *et al.* 2004), and general increases in total WBC count (Jain 1986, Latimer *et al.* 1988, Jain 1993, Thrall 2004). In addition, Jain (1986) reports that a reduction in relative eosinophil numbers is more often a stress reaction than a response to disease (discussed in detail later). Thus, by considering the relative number of both monocytes and eosinophils, as well as the total leukocyte count (and see below), it may be possible to dissociate the effects of infection from those of stressors. The identification of parameters associated solely with one or the other that could be incorporated into standard hematological screenings represents an area where more work is needed.

Other leukocyte stress indicators

Leukocytosis, or general increases in total WBC numbers, has been occasionally used as a measure of stress (e.g. Ots *et al.* 1998), although using this parameter to infer stress is problematic. First, WBC counts are inherently widely variable among individuals (Jain 1986). Second, as is the case for H:L ratios in the plumage brightness literature, some authors have attempted to connect this measure to immunocompetence, but there appears to be inconsistency in the ecological literature regarding the definition of immunocompetency in terms of total white blood cell counts. For example, Nunn *et al.* (2000) argued that higher 'baseline' counts indicate stronger immune systems in animals, whereas Pap (2002) suggested the opposite. Forson & Storfer (2006) found lower numbers of leukocytes in salamanders exposed to a herbicide (compared to a control group), and interpreted this result as a suppression of the innate immune system (implying that higher counts are the norm). Murad & Houston (1988) observed lowered

leukocyte counts in fish exposed to cadmium, and Wedemeyer *et al.* (1990) state that lowered WBC counts are an indication of acute stress in all fish. In fact, the effect of stress on WBC counts in other animals seems only to add to the confusion. In house finches, capture followed by 1 hour of handling leads to decreases in total leukocytes (Davis 2005); transport of wild and domestic birds also causes reductions in total leukocytes over 1 to 3 hours (Parga *et al.* 2001, Scope *et al.* 2002). In the example of the exercised horse shown in Fig. 2.3, total leukocyte numbers *increased* until the end of the exercise period (Cardinet *et al.* 1964). Early work by Dougherty & White (1944) demonstrated that total leukocyte numbers dropped in mice and rats after injection with ACTH, reaching a low at 9 hours post-injection and returning to normal levels at 24 hours. Similar trends were seen in rats subjected to restraint for 2 hours (Dhabhar *et al.* 1996). Injections of steroid hormones in horses, however, led to a doubling of total leukocytes within 2 hours (Jain 1986).

Part of the problem with interpreting total leukocyte counts, especially in little-studied species, is that prior information regarding the 'normal' range of leukocyte numbers in such species, like information on leukocyte profiles (discussed previously), is rarely available. Without this information one cannot know whether the counts obtained are 'high' or 'low'. If the normal range *is* known, as it is for humans and some domesticated animals, then higher than normal white blood cell numbers are most commonly interpreted as a sign of inflammation (Jain 1986, Latimer *et al.* 1988, Ford 2002), which in itself is informative because of its prognostic value. In fact, in the medical literature there is a growing body of evidence demonstrating that high white blood cell counts are an important predictor of patient mortality from a variety of ailments, including heart disease, stroke and diabetes (Do Lee *et al.* 2001, Ford 2002, Ha Jee *et al.* 2005, Núñez *et al.* 2006). Interestingly, however, other medical researchers found that the

neutrophil-lymphocyte ratio was even more predictive of patient mortality than their total leukocyte count (Horne *et al.* 2005)!

A final leukocyte parameter to consider as a measure of stress is the eosinophil count. Although not commonly cited in contemporary work, historic research on humans and mammals demonstrated that glucocorticoid-induced stress leads to a reduction in eosinophil numbers (Hills *et al.* 1948, Gordon 1955, Cardinet *et al.* 1964). This phenomenon is also listed as in indicator of stress in veterinary haematology texts (Jain 1986, 1993). Oddly, this parameter is not routinely reported in modern stress research, even in studies involving mammals (Dhabhar *et al.* 1995, 1996, López-Olvera *et al.* 2007), although it has been reported in fish that were administered cortisol (Wojtaszek *et al.* 2002). Within the amphibians, there is also some experimental evidence that stress hormones reduce eosinophil numbers in amphibians (Belden & Kiesecker 2005), and that this reduction leads to reduced resistance to parasites. Since eosinophil numbers may help distinguish leukocyte responses to stress from those caused by infection (Jain 1986), this variable should be incorporated more often into hematological measurements.

Conclusions

Research conducted over the past several decades indicates that the quantification of hematological parameters such as neutrophilia/heterophilia, lymphopenia, or H:L ratio (but not total WBC counts), may be measured as a compliment to, or even in lieu of, the measurement of adrenal hormones in the study of vertebrate stress responses. A large body of evidence demonstrates that the adrenal and leukocyte responses to stress are tightly linked, and are similar across vertebrate taxa. The leukocyte approach offers several advantages, such as a longer period of time within which to obtain initial blood samples (especially in ectotherms), low cost and

feasibility when working with small animals. Moreover, work currently being conducted in humans and birds shows that leukocyte profiles can help predict an individual's future performance and viability. Regardless of the taxon under study, an understanding of any animal's leukocyte parameters and what they mean ultimately comes from immunology, a field in which the majority of research is conducted in a biomedical context and published in biomedical journals. As is the case with any physiological measure, ecologists must be aware of and consider this body of research when designing their studies and interpreting their data (Romero 2004). With that effort, along with an increasing understanding of how to apply biomedical knowledge to novel species, hematological measures will no doubt become more established in ecology. Considering all of the issues discussed here, we conclude that counts of leukocytes can provide a reliable method in ecological research to study vertebrate responses to stress, and this approach can be used to help ascertain the current and future welfare of study subjects.

Acknowledgements

This review was born from discussions with multiple colleagues and from many reviewers of our hematological research, whom we thank for providing the impetus for writing it. We thank the members of the Maerz herpetology laboratory at the University of Georgia for stimulating discussions of the subject. We also thank Karin Lindström and two anonymous referees for providing helpful comments on the manuscript. Funding for A.K.D. was provided by the Warnell School of Forestry and Natural Resources at UGA, and for D.L.M. by the Center for Behavioral Neuroscience.

Literature cited

Acanfora, D., M. Gheorghiade, L. Trojano, G. Furgi, E. Pasini, C. Picone, A. Papa, G. L. Iannuzzi, R. O. Bonow, and F. Rengo. 2001. Relative lymphocyte count: A prognostic indicator of mortality in elderly patients with congestive heart failure. American Heart Journal 142: 167-173.

Al-Murrani, W. K., I. K. Al-Rawi, and N. M. Raof. 2002. Genetic resistance to *Salmonella typhimurium* in two lines of chickens selected as resistant and sensitive on the basis of heterophil/lymphocyte ratio. British Poultry Science 43: 501-507.

Al-Murrani, W. K., A. J. Al-Rawi, M. F. Al-Hadithi, and B. Al-Tikriti. 2006. Association between heterophil/lymphocyte ratio, a marker of 'resistance' to stress, and some production and fitness traits in chickens. British Poultry Science 47: 443-448.

Altan, O., A. Altan, M. Cabuk, and H. Bayraktar. 2000. Effects of heat stress on some blood parameters in broilers. Turkish Journal of Veterinary Animal Science 24: 145-148.

Anderson, B. H., D. L. Watson, and I. G. Colditz. 1999. The effect of dexamethasone on some immunological parameters in cattle. Veterinary Research Communications 23: 399-413.

Baker, M. L., E. Gemmell, and R. T. Gemmell. 1998. Physiological changes in brushtail possums, *Trichosurus vulpecula*, transferred from the wild to captivity. Journal of Experimental Zoology 280: 203-212.

Belden, L. K., and J. M. Kiesecker. 2005. Glucocorticosteroid hormone treatment of larval treefrogs increases infection by *Alaria* sp trematode cercariae. Journal of Parasitology 91: 686-688.

Bennett, M. F. 1986. Stress and changes in the blood of newts, *Notophthalmus viridescens*, during early regeneration. Journal of Comparative Physiology A 159: 823-826.

Bennett, M. F., and J. K. Alspaugh. 1964. Some changes in the blood of frogs following administration of hydrocortisone. The Virginia Journal of Science 15: 76-79.

Bennett, M. F., and J. A. Harbottle. 1968. The effects of hydrocortisone on the blood of tadpoles and frogs, *Rana catesbeiana*. Biological Bulletin 135: 92-95.

Bennett, M. F., and A. O. Johnson. 1973. Osmotic stress, ACTH and the white blood cell picture in newts, *Notophthalmus viridescens*. Journal of Comparative Physiology A 82: 333-338.

Bennett, M. F., and C. Gaudio Neville. 1975. Effects of cold shock on the distribution of leukocytes in goldfish, *Carassius auratus*. Journal of Comparative Physiology A 98: 213-216.

Bennett, M. F., and L. E. Reap. 1978. Photoperiod, stress and the distribution of leukocytes in the peripheral blood of *Notophthalmus viridescens*. Journal of Comparative Physiology A 125: 205-207.

Bennett, M. F., C. A. Gaudio, A. O. Johnson, and J. H. Spisso. 1972. Changes in the blood of newts, *Notophthalmus viridescens*, following administration of hydrocortisone. Journal of Comparative Physiology A 80: 233-237.

Bilandžič, N., M. Žurič, M. Lojkič, B. Šimič, D. Milić, and I. Barač. 2006. Cortisol and immune measures in boars exposed to three-day administration of exogenous adrenocorticotropic hormone. Veterinary Research Communications 30: 433-444.

Bishop, C. R., J. W. Athens, D. R. Boggs, H. R. Warner, Cartwrig.Ge, and M. M. Wintrobe. 1968. Leukokinetic Studies 13. A non-steady-state kinetic evaluation of mechanism of cortisone-induced granulocytosis. Journal of Clinical Investigation 47: 249-&.

Bly, J. E., N. W. Miller, and L. W. Clem. 1990. A monoclonal antibody specific for neutrophils in normal and stressed catfish. Developmental and Comparative Immunology 14: 211-221.

Brenner, I., P. N. Shek, J. Zamecnik, and R. J. Shephard. 1998. Stress hormones and the immunological responses to heat and exercise. International Journal of Sports Medicine 19: 130-143.

Burguez, P. N., J. Ousey, R. S. G. Cash, and P. D. Rossdale. 1983. Changes in blood neutrophil and lymphocyte counts following administration of cortisol to horses and foals. Equine Veterinary Journal 15: 58-60.

Cabagna, M. C., R. C. Lajmanovich, G. Stringhini, J. C. Sanchez-Hernandez, and P. M. Peltzer. 2005. Hematological parameters of health status in the common toad *Bufo arenarum* in agroecosystems of Santa Fe Province, Argintina. Applied Herpetology 2: 373-380.

Campbell, T. W. 1995. Avian Hematology and Cytology. Iowa State University Press, Ames.

Campbell, T. W. 1996. Clinical pathology, pp. 248-257. *In* D. R. Mader [ed.], Reptile medicine and surgery. W.B. Saunders Company, Philadelphia.

Camplani, A., N. Saino, and A. P. Moller. 1999. Carotenoids, sexual signals and immune function in barn swallows from Chernobyl. Proceedings of the Royal Society of London Series B-Biological Sciences 266: 1111-1116.

Cardinet, G. H., J. F. Littrell, and O. W. Schalm. 1964. Effects of sustained muscular activity upon blood morphology of the horse. The California Veterinarian 18: 31-35.

Case, B. C., G. A. Lewbart, and P. D. Doerr. 2005. The physiological and behavioural impacts of and preference for an enriched environment in the eastern box turtle (*Terrapene carolina carolina*). Applied Animal Behaviour Science 92: 353-365.

Cathers, T., G. A. Lewbart, M. Correa, and J. B. Stevens. 1997. Serum chemistry and hematology values for anesthetized American bullfrogs (*Rana catesbeiana*). Journal of Zoo and Wildlife Medicine 28: 171-174.

Chen, X., C. Niu, and L. Pu. 2007. Effects of stocking density on growth and non-specific immune responses in juvenile soft-shelled turtle, *Pelodiscus sinensis*. Aquaculture Research 38: 1380-1386.

Cohen, J. J. 1972. Thymus-derived lymphocytes sequestered in bone marrow of hydrocortisone-treated mice. Journal of Immunology 108: 841-&.

Coppo, J. A., N. B. Mussart, S. A. Fioranelli, and P. A. Zeinsteger. 2005. Blood and urine physiological values in captive bullfrog, *Rana catesbeiana* (Anura: Ranidae). Analecta Veterinaria 25: 15-17.

Cox, J. H., and W. L. Ford. 1982. The migration of lymphocytes across specialized vascular endothelium 4: Prednisolone acts at several points on the re-circulation pathways of lymphocytes. Cellular Immunology 66: 407-422.

Dale, D. C., A. S. Fauci, D. Guerry, and S. M. Wolff. 1975. Comparison of agents producing a neutrophilic leukocytosis in man - hydrocortisone, prednisone, endotoxin, and etiocholanolone. Journal of Clinical Investigation 56: 808-813.

Davis, A. K. 2005. Effects of handling time and repeated sampling on avian white blood cell counts. Journal of Field Ornithology 76: 334-338.

Davis, A. K., and J. C. Maerz. 2008a. Sex-related differences in hematological stress indices of breeding, paedomorphic mole salamanders. Journal of Herpetology 42: 197-201.

Davis, A. K., and J. C. Maerz. 2008b. Comparison of hematological stress indicators in recently captured and captive paedomorphic mole salamanders, *Ambystoma talpoideum*. Copeia 2008: 613-617.

Davis, A. K., K. C. Cook, and S. Altizer. 2004. Leukocyte profiles of House Finches with and without mycoplasmal conjunctivitis, a recently emerged bacterial disease. Ecohealth 1: 362-373.

Davis, G. S., K. E. Anderson, and A. S. Carroll. 2000. The effects of long-term caging and molt of single comb white leghorn hens on heterophil to lymphocyte ratios, corticosterone and thyroid hormones. Poultry Science 79: 514-518.

de la Balze, F. A., E. C. Reifenstein, and F. Albright. 1946. Differential blood counts in certain adrenal cortical disorders (Cushing's syndrome, Addison's disease and panhypopituitarism). Journal of Clinical Endocrinology 6: 312-319.

Dethloff, G. M., D. Schlenk, J. T. Hamm, and H. C. Bailey. 1999. Alterations in physiological parameters of rainbow trout (*Oncorhynchus mykiss*) with exposure to copper and copper/zinc mixtures. Ecotoxicology and Environmental Safety 42: 253-264.

Dhabhar, F. S. 2002. A hassle a day may keep the doctor away: Stress and the augmentation of immune function. Integrative and Comparative Biology 42: 556-564.

Dhabhar, F. S., A. H. Miller, B. S. McEwen, and R. L. Spencer. 1995. Effects of stress on immune cell distribution - Dynamics and hormonal mechanisms. Journal of Immunology 154: 5511-5527.

Dhabhar, F. S., A. H. Miller, B. S. McEwen, and R. L. Spencer. 1996. Stress-induced changes in blood leukocyte distribution - Role of adrenal steroid hormones. Journal of Immunology 157: 1638-1644.

Dhabhar, F. S., A. H. Miller, M. Stein, B. S. McEwen, and R. L. Spencer. 1994. Diurnal and acute stress-induced changes in distribution of peripheral blood leukocyte subpopulations. Brain Behavior and Immunity 8: 66-79.

Dick, P. T., and D. G. Dixon. 1985. Changes in circulating blood cell levels of rainbow trout, *Salmo gairdneri* Richardson, following acute and chronic exposure to copper. Journal of Fish Biology 26: 475-481.

Do Lee, C., A. R. Folsom, F. J. Nieto, L. E. Chambless, E. Shahar, and D. A. Wolfe. 2001. White blood cell count and incidence of coronary heart disease and ischemic stroke and mortality from cardiovascular disease in African-American and White men and women - Atherosclerosis risk in communities study. American Journal of Epidemiology 154: 758-764.

Dougherty, T. F., and A. White. 1944. Influence of hormones on lymphoid tissue structure and function. The role of the pituitary adrenotrophic hormone in the regulation of the lymphocytes and other cellular elements of the blood. Endocrinology 35: 1-14.

Dufva, R., and K. Allander. 1995. Intraspecific variation in plumage coloration reflects immune response in Great Tit (*Parus major*) males. Functional Ecology 9: 785-789.

Eliman, M. M. 1997. Hematology and plasma chemistry of the inland bearded dragon, *Pogona vitticeps*. Bulletin of the Association of Reptile and Amphibian Veterinarians 7.

Ellis, A. E. 1977. The leukocytes of fish: a review. Journal of Fish Biology 11: 453-491.

Ellsaesser, C. F., and L. W. Clem. 1986. Hematological and immunological changes in channel catfish stressed by handling and transport. Journal of Fish Biology 28: 511-521.

Elston, J. J., M. Beck, M. A. Alodan, and V. Vega-Murillo. 2000. Laying hen behavior 2. Cage type preference and heterophil to lymphocyte ratios. Poultry Science 79: 477-482.

Fauci, A. S. 1975. Mechanisms of corticosteroid action on lymphocyte subpopulations 1. Redistribution of circulating T-lymphocytes and B-lymphocytes to bone marrow. Immunology 28: 669-680.

Fauci, A. S., and D. C. Dale. 1974. Effect of in-vivo hydrocortisone on subpopulations of human lymphocytes. Journal of Clinical Investigation 53: 240-246.

Figuerola, J., E. Munoz, R. Gutierrez, and D. Ferrer. 1999. Blood parasites, leucocytes and plumage brightness in the Cirl Bunting, *Emberiza cirlus*. Functional Ecology 13: 594-601.

Fisse, A., M. Draud, B. Raphael, and K. Melkonian. 2004. Differential leukocyte counts of critically endangered grand cayman blue iguanas, *Cyclura nubila lewisi*. Journal of Herpetological Medicine and Surgery 14: 19-21.

Forbes, M. R., D. L. McRuer, and D. Shutler. 2006. White blood cell profiles of breeding American toads (*Bufo americanus*) relative to sex and body size. Comparative Clinical Pathology 15: 155-159.

Ford, E. S. 2002. Leukocyte count, erythrocyte sedimentation rate, and diabetes incidence in a national sample of US adults. American Journal of Epidemiology 155: 57-64.

Forson, D., and A. Storfer. 2006. Atrazine increases ranavirus susceptibility in the tiger salamander, *Ambystoma tigrinum*. Ecological Applications 16: 2325-2332.

Frank, D., A. Gauthier, and R. Bergeron. 2006. Placebo-controlled double-blind clomipramine trial for the treatment of anxiety or fear in beagles during ground transport. Canadian Veterinary Journal-Revue Veterinaire Canadienne 47: 1102-1108.

Gordon, A. S. 1955. Some aspects of hormonal influences upon the leukocytes. Annals of the New York Academy of Science 59: 907-927.

Groombridge, J. J., J. G. Massey, J. C. Brunch, T. R. Malcolm, C. N. Brosius, M. M. Okada, and B. Sparklin. 2004. Evaluating stress in a Hawaiian honeycreeper, *Paroreomyza montana*, following translocation. Journal of Field Ornithology 75: 183-187.

Gross, W. B., and H. S. Siegel. 1983. Evaluation of the heterophil/lymphocyte ratio as a measure of stress in chickens. Avian Diseases 27: 972-979.

Ha Jee, S., J. Y. Park, H. S. Kim, T. Y. Lee, and J. M. Samet. 2005. White blood cell count and risk for all-cause, cardiovascular, and cancer mortality in a cohort of Koreans. American Journal of Epidemiology 162: 1062-1069.

Harmon, B. G. 1998. Avian heterophils in inflammation and disease resistance. Poultry Science 77: 972-977.

Harris, J., and D. J. Bird. 2000. Modulation of the fish immune system by hormones. Veterinary Immunology and Immunopathology 77: 163-176.

Hawkey, C. M., and T. B. Dennett. 1989. Color Atlas of Comparative Veterinary Hematology. Iowa State University Press, Ames, Iowa.

Hills, A. G., P. H. Forsham, and C. A. Finch. 1948. Changes in circulating leukocytes induced by the administration of pituitary adrenocorticotrophic hormone (ACTH) in man. Blood 3: 755-768.

Horne, B. D., J. L. Anderson, J. M. John, A. Weaver, T. L. Bair, K. R. Jensen, D. G. Renlund, and J. B. Muhlestein. 2005. Which white blood cell subtypes predict increased cardiovascular risk? Journal of the American College of Cardiology 45: 1638-1643.

Hrubec, T. C., J. L. Cardinale, and S. A. Smith. 2000. Hematology and plasma chemistry reference intervals for cultured tilapia (*Oreochromis hybrid*). Veterinary Clinical Pathology 29: 7-12.

Jain, N. C. 1986. Schalm's Veterinary Hematology. Lea & Febiger, Philadelphia.

Jain, N. C. 1993. Essentials of Veterinary Hematology. Blackwell Publishing, Philadelphia.

Kiesecker, J. M. 2002. Synergism between trematode infection and pesticide exposure: a link to amphibian deformities in nature? Proceedings of the National Academy of Sciences 99: 9900-9904.

Kilgas, P., V. Tilgar, and R. Mand. 2006. Hematological health state indices predict local survival in a small passerine bird, the great tit (*Parus major*). Physiological and Biochemical Zoology 79: 565-572.

Kock, R. A., S. R. O. Mihok, J. Wambua, J. Mwanzia, and K. Saigawa. 1999. Effects of translocation on hematologic parameters of free-ranging black rhinoceros (*Diceros bicornis michaeli*) in Kenya. Journal of Zoo and Wildlife Medicine 30: 389-396.

Kranendonk, G., H. Hopster, F. van Eerdenburg, K. van Reenen, M. Fillerup, J. de Groot, M. Korte, and M. Taverne. 2005. Evaluation of oral administration of cortisol as a model for prenatal stress in pregnant sows. American Journal of Veterinary Research 66: 780-790.

Kronfol, Z., R. Turner, H. Nasrallah, and G. Winokur. 1984. Leukocyte regulation in depression and schizophrenia. Psychiatry Research 13: 13-18.

Lance, V. A., and R. M. Elsey. 1999. Plasma catecholamines and plasma corticosterone following restraint stress in juvenile alligators. Journal of Experimental Zoology 283: 559-565.

Langkilde, T., and R. Shine. 2006. How much stress do researchers inflict on their study animals? A case study using a scincid lizard, *Eulamprus heatwolei*. Journal of Experimental Biology 209: 1035-1043.

Larsson, A., K. J. Lehtinen, and C. Haux. 1980. Biochemical and hematological effects of a titanium dioxide industrial effluent on fish. Bulletin of Environmental Contamination and Toxicology 25: 427-435.

Latimer, K. S., K. N. Tang, M. A. Goodwin, W. L. Steffens, and J. Brown. 1988. Leukocyte changes associated with acute inflammation in chickens. Avian Diseases 32: 760-772.

LeBlanc, C. J., J. J. Heatley, and E. B. Mack. 2000. A review of the morphology of lizard leukocytes with a discussion of the clinical differentiation of bearded dragon, *Pogona vitticeps*, leukocytes. Journal of Herpetological Medicine and Surgery 10: 27-30.

Lindström, K., D. Hawley, A. K. Davis, and M. Wikelski. 2005. Stress responses and disease in three wintering house finch (*Carpodacus mexicanus*) populations along a latitudinal gradient. General and Comparative Endocrinology 143: 231-239.

Lobato, E., J. Moreno, S. Merino, J. J. Sanz, and E. Arriero. 2005. Haematological variables are good predictors of recruitment in nestling pied flycatchers (*Ficedula hypoleuca*). Ecoscience 12: 27-34.

López-Olvera, J. R., I. Marco, J. Montané, E. Casas-Díaz, and S. Lavín. 2007. Effects of acepromazine on the stress response in Southern chamois (*Rupicapra pyrenaica*) captured by means of drive-nets. Canadian Journal of Veterinary Research 71: 41-51.

Maney, D. L., A. K. Davis, C. T. Goode, A. Reid, and C. Showalter. 2008. Carotenoid-based plumage coloration predicts leukocyte parameters during the breeding season in northern cardinals (*Cardinalis cardinalis*) Ethology 114: 369-380.

Martinez-Silvestre, A., I. Marco, M. A. Rodriguez-Dominguez, S. Lavin, and R. Cuenca. 2005. Morphology, cytochemical staining, and ultrastructural characteristics of the blood cells of the giant lizard of El Hierro (Gallotia simonyi). Research in Veterinary Science 78: 127-134.

Maxwell, M. H. 1987. The avian eosinophil: a review. Worlds Poultry Science Journal 43: 190-207.

Maxwell, M. H. 1993. Avian blood leukocyte responses to stress. Worlds Poultry Science Journal 49: 34-43.

Maxwell, M. H., and G. W. Robertson. 1998. The avian heterophil leucocyte: a review. Worlds Poultry Science Journal 54: 155-178.

Mazerolle, D. F., and K. A. Hobson. 2002. Physiological ramifications of habitat selection in territorial male ovenbirds: consequences of landscape fragmentation. Oecologia 130: 356-363.

McFarlane, J. M., S. E. Curtis, J. Simon, and O. A. Izquierdo. 1989. Multiple concurrent stressors in chicks 2. Effects on hematologic, body composition, and pathologic traits. Poultry Science 68: 510-521.

McLeay, D. J. 1973. Effects of ACTH on pituitary interrenal axis and abundance of white blood cell types in juvenile coho salmon, *Oncorhynchus kisutch*. General and Comparative Endocrinology 21: 431-440.

Mishra, S., and A. K. Srivastava. 1979. Hematology as index of sublethal toxicity of zinc in a freshwater teleost. Bulletin of Environmental Contamination and Toxicology 22: 695-698.

Moreno, J., S. Merino, J. Martinez, J. J. Sanz, and E. Arriero. 2002. Heterophil/lymphocyte ratios and heat-shock protein levels are related to growth in nestling birds. Ecoscience 9: 434-439.

Morici, L. A., R. M. Elsey, and V. A. Lance. 1997. Effects of long-term corticosterone implants on growth and immune function in juvenile alligators, *Alligator mississippiensis*. Journal of Experimental Zoology 279: 156-162.

Morrow-Tesch, J. L., J. J. McGlone, and R. L. Norman. 1993. Consequences of restraint stress on natural killer cell activity, behavior, and hormone levels in Rhesus Macaques (*Macaca mulatta*). Psychoneuroendocrinology 18: 383-395.

Muir, J. L., and H. P. Pfister. 1987. Time course of the corticosterone and prolactin response following predictable and unpredictable novelty stress in *Rattus norvegicus*. Physiology & Behavior 40: 103-107.

Murad, A., and A. H. Houston. 1988. Leukocytes and leukopoietic capacity in goldfish, *Carassius auratus*, exposed to sublethal levels of cadmium. Aquatic Toxicology 13: 141-154.

Nicol, C. J., S. N. Brown, E. Glen, S. J. Pope, F. J. Short, P. D. Warriss, P. H. Zimmerman, and L. J. Wilkins. 2006. Effects of stocking density, flock size and management on the welfare of laying hens in single-tier aviaries. British Poultry Science 47: 135-146.

Noda, K., H. Akiyoshi, M. Aoki, T. Shimada, and F. Ohashi. 2007. Relationship between transportation stress and polymorphonuclear cell functions of bottlenose dolphins, *Tursiops truncatus*. Journal of Veterinary Medical Science 69: 379-383.

Núñez, J., E. Núñez, J. Sanchis, V. Bodí, and A. Llàcer. 2006. Prognostic value of leukocytosis in acute coronary syndromes: The cinderella of the inflammatory markers. Current Medicinal Chemistry 13: 2113-2118.

Nunn, C. L., J. L. Gittleman, and J. Antonovics. 2000. Promiscuity and the primate immune system. Science 290: 1168-1170.

Nussey, G., J. H. J. Vanvuren, and H. H. Dupreez. 1995. Effect of copper on the differential white blood cell counts of the Mozambique tilapia (*Oreochromis mossambicus*). Comparative Biochemistry and Physiology C 111: 381-388.

Obernier, J. A., and R. L. Baldwin. 2006. Establishing an appropriate period of acclimatization following transportation of laboratory animals. Ilar Journal 47: 364-369.

Ommen, S. R., S. C. Hammill, and R. J. Gibbons. 2002. The relative lymphocyte count predicts death in patients receiving implantable cardioverter defibrillators. Pacing and Clinical Electrophysiology 25: 1424-1428.

Ommen, S. R., R. J. Gibbons, D. O. Hodge, and S. P. Thomson. 1997. Usefulness of the lymphocyte concentration as a prognostic marker in coronary artery disease. American Journal of Cardiology 79: 812-&.

Ommen, S. R., D. O. Hodge, R. J. Rodeheffer, C. G. A. McGregor, S. P. Thomson, and R. J. Gibbons. 1998. Predictive power of the relative lymphocyte concentration in patients with advanced heart failure. Circulation 97: 19-22.

Onbasilar, E. E., and F. T. Aksoy. 2005. Stress parameters and immune response of layers under different cage floor and density conditions. Livestock Production Science 95: 255-263.

Ots, I., A. Murumagi, and P. Horak. 1998. Haematological health state indices of reproducing Great Tits: methodology and sources of natural variation. Functional Ecology 12: 700-707.

Ottaway, C. A., and A. J. Husband. 1994. The influence of neuroendocrine pathways on lymphocyte migration. Immunology Today 15: 511-517.

Owen, J. C., and F. R. Moore. 2006. Seasonal differences in immunological condition of three species of thrushes. Condor 108: 389-398.

Pap, T. L. 2002. Breeding time and sex-specific health status in the barn swallow (*Hirundo rustica*). Canadian Journal of Zoology-Revue Canadienne De Zoologie 80: 2090-2099.

Parga, M. L., H. Pendl, and N. A. Forbes. 2001. The effect of transport on hematologic parameters in trained and untrained Harris's Hawks (*Parabuteo unicinctus*) and Perigrine Falcons (*Falco peregrinus*). Journal of Avian Medicine and Surgery 15: 162-169.

Pfaff, J. A., L. Zanette, S. A. MacDougall-Shackleton, and E. A. MacDougall-Shackleton. 2007. Song repertoire size varies with HVC volume and is indicative of male quality in song sparrows (*Melospiza melodia*). Proceedings of the Royal Society B 274: 2035-2040.

Pough, F. H. 1980. Advantages of ectothermy for tetrapods. American Naturalist 115: 92-112.

Pulsford, A. L., S. Lemairegony, M. Tomlinson, N. Collingwood, and P. J. Glynn. 1994. Effects of acute stress on the immune system of the Dab, *Limanda limanda*. Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology 109: 129-139.

Reddan, D. N., P. S. Klassen, L. A. Szczech, J. A. Coladonato, S. O'Shea, W. F. Owen, and E. G. Lowrie. 2003. White blood cells as a novel mortality predictor in haemodialysis patients. Nephrology Dialysis Transplantation 18: 1167-1173.

Reidarson, T. H., and J. F. McBain. 1999. Hematologic, biochemical, and endocrine effects of dexamethasone on bottlenose dolphins (*Tursiops truncatus*). Journal of Zoo and Wildlife Medicine 30: 310-312.

Romero, L. M. 2004. Physiological stress in ecology: lessons from biomedical research. Trends in Ecology & Evolution 19: 249-255.

Romero, L. M., and M. Wikelski. 2001. Corticosterone levels predict survival probabilities of Galapagos marine iguanas during El Nino events. Proceedings of the National Academy of Sciences of the United States of America 98: 7366-7370.

Romero, L. M., and R. C. Romero. 2002. Corticosterone responses in wild birds: the importance of rapid initial sampling. Condor 104: 129-135.

Romero, L. M., and J. M. Reed. 2005. Collecting baseline corticosterone samples in the field: is under 3 min good enough? Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology 140: 73-79.

Rossdale, P. D., P. N. Burguez, and R. S. G. Cash. 1982. Changes in blood neutrophil/lymphocyte ratio related to adrenocortical function in the horse. Equine Veterinary Journal 14: 293-298.

Rouf, M. A. 1969. Hematology of the leopard frog, *Rana pipiens*. Copeia 1969: 682-687.

Rupley, A. E. 1997. Manual of Avian Practice. W.B. Saunders Company, Philadelphia PA, USA.

Saad, A. H., and R. Elridi. 1988. Endogenous corticosteroids mediate seasonal cyclic changes in immunity of lizards. Immunobiology 177: 390-403.

Sacchi, R., F. Pupin, M. A. L. Zuffi, S. Scali, E. Boncompagni, A. Binda, P. Galeotti, and M. Fasola. 2007. Blood cell morphology of the Moorish gecko, *Tarentola mauritanica*. Amphibia-Reptilia 28: 503-508.

Saks, L., I. Ots, and P. Horak. 2003. Carotenoid-based plumage coloration of male greenfinches reflects health and immunocompetence. Oecologia 134: 301-307.

Saunders, D. C. 1968. Differential blood cell counts of 50 species of fishes from the Red Sea. Copeia 1968: 491-498.

Scope, A., T. Filip, C. Gabler, and F. Resch. 2002. The influence of stress from transport and handling on hematologic and clinical chemistry blood parameters of racing pigeons (*Columba livia domestica*). Avian Diseases 46: 224-229.

Silveira-Coffigny, R., A. Prieto-Trujillo, and F. Ascencio-Valle. 2004. Effects of different stressors in haematological variables in cultured *Oreochromis aureus* S. Comparative Biochemistry and Physiology, Part C 139: 245-250.

Stevenson, R. D., S. R. Tuberty, P. L. DeFur, and J. C. Wingfield. 2005. Ecophysiology and conservation: the contribution of endocrinology and immunology - Introduction to the symposium. Integrative and Comparative Biology 45: 1-3.

Thomson, S. P., R. J. Gibbons, P. A. Smars, V. J. Suman, R. V. Pierre, P. J. Santrach, and N. S. Jiang. 1995. Incremental value of the leukocyte differential and the rapid creatine

kinase-MB isoenzyme for the early diagnosis of myocardial infarction. Annals of Internal Medicine 122: 335-341.

Thrall, M. A. 2004. Hematology of amphibians. *In* M. A. Thrall, D. C. Baker and E. D. Lassen [eds.], Veterinary Hematology and Clinical Chemistry: Text and Clinical Case Presentations. Lippincott Williams & Wilkins, Philadelphia, PA.

Valenzuela, A. E., V. M. Silva, and A. E. Klempau. 2008. Effects of different artificial photoperiods and temperatures on haematological parameters of rainbow trout (*Oncorhynchus mykiss*). Fish Physiology and Biochemistry 34: 159-167.

Van Dijk, H., N. Bloksma, P. M. Rademaker, W. J. Schouten, and J. M. Willers. 1979. Differential potencies of corticosterone and hydro-cortisone in immune and immune-related processes in the mouse. International Journal of Immunopharmacology 1: 285-292.

Washburn, B. E., D. L. Morris, J. J. Millspaugh, J. Faaborg, and J. H. Schulz. 2002. Using a commercially available radioimmunoassay to quantify corticosterone in avian plasma. Condor 104: 558-563.

Wedemeyer, G. A., B. A. Barton, and D. J. McLeay. 1990. Stress and acclimation, pp. 451-489. *In* C. B. Schreck and P. B. Moyle [eds.], Methods for fish biology. American Fisheries Society, Bethesda, MD.

Werner, R. E. 2007. Reference hematology and serum chemistry values for the diamondback terrapin (*Malaclemys terrapin*). Herpetological Review 38: 17-22.

Wikelski, M., and S. J. Cooke. 2006. Conservation physiology. Trends in Ecology & Evolution 21: 38-46.

Witeska, M. 2005. Stress in fish: hematological and immunological effects of heavy metals. Electronic Journal of Ichthyology 1: 35-41.

Wojtaszek, J., and A. Adamowicz. 2003. Hematology of the fire-bellied toad, *Bombina bombina* L. Comparative Clinical Pathology 12: 129-134.

Wojtaszek, J., D. Dziewulska-Szwajkowska, M. Lozinska-Gabska, A. Adamowicz, and A. Dzugaj. 2002. Hematological effects of high dose of cortisol on the carp (*Cyprinus carpio* L.): Cortisol effect on the carp blood. General and Comparative Endocrinology 125: 176-183.

Table 2.1 Comparison of leukocyte profiles (percentage of total leukocytes) across vertebrate taxa. For reptile species, azurophils, if reported, were pooled with monocytes. Parameters from wild or control animals used if reported.

Taxon	Species	Lymphocyte	Neutro/ Heterophil	Eosinophil	Basophil	Monocyte	Source
Mammals	Dog (*Canis lupus familiaris*)	23.1	66.4	6.3	0.1	4.0	(Jain 1986)
	Human (*Homo sapien*)	34.0	59.0	2-7	0.5	4.0	(Albritton 1952)
	Horse (*Equus caballus*)	38.7	52.6	3.4	0.5	4.3	(Jain 1986)
Birds	Chicken (*Gallus gallus*)	63.0	30.1	2.5	1.3	3.1	(Branton *et al.* 1997)
	Great tit (*Parus major*)	68.5	19.6	5.6	5.6	1.0	(Hauptmanova *et al.* 2002)
	Glaucous-winged gull (*Larus glaucescens*)	43.0	53.0	3.0	0.0	1.0	(Newman *et al.* 1997)
Amphibians	Red-spotted newt (*Notopthalmus viridescens*)	63.5	24.3	6.2	3.2	2.8	(Bennett & Daigle 1983)
	Bullfrog (*Rana catesbeiana*)	62.9	22.0	8.9	2.5	0.6	(Cathers *et al.* 1997)
	American toad (*Bufo americanus*)	20.0	68.0	3.3	7.4	1.5	(Forbes *et al.* 2006)
Fish	North African catfish (*Clarias gariepinus*)	58.8	39.4	0.0	0.0	2.6	(Gabriel *et al.* 2004)
	Channel catfish (*Ictalurus punctatus*)	43.0	3.5	0.0	0.0	1.6	(Ellsaesser & Clem 1986)
	Tilapia (*Oreochromis mossambicus*)	69.5	7.8	1.3	0.0	21.5	(Nussey *et al.* 1995)
Reptiles	Diamondback terrapin (*Malaclemys terrapin*)	17.7	74.6	1.1	1.6	6.1	(Werner 2007)
	Russian tortoise (*Agrionemys horsfieldi*)	46.7	37.2	4.8	5.0	6.3	(Knotkova *et al.* 2002)
	Inland bearded dragon (*Pogona vitticeps*)	59.0	27.0	0.0	9.0	5.0	(Eliman 1997)

CHAPTER 2 FIGURES

2.1 Photomicrographs of amphibian (a) and mammalian (b) neutrophils, and avian (c) and reptilian (d) heterophils.

2.2 Plasma corticosterone levels in (a) female water skinks (*Eulamprus heatwolei*) reproduced from Langkilde & Shine (2006); and (b) rats (reproduced from Muir & Pfister 1987). Skinks were stressed by chasing for 30 seconds. Rats were subjected to restraint stress and compared with a control group (no restraint).

2.3 Lymphocyte (a) and neutrophil (b) responses of a horse to exercise (Exercise day: a 32-mile (51.5-km) track traversed over 8 hours; control day: no exercise). Blood samples were collected at 2-h intervals throughout. Graphs reproduced from Cardinet *et al.* (1964). Arrows indicate end of exercise.

2.4 Plasma corticosterone levels (a) and heterophil-lymphocyte ratios (b) in house finches (*Carpodacus mexicanus*) held for 1 h and sampled at 3 min, 30 min and 60 min after capture. Number of birds sampled is indicated in each column. (a) modified from Lindström *et al.* (2005) and (b) from Davis (2005). Fourth column in lower chart (60*) indicates average H : L ratios of birds held for 1 h and sampled once at time 60.

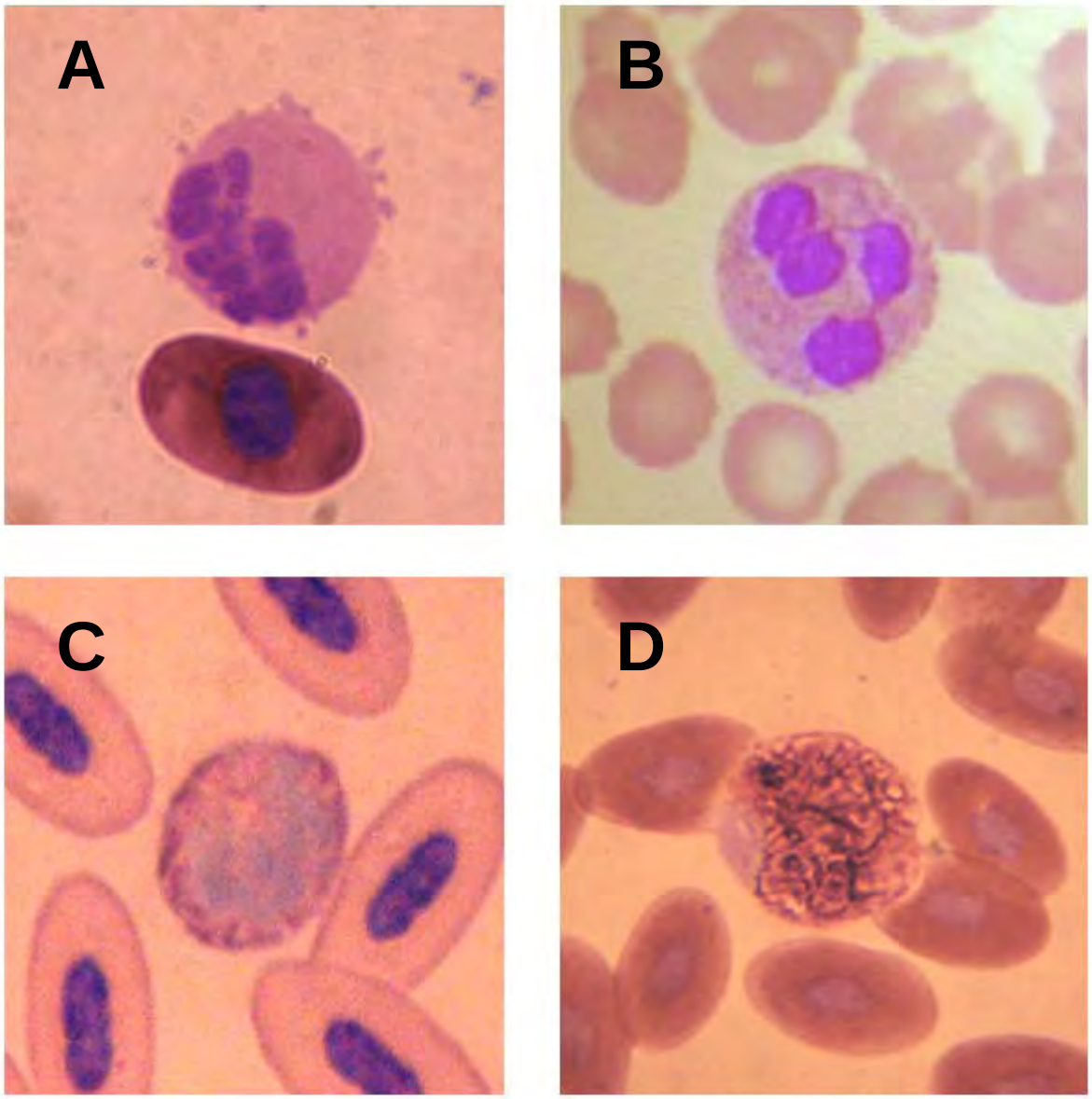

Figure 2.1

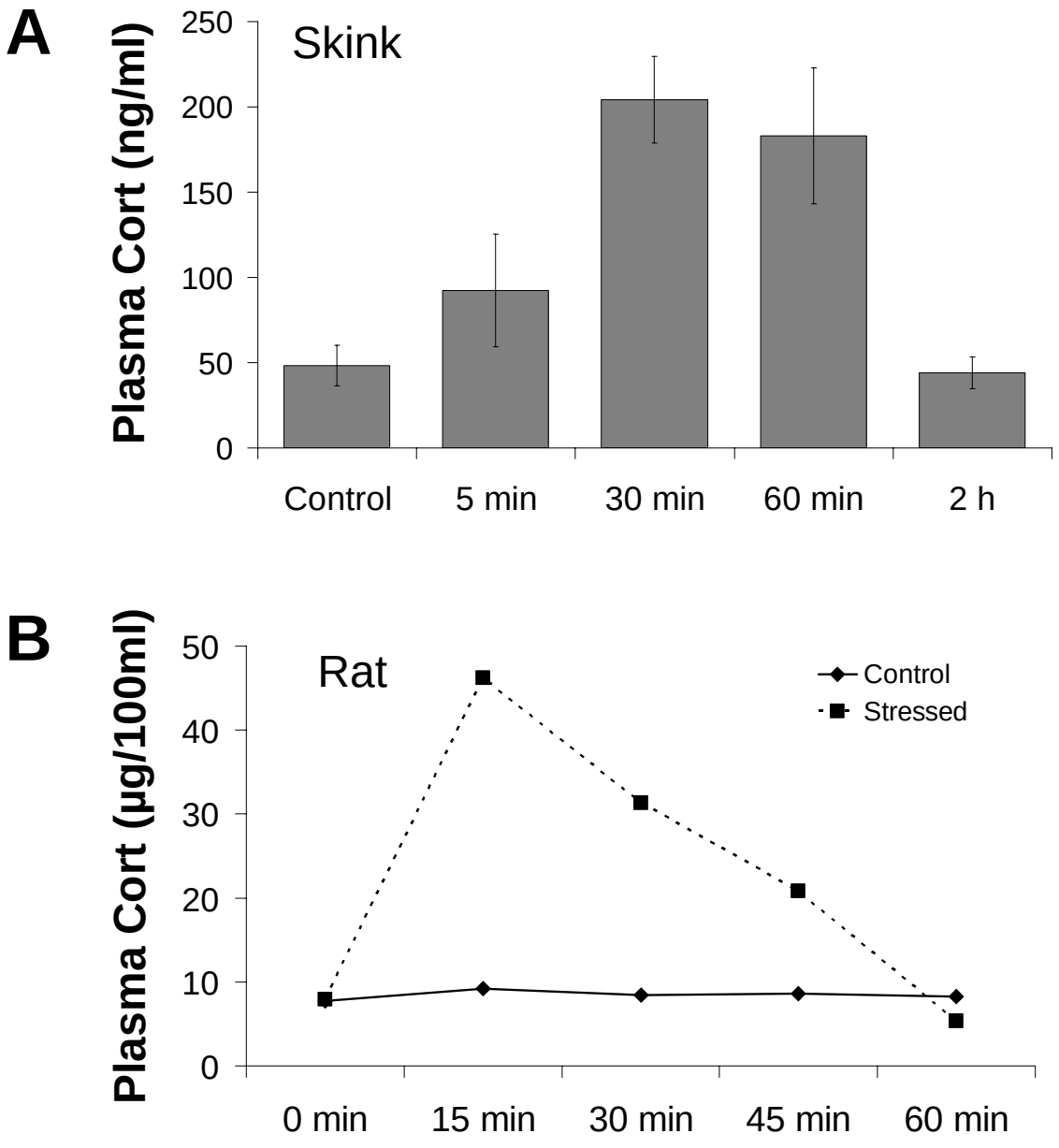
A
Skink
Plasma Cort (ng/ml)
250
200
150
100
50
0
Control
5 min
30 min
60 min
2 h
B
Rat
Plasma Cort (µg/100ml)
50
40
30
20
10
0
Control
Stressed
0 min
15 min
30 min
45 min
60 min

Figure 2.2

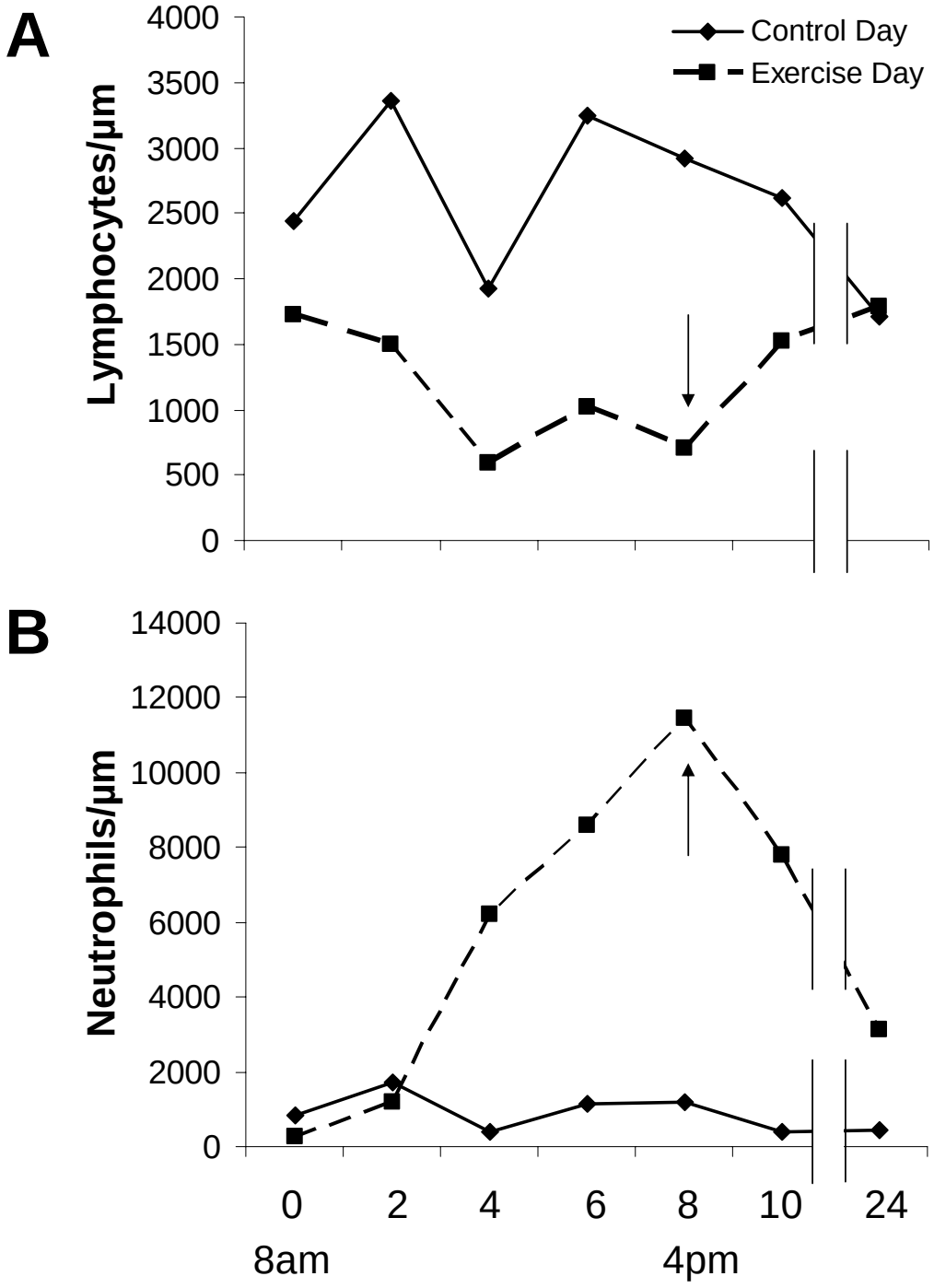
A
Lymphocytes/µm
4000
3500
3000
2500
2000
1500
1000
500
0
Control Day
Exercise Day
B
Neutrophils/µm
14000
12000
10000
8000
6000
4000
2000
0
0
2
4
6
8
10
24
8am
4pm

Figure 2.3

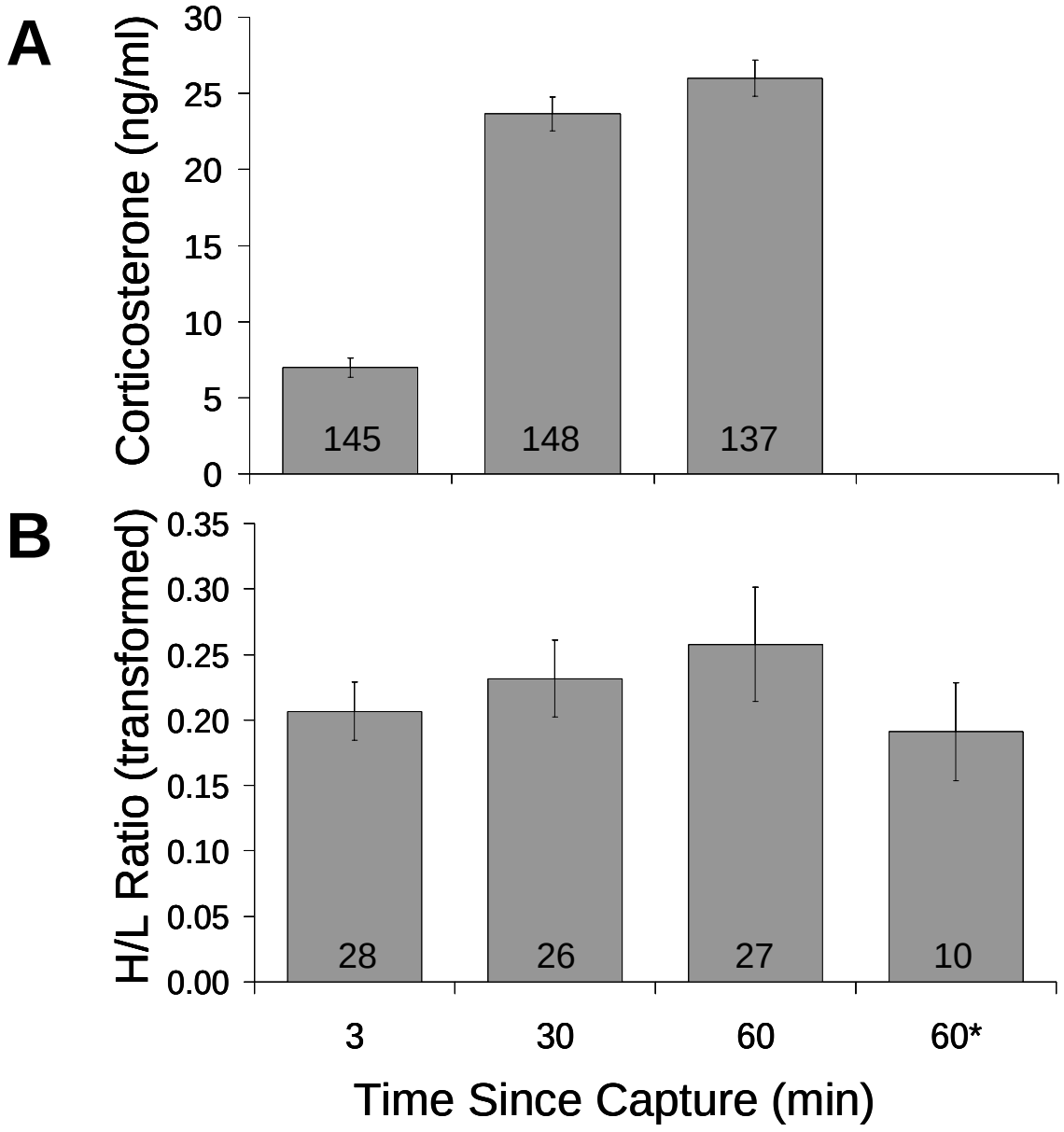
A
Corticosterone (ng/ml)
30
25
20
15
10
5
0
145
148
137
B
H/L Ratio (transformed)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
28
26
27
10
3
30
60
60*
Time Since Capture (min)

Figure 2.4

CHAPTER 3

EFFECTS OF EXOGENOUS CORTICOSTERONE ON CIRCULATING LEUKOCYTES OF A SALAMANDER (*AMBYSTOMA TALPOIDEUM*) WITH UNUSUALLY ABUNDANT EOSINOPHILS[1]

[1]Davis, A.K., and Maerz, J.C. 2010. *International Journal of Zoology* 2010. DOI:10.1155/2010/735937. Reprinted here with permission of publisher.

Abstract

When animals become stressed, their levels of glucocorticoid hormones increase, and these hormones orchestrate a number of physiological changes to prepare the animal to deal with the stress event, one of which is to cause certain white blood cells to move from tissues to circulation or vice versa. The primary alteration is an increase in the abundance of circulating neutrophils and a decrease in lymphocytes in circulation such that the ratio of these cells (N:L) is related to the magnitude of the stress event. A lesser-known effect of glucocorticoids is also a decrease the number of circulating eosinophils, although since eosinophils make up less than 5% of white blood cells in most animals, this effect is often overlooked. Recent evidence indicates that salamanders in the genus *Ambystoma* have unusually high numbers of circulating eosinophils (over one third of all white blood cells), and as such, any effect of stress hormones on circulating leukocytes (especially eosinophils) of these species should be especially pronounced. We conducted an experiment to determine the effect of corticosterone administration on counts (from blood smears) of neutrophils, lymphocytes and eosinophils in a salamander in this genus, *A. talpoideum*. Forty-seven salamanders were captured and either immediately sampled as reference animals (n=11), given a sham injection (n=8), or injected with 0.1cc of a 100µg/ml corticosterone solution (n=28). After 24 hours, average N:L ratios of sham and corticosterone groups were 2.17 and 4.12, respectively, compared to 0.56 for reference salamanders. However, absolute counts of neutrophils and lymphocytes showed that this effect was driven by a reduction in lymphocytes, since neutrophil counts were statistically similar across treatments. Importantly, relative and absolute numbers of eosinophils decreased in the sham and corticosterone groups, confirming the sensitivity of this cell to stress in amphibians.

Given the recent increase in the use of eosinophil counts to measure parasite susceptibility in amphibians, these results should be considered when interpreting white blood cell data, especially if amphibian subjects are exposed to potentially stressful conditions.

Introduction

In all vertebrates, acute stress-related increases in glucocorticoid hormones affect multiple physiological systems in the body, such as increasing heart rate to speed delivery of blood to tissues and promoting gluconeogenesis to increase energy reserves, actions which are thought to promote survival during stress events (reviewed in Wingfield and Romero 2001). Glucocorticoids also influence the numbers of circulating white blood cells. This effect has been well-studied in many taxa including mammals (Rossdale et al. 1982, Dhabhar et al. 1996, Anderson et al. 1999, Kim et al. 2005), amphibians (Bennett and Alspaugh 1964, Bennett and Harbottle 1968, Bennett et al. 1972), reptiles (Wojtaszek 1993), fish (Bennett and Gaudio Neville 1975, Wojtaszek et al. 2002) and birds (Gross and Siegel 1983, Moreno et al. 2002, Davis 2005). In most cases, increases in glucocorticoid hormones leads to an increase in the number of neutrophils (or heterophils in birds and reptiles) in circulation and a decrease in the number of lymphocytes in circulation. The increase in neutrophil abundance occurs via a combination of cell migration from tissues into circulation along with increased production from hemopoeitic tissue and extension of cell lifespan, while lymphocytes are thought to migrate from circulation into lymphoid tissue (Engler et al. 2004, Trottier et al. 2008). This process is thought to temporarily redistribute important cell types to where they would be most needed during particularly harsh or ‘stressful’ conditions (Dhabhar et al. 1994, Dhabhar et al. 1995, 1996).

Since neutrophils are phagocytic and target foreign particles and microbes, their accumulation in the bloodstream during stress events would allow them to be rapidly mobilized in response to injuries or infections. The significance of the reduction in circulating lymphocytes, which are responsible for cell-mediated immunity and cytokine responses (Thrall et al. 2006), is less clear. Regardless of the reason however, this effect conveniently provides a way for animal ecologists to use the ratio of neutrophils (or heterophils) to lymphocytes (N:L or H:L ratio) as an indirect index of the level of plasma glucocorticoids in study animals (reviewed in Davis et al. 2008a), since the ratio is positively related to the magnitude of the stress event (Gross and Siegel 1983).

There is a third white blood cell type that has been shown in some taxa to be sensitive to hormones involved in the stress response – the eosinophil, although this effect is often overlooked, perhaps because this cell comprises less than 5% of the circulating leukocyte population in most species (Jain 1993, Thrall et al. 2006). The function of eosinophils in the innate immune system has not been fully elucidated, but they are known to respond to metazoan parasite infections (Jain 1993, Stockham and Scott 2002, Thrall et al. 2006), and in amphibians they have a role in metamorphosis (Ussing and Rosenkilde 1995, Davis 2009b), although the nature of this role is not known. With respect to their sensitivity to glucocorticoid hormones, the majority of studies into this effect have focused only on mammalian subjects (e.g. McGarry 1977, Anderson et al. 1999, Noda et al. 2006). Nevertheless, these studies show that increases in glucocorticoid hormones can often cause a reduction in circulating eosinophil numbers in addition to the change in neutrophils and lymphocytes, although the reason for the reduction of circulating eosinophils (termed ‘eosinopenia’ in veterinary and biomedical textbooks) is not known. It is also not clear how prevalent this phenomenon is in the animal kingdom. The effects of glucocorticoids on leukocytes of birds has been well-studied, especially by poultry researchers

(e.g. Gross and Siegel 1983, McFarlane et al. 1989), but a review of the early literature on this subject, covering 66 investigations into stress and leukocytes, made no mention of an effect on circulating eosinophil numbers (Maxwell 1993). Furthermore, the early studies of the effects of glucocorticoids on leukocytes of amphibians mostly opted not to report the effects on eosinophils because of their low numbers in the frogs and newts studied (Bennett and Newell 1965, Bennett and Reap 1978).

There is recent evidence that eosinophil numbers in certain amphibians can be affected by glucocorticoid hormones, and in fact this susceptibility may even have serious consequences for amphibian populations because of their role in the immune system. With their role in host defense against metazoan parasites in mind, Belden and Kiesecker (2005) showed that exogenous administration of a glucocorticoid hormone to larval amphibians (gray treefrogs, *Hyla versicolor*) causes reductions in circulating eosinophil numbers which then leads to increases in trematode parasite infections, which are thought by some to be responsible for the increasing incidence of limb deformities reported in many populations (e.g. Johnson et al. 2006, Rajakaruna et al. 2008). In addition, while not specifically examining effects of hormones, other work by Kiesecker (2002) and Rohr et al. (2008) confirmed the link between eosinophil numbers and trematode susceptibility by showing how amphibians (frog species in the genus *Rana*) exposed to agrochemicals have reduced eosinophil production and abundance and are more susceptible to trematode infection. We point out that while these studies highlight the importance of eosinophils in the amphibian defense against these and other parasites, in each study above, the species that were examined typically have less than 5% eosinophils in circulation (Davis 2009a). Interestingly, a recent report showed that up to 50% of the circulating white blood cells in salamanders within the genus *Ambystoma* are eosinophils (Davis and Durso 2009), which may be

the highest of any species in the animal kingdom. While the reasons for this unusual abundance of eosinophils are not clear, it would be of interest to know if and to what degree the eosinophils of these animals are susceptible to hormones involved in the stress response, and how this compares to that of neutrophils and lymphocytes.

Here we describe the results of an experiment designed to answer the question above, using wild-caught, paedogenic mole salamanders, *Ambystoma talpoideum* (Fig. 3.1A), which are common in ponds and wetlands in the southeastern United States. Eosinophils make up between 25% to 45% of the normal circulating white blood cell population in this species in the wild (Davis and Maerz 2008a, b). In this experiment, exogenous corticosterone (cort) was administered (via injection) to recently captured individuals and after a 24hr period their circulating level of neutrophils, lymphocytes and eosinophils was compared to levels in untreated (reference) individuals, and to those injected with a sham treatment. The results of this study will be of importance to researchers who collect white blood cell data (especially eosinophil counts) in these and other amphibians, and for further elucidating the range of effects of glucocorticoids on animal physiology.

Methods

Experimental setup.- On August 12, 2008, we captured 47 paedogenic mole salamanders from a single permanent pond in the Whitehall Experimental Forest at the University of Georgia (Athens, GA). All salamanders were captured within 20 minutes, and transported to our lab (10 minutes away) in a container of pond water. In the lab, salamanders were haphazardly divided into 3 groups: reference salamanders (n=11), which provided a baseline for hematological

profiles, sham injected salamanders (n=8), and corticosterone injected salamanders (n=28). We assigned more animals to the corticosterone injected treatment so we could evaluate the extent of variation in leukocyte response to the stress hormone injection. The haphazard assignment of salamanders did not result in biased body sizes within any treatment; the mean body mass of each group was 2.31 (± 0.25 SD), 2.17 (± 0.34 SD) and 2.09 (± 0.32 SD) grams, for the reference, sham and corticosterone groups, respectively, and these means were not significantly different (one way ANOVA: $F_{2,44}$=1.918, p=0.159).

The reference group was processed (described below) immediately to obtain control leukocyte profiles. It is important to point out here that the effects of glucocorticoid hormones on leukocytes of ectothermic animals take hours to manifest (reviewed in Davis et al. 2008a) such that effects of capture, handling or transport are negligible for this group since all individuals were processed within 1 hour of capture from the pond. The salamanders from the other two groups were injected with their respective solutions (described below) then held for 24 hours in 40 L plastic containers (7-8 individuals per container) filled with 10 L of tap water treated with Tetra AquaSafe water conditioner. Each container also had a layer of leaves (collected from the pond) covering the bottom, to provide the salamanders with natural cover for hiding.

Processing the salamanders from all groups was the same and followed established procedures (Davis and Maerz 2008a, b, 2009). For this, each individual was euthanized in a 2 % MS-222 solution, and then weighed with an electronic balance. The animal was decapitated and blood from the heart region was dripped onto a clean microscope slide and a standard blood smear made. Finally, the carcass was dissected open and the sex assigned confirmed based on inspection of the reproductive organs.

Injection solutions.- Prior to collecting the salamanders, we prepared two solutions for the experimental injections. The first was a sham solution of buffered saline. The second was a solution of 100µg/ml corticosterone (Sigma Aldrich) in buffered saline. Salamanders in both injection groups received an intraperitoneal injection near the left hind leg of 0.1cc of the designated treatment solution. This dosage of corticosterone is within the range commonly used by other amphibian researchers who study the effects of stress; Moore and Miller (1984) administered corticosterone doses ranging from 10 to 250µg/ml to sets of newts, and Burmeister et al. (2001) administered between 100-400µg/ml of corticosterone to treefrogs. Treatment animals were replaced into their container and left for 24 hours, which is the time required for any glucocorticoid hormone-induced changes in leukocyte populations to occur in amphibians (Bennett and Alspaugh 1964, Bennett and Newell 1965, Bennett and Harbottle 1968, Bennett et al. 1972). All procedures (transport, sampling the pond animals, injections, etc.) up to this point were completed within 1 hour of capture from the pond. After 24 hours, all salamanders in the sham and corticosterone groups were processed in the same manner as the reference salamanders the day before. We note that since we did not measure endogenous levels of corticosterone in this experiment, we do not know for certain if and to what degree the final hormone level of the corticosterone salamanders was greater than the sham and reference group. However, based on results from prior work where similar hormone doses were administered to similarly-sized amphibians and where endogenous levels were in fact measured (Burmeister et al. 2001), we logically assume that the administration of corticosterone here acted to raise endogenous corticosterone above resting levels, thereby mimicking a stress response in the target salamanders.

Leukocyte counting.- Procedures for reading salamander blood smears and counting leukocytes were typical of those used by us in prior studies of birds (Davis et al. 2004, Davis 2005, Davis et al. 2008b) and amphibians (Davis and Maerz 2008a, b, Davis 2009b). Briefly, dried blood smears were stained with giemsa, and then examined under 1000X with a standard light microscope. The smear was scanned in a standard zig-zag pattern and fields of view with even distributions of red blood cells were selected for counting. At this magnification and for this species, the average number of erythrocytes per field was 32.2 ±8.7SD (based on counts from 25 random fields; Davis, *unpubl. data*). All white blood cells were tallied in each field until at least 100 cells had been counted. Cells were identified as neutrophils, lymphocytes, eosinophils, basophils and monocytes (Figs. 3.1B-G), following Thrall (2004), Turner (1988) and Hadji-Azimi et al. (1987), although the focus here was on the first three cell types. The relative number of each cell type was calculated (i.e. the percentage, based on the total number of white blood cells counted), and we calculated the ratio of neutrophils to lymphocytes (N:L) for each salamander based on the percentages of these cells (Davis 2005, Davis et al. 2008b, Davis and Maerz 2008a, b). We also calculated the number of each cell per 10 fields of view to use as an absolute estimate of the number of each cell type in circulation (Forson and Storfer 2006, Davis et al. 2009).

Data Analysis.- We examined the effect of corticosterone on absolute numbers of neutrophils, lymphocytes and eosinophils (all log-transformed +1) using ANCOVA, where the cell count was the response variable, treatment (reference, sham or cort) and sex were the categorical explanatory variables, and body mass was a continuous covariate, to account for any possible size or ontogenetic-related effects on leukocyte profiles. Interaction terms were initially

included in the models, but removed if not significant. Analyses were conducted using Statistica 6.1 software (Statistica 2003).

Results

Relative cell counts.- Across all 47 individuals, the relative numbers of each white blood cell type showed a profile typical of Ambystomatid salamanders, in that eosinophils made up nearly a third of all white blood cells (Table 3.1, Davis and Durso 2009). While we did not perform statistical analyses on relative cell counts (only absolute numbers were analyzed), it is evident from Table 3.1 that the relative numbers of neutrophils, lymphocytes and eosinophils differed visibly across treatments. The mean percentage of neutrophils increased from 21.3% in the salamanders sampled within one hour of capture, to 44.8% in the sham injection group, and 54.9% in the corticosterone injection group. The mean percentage of lymphocytes decreased from 40.5% in the reference animals to 17.7% and 17.2% in the experimental groups. The mean percentage of eosinophils also decreased from 32% in the reference group to 27.5% in the sham and 19.8% in the cort group. Interestingly, one salamander in the sham group appeared to have an extremely atypical leukocyte profile, as 75.8% of its white blood cells were neutrophils and only 4.2% were lymphocytes. There was nothing unusual about this individual noted during processing that we can attribute to this deviation, such as an injury or missing appendages. Nevertheless, these extreme values led to an especially high N:L ratio for this individual (18.0), which was over two standard deviations above the mean for that group (2.17, Table 3.1). We conducted subsequent analyses of absolute neutrophil and lymphocyte counts with and without this individual so we could characterize the effect of this individual on subsequent inferences.

Regarding the N:L ratios of the corticosterone group, there was considerable variation in N:L ratios among salamanders. Of the 28 individuals receiving the single dose of corticosterone (0.1cc of a 100µg/ml concentration), N:L ratios varied from 0.13 to 11.0 and showed an approximate normal distribution (Fig. 3.2).

Effects on absolute cell counts.- With the one outlier included, the initial ANCOVA model examining factors influencing neutrophil abundance revealed no significant interaction effects between treatment, sex or body mass ($p>0.05$ for all). In a model with main effects only, there was no significant effect of treatment ($F_{2,42}=0.263$, $p=0.770$), nor of sex ($F_{1,42}=2.734$, $p=0.106$), but an effect of body size ($F_{1,42}=4.566$, $p=0.038$). With the outlier excluded, there were no significant interaction terms, and the main effects model showed similar results: there was no effect of treatment ($F_{2,41}=0.760$, $p=0.474$; Fig. 3.3A) or sex ($F_{1,41}=2.411$, $p=0.128$), but an effect of body size ($F_{1,41}=5.038$, $p=0.030$). The relationship between body size and neutrophil abundance was positive, but weakly so (Pearson correlation, $r=0.26$, $p=0.084$). With the outlier included, the model examining lymphocyte abundance showed no significant interaction terms ($p>0.05$ for all), and the model with main effects only showed no significant variation between sexes ($F_{1,42}=3.277$, $p=0.077$), but significant effects of treatment ($F_{2,42}=33.031$, $p<0.001$) and body size ($F_{1,42}=4.459$, $p=0.041$). The results were similar with the outlier excluded; the main effects model showed significant effects of body size ($F_{1,41}=4.429$, $p=0.041$) and treatment ($F_{2,41}=32.431$, $p<0.001$). In the treatment effect, Tukey's HSD tests showed that the sham and cort groups had significantly lower numbers of lymphocytes than the reference group ($p<0.001$ for both), but they were not significantly different themselves ($p=0.566$; Fig. 3.3B). The effect of body size on lymphocyte abundance was positive (Pearson correlation, $r=0.37$, $p=0.012$). Finally, in the initial ANCOVA model examining variation in eosinophil abundance there was no support

for inclusion of any interaction term ($p>0.05$ for all). In the main effects only model, there was no variation between sexes ($F_{1,42}=0.060$, $p=0.807$), a weak trend with body size ($F_{1,42}=3.612$, $p=0.064$), and importantly, a significant effect of treatment ($F_{2,42}=3.536$, $p=0.038$). Tukey's HSD tests showed that the sham-injected salamanders had statistically similar numbers of eosinophils as did the reference salamanders ($p=0.180$), but cort-injected salamanders had significantly lower numbers of eosinophils than did the reference individuals ($p=0.005$). The numbers of eosinophils in corticosterone-injected salamanders were statistically similar to those of the sham-injected salamanders ($p=0.642$; Fig. 3.3C).

Discussion

The results of this experiment demonstrated that both capture and administration of exogenous corticosterone causes alterations in the leukocyte profile of paedogenic mole salamanders. Consistent with studies of most other animal taxa (Davis et al. 2008a), the ratio of neutrophils to lymphocytes reflected the different levels of stress in each treatment; the average N:L ratio was higher among sham-injected salamanders than among reference salamanders (presumably reflecting the natural rise in corticosterone after these activities), and higher still among corticosterone injected salamanders than among sham and reference salamanders (Table 3.1). Interestingly though, comparing absolute numbers of both neutrophils and lymphocytes showed that this trend was being driven largely by a decrease in circulating lymphocyte numbers (Fig. 3.3B), while absolute neutrophil levels stayed more or less constant across treatments (Fig. 3.3A). In fact, the estimated count of lymphocytes decreased dramatically, from a mean of 3.1 cells per 10 microscope fields in the reference salamanders (i.e. before log-transformation) to 0.7

in the sham and 0.5 in the cort groups, which is equivalent to an 84% drop overall in the circulating numbers of this cell.

The numbers of circulating eosinophils in this amphibian species appeared to be influenced by the stress treatments as well, although the only significant difference among treatment groups was between the reference and corticosterone groups. Still, the average counts of eosinophils dropped from a mean of 3.8 cells per 10 microscope fields in the reference group, to 1.4 cells in the sham group and 0.8 cells in the corticosterone group (Fig. 3.3C). Thus, the magnitude of the overall difference between the eosinophil counts in reference salamanders and cort-injected salamanders was equivalent to a 79% drop in circulating numbers, which is of similar magnitude as the drop in estimated lymphocyte numbers. Interestingly, since eosinophils make up such a large proportion of the white blood cell population in this species, the large drop in eosinophil numbers combined with the equally-large decrease in lymphocyte numbers no doubt both contributed to the apparent increase in *relative* neutrophil numbers in the sham and cort treatments (Table 3.1), when in fact the absolute numbers of this cell did not necessarily increase (Fig. 2.3A). In this case, the fewer numbers of lymphocytes and eosinophils in these treatments made neutrophils the most abundant cell type (Table 3.1), even though absolute neutrophils numbers did not increase. Thus, future studies involving white blood cell counts in this and other Ambystomatid species with abundant eosinophils should consider the impact of the eosinophil numbers on the commonly-used neutrophil-lymphocyte ratio.

From an additional methodological standpoint, the sensitivity of eosinophils to variations in stress hormones could be of utility to researchers who routinely collect white blood cell data, in that it could aid in the interpretation of such data and help discriminate between a stress response and an inflammation or disease response (Davis et al. 2008a). For example, since

neutrophils (or heterophils in birds and reptiles) can increase in number in response to infection (Jain 1993, Stockham and Scott 2002, Thrall et al. 2006) but also in response to general stress (Davis et al. 2008a), interpreting high neutrophil counts in study subjects can be particularly challenging. However, if the increased neutrophil count is seen along with reductions in lymphocytes and/or eosinophils, a researcher could more easily interpret this to be a stress response, consistent with increasing levels of glucocorticoid hormones. An example of this challenge within amphibians is a recent study of the effects of the fungal disease, chytridiomycosis, on leukocyte counts in frog larvae (Davis et al. 2009). Results of that study indicated that infected larvae had increased neutrophil numbers in circulation, consistent with either inflammation or stress. There was no change in lymphocyte numbers, but infected larvae did have a significant reduction in circulating eosinophils, which based on the current results, is consistent with increased stress in the infected group, as opposed to an inflammation response, which is not usually associated with reductions in eosinophil counts (Jain 1993, Stockham and Scott 2002, Thrall et al. 2006). Since infections can compromise mouthparts and therefore feeding ability in anuran larvae (Rowe et al. 1996), it is possible that infection of this disease in larvae leads to reductions in food intake, which is known to cause increases in glucocorticoid hormones (Kubíková et al. 2001).

As a final point, our results indicate that the sensitivity of eosinophils to stress should be kept in mind in studies where these cells are counted to serve as a measure of parasite susceptibility, especially if the amphibians under study inhabit, or are reared under, potentially stressful conditions or environments, such as could occur with exposure to pesticides and agrochemicals (e.g. Kiesecker 2002, Rohr et al. 2008). In these studies, chemical exposure tends to reduce eosinophil production or circulating abundance, and the assumption is that the

chemicals caused the reduction (i.e. by impairing or 'suppressing' immune function). Results from the current study indicate that an endogenous stress response to the chemicals, not the chemicals *per se*, can cause the same result. In fact, given the unusual abundance of eosinophils in Ambystomatid salamanders, the hormonally-induced reductions of both lymphocytes and eosinophils (which combined make up at least 50% of the white blood cell population in these amphibians) could even explain the reduction in total white blood cell numbers seen recently in *Ambystoma tigrinum* salamanders after exposure to the herbicide, atrazine (Forson and Storfer 2006). Lastly, while not specifically addressing parasite susceptibility, field surveys by Raffel et al. (2006) showed how counts of both lymphocytes and eosinophils in newts decreased with decreasing water temperature, while there was little effect on neutrophil abundance. The idea that stress could cause these patterns was dismissed in that paper, but the similarity of those results to the current study may not be coincidental. In any case, these examples emphasize how important it is that animal and wildlife researchers have a thorough understanding of the effects of stress on leukocyte counts.

Acknowledgements

We thank two anonymous reviewers for providing helpful comments on the manuscript. AKD was supported by funding from the D.B. Warnell School of Forestry and Natural Resources at the University of Georgia and the Morris Animal Foundation during this project. All procedures in this study were approved by the UGA Animal Care and Use Committee (AUP #A2009-10120).

Literature cited

Anderson, B. H., D. L. Watson, and I. G. Colditz. 1999. The effect of dexamethasone on some immunological parameters in cattle. Veterinary Research Communications 23: 399-413.

Belden, L. K., and J. M. Kiesecker. 2005. Glucocorticosteroid hormone treatment of larval treefrogs increases infection by *Alaria* sp trematode cercariae. Journal of Parasitology 91: 686-688.

Bennett, M. F., and J. K. Alspaugh. 1964. Some changes in the blood of frogs following administration of hydrocortisone. The Virginia Journal of Science 15: 76-79.

Bennett, M. F., and N. C. Newell. 1965. A further study of the effects of hydrocortisone on the blood of frogs. The Virginia Journal of Science 16: 128-130.

Bennett, M. F., and J. A. Harbottle. 1968. The effects of hydrocortisone on the blood of tadpoles and frogs, *Rana catesbeiana*. Biological Bulletin 135: 92-95.

Bennett, M. F., and C. Gaudio Neville. 1975. Effects of cold shock on the distribution of leukocytes in goldfish, *Carassius auratus*. Journal of Comparative Physiology A 98: 213-216.

Bennett, M. F., and L. E. Reap. 1978. Photoperiod, stress and the distribution of leukocytes in the peripheral blood of *Notophthalmus viridescens*. Journal of Comparative Physiology A 125: 205-207.

Bennett, M. F., C. A. Gaudio, A. O. Johnson, and J. H. Spisso. 1972. Changes in the blood of newts, *Notophthalmus viridescens*, following administration of hydrocortisone. Journal of Comparative Physiology A 80: 233-237.

Burmeister, S., C. Somes, and W. Wilczynski. 2001. Behavioral and hormonal effects of exogenous vasotocin and corticosterone in the green treefrog. General and Comparative Endocrinology 122: 189-197.

Davis, A. K. 2005. Effects of handling time and repeated sampling on avian white blood cell counts. Journal of Field Ornithology 76: 334-338.

Davis, A. K. 2009a. The Wildlife Leukocytes Website: The ecologist's source for information about leukocytes of wildlife species. www.wildlifehematology.uga.edu.

Davis, A. K. 2009b. Metamorphosis-related changes in leukocyte profiles of larval bullfrogs (*Rana catesbeiana*). Comparative Clinical Pathology 18: 181-186.

Davis, A. K., and J. C. Maerz. 2008a. Sex-related differences in hematological stress indices of breeding, paedomorphic mole salamanders. Journal of Herpetology 42: 197-201.

Davis, A. K., and J. C. Maerz. 2008b. Comparison of hematological stress indicators in recently captured and captive paedomorphic mole salamanders, *Ambystoma talpoideum*. Copeia 2008: 613-617.

Davis, A. K., and J. C. Maerz. 2009. Effects of larval density on hematological stress indices in salamanders. Journal of Experimental Zoology Part A-Ecological Genetics and Physiology 311A: 697-704.

Davis, A. K., and A. M. Durso. 2009. Leukocyte differentials of northern cricket frogs (*Acris c. crepitans*) with a compilation of published values from other amphibians. Herpetologica 65: 260-267.

Davis, A. K., K. C. Cook, and S. Altizer. 2004. Leukocyte profiles of House Finches with and without mycoplasmal conjunctivitis, a recently emerged bacterial disease. Ecohealth 1: 362-373.

Davis, A. K., D. L. Maney, and J. C. Maerz. 2008a. The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. Functional Ecology 22: 760-772.

Davis, A. K., N. E. Diggs, P. P. Marra, and R. J. Cooper. 2008b. Hematological stress indices show no effect of radio-transmitters on wintering Hermit Thrushes. Journal of Field Ornithology 79: 293-297.

Davis, A. K., M. K. Keel, A. Ferreira, and J. C. Maerz. 2009. Effects of chytridiomycosis on circulating white blood cell distributions of bullfrog larvae (*Rana catesbeiana*). Comparative Clinical Pathology Online Early. DOI: 10.1007/s00580-009-0914-8.

Dhabhar, F. S., A. H. Miller, B. S. McEwen, and R. L. Spencer. 1995. Effects of stress on immune cell distribution - Dynamics and hormonal mechanisms. Journal of Immunology 154: 5511-5527.

Dhabhar, F. S., A. H. Miller, B. S. McEwen, and R. L. Spencer. 1996. Stress-induced changes in blood leukocyte distribution - Role of adrenal steroid hormones. Journal of Immunology 157: 1638-1644.

Dhabhar, F. S., A. H. Miller, M. Stein, B. S. McEwen, and R. L. Spencer. 1994. Diurnal and acute stress-induced changes in distribution of peripheral blood leukocyte subpopulations. Brain Behavior and Immunity 8: 66-79.

Engler, H., M. T. Bailey, A. Engler, and J. F. Sheridan. 2004. Effects of repeated social stress on leukocyte distribution in bone marrow, peripheral blood and spleen. Journal of Neuroimmunology 148: 106-115.

Forson, D., and A. Storfer. 2006. Atrazine increases ranavirus susceptibility in the tiger salamander, *Ambystoma tigrinum*. Ecological Applications 16: 2325-2332.

Gross, W. B., and H. S. Siegel. 1983. Evaluation of the heterophil/lymphocyte ratio as a measure of stress in chickens. Avian Diseases 27: 972-979.

Hadji-Azimi, I., V. Coosemans, and C. Canicatti. 1987. Atlas of adult *Xenopus laevis laevis* hematology. Developmental and Comparative Immunology 11: 807-874.

Jain, N. C. 1993. Essentials of Veterinary Hematology. Blackwell Publishing, Philadelphia.

Johnson, P. T. J., E. R. Preu, D. R. Sutherland, J. M. Romansic, B. Han, and A. R. Blaustein. 2006. Adding infection to injury: Synergistic effects of predation and parasitism on amphibian malformations. Ecology 87: 2227-2235.

Kiesecker, J. M. 2002. Synergism between trematode infection and pesticide exposure: a link to amphibian deformities in nature? Proceedings of the National Academy of Sciences 99: 9900-9904.

Kim, C. Y., J. S. Han, T. Suzuki, and S. S. Han. 2005. Indirect indicator of transport stress in hematological values in newly acquired cynomolgus monkeys. Journal of Medical Primatology 34: 188-192.

Kubíková, L., P. Výboh, and L. Koštál. 2001. Behavioural, endocrine and metabolic effects of food restriction in broiler breeder hens. Acta Veterinaria Brno 70: 247-257.

Maxwell, M. H. 1993. Avian blood leukocyte responses to stress. Worlds Poultry Science Journal 49: 34-43.

McFarlane, J. M., S. E. Curtis, J. Simon, and O. A. Izquierdo. 1989. Multiple concurrent stressors in chicks 2. Effects on hematologic, body composition, and pathologic traits. Poultry Science 68: 510-521.

McGarry, M. P. 1977. Hydrocortisone acetate-induced eosinopenia in mice: independence from the thymus. Cellular Immunology 29: 347-352.

Moore, F. L., and L. J. Miller. 1984. Stress-induced inhibition of sexual-behavior: corticosterone inhibits courtship behaviors of a male amphibian (T*aricha granulosa*). Hormones and Behavior 18: 400-410.

Moreno, J., S. Merino, J. Martinez, J. J. Sanz, and E. Arriero. 2002. Heterophil/lymphocyte ratios and heat-shock protein levels are related to growth in nestling birds. Ecoscience 9: 434-439.

Noda, K., M. Aoki, H. Akiyoshi, H. Asaki, T. Ogata, K. Yamauchi, T. Shimada, and F. Ohashi. 2006. Effect of bovine lactoferrin on the immune responses of captive bottlenosed dolphins (*Tursiops truncates*) being transported over long distances. Veterinary Record 159: 885-888.

Raffel, T. R., J. R. Rohr, J. M. Kiesecker, and P. J. Hudson. 2006. Negative effects of changing temperature on amphibian immunity under field conditions. Functional Ecology 20: 819-828.

Rajakaruna, R. S., P. Piyatissa, U. A. Jayawardena, A. N. Navaratne, and P. H. Amerasinghe. 2008. Trematode infection induced malformations in the common hourglass treefrogs. Journal of Zoology 275: 89-95.

Rohr, J. R., A. M. Schotthoefer, T. R. Raffel, H. J. Carrick, N. Halstead, J. T. Hoverman, C. M. Johnson, L. B. Johnson, C. Lieske, M. D. Piwoni, P. K. Schoff, and V. R. Beasley. 2008. Agrochemicals increase trematode infections in a declining amphibian species. Nature 455: 1235-U50.

Rossdale, P. D., P. N. Burguez, and R. S. G. Cash. 1982. Changes in blood neutrophil/lymphocyte ratio related to adrenocortical function in the horse. Equine Veterinary Journal 14: 293-298.

Rowe, C. L., O. M. Kinney, A. P. Fiori, and J. D. Congdon. 1996. Oral deformities in tadpoles (*Rana catesbeiana*) associated with coal ash deposition: Effects on grazing ability and growth. Freshwater Biology 36: 723-730.

Statistica. 2003. Statistica version 6.1, Statsoft Inc.

Stockham, S. L., and M. A. Scott. 2002. Fundamentals of veterinary clinical pathology. Blackwell Publishing, Ames, Iowa USA.

Thrall, M. A. 2004. Hematology of amphibians. *In* M. A. Thrall, D. C. Baker and E. D. Lassen [eds.], Veterinary Hematology and Clinical Chemistry: Text and Clinical Case Presentations. Lippincott Williams & Wilkins, Philadelphia, PA.

Thrall, M. A., D. C. Baker, E. D. Lassen, T. W. Campbell, D. DeNicola, A. Rebar, M. J. Fettman, and G. Weiser. 2006. Veterinary hematology and clinical chemistry. Blackwell Publishing, Ames, Iowa USA.

Trottier, M. D., M. M. Newsted, L. E. King, and P. J. Fraker. 2008. Natural glucocorticoids induce expansion of all developmental stages of murine bone marrow granulocytes without inhibiting function. Proceedings of the National Academy of Sciences of the United States of America 105: 2028-2033.

Turner, R. J. 1988. Amphibians, pp. 129-209. *In* A. F. Rawley and N. A. Ratcliffe [eds.], Vertebrate blood cells. Cambridge University Press, Cambridge.

Ussing, A. P., and P. Rosenkilde. 1995. Effect of induced metamorphosis on the immune system of the Axolotl, *Ambystoma mexicanum*. General and Comparative Endocrinology 97: 308-319.

Wingfield, J. C., and L. M. Romero. 2001. Adrenocortical responses to stress and their modulation in free-living vertebrates, pp. 211-234. *In* B. S. McEwen and H. M. Goodman

[eds.], Handbook of physiology, section 7: The endocrine system, Vol. IV: Coping with the environment: neural and endocrine mechanisms. Oxford University Press, New York.

Wojtaszek, J., D. Dziewulska-Szwajkowska, M. Lozinska-Gabska, A. Adamowicz, and A. Dzugaj. 2002. Hematological effects of high dose of cortisol on the carp (*Cyprinus carpio* L.): Cortisol effect on the carp blood. General and Comparative Endocrinology 125: 176-183.

Wojtaszek, J. S. 1993. The effect of cortisol on the circulating blood parameters and on the activity of alanine and aspartate aminotransferases in the grass snake *Natrix natrix natrix* L. Comparative Biochemistry and Physiology A 105: 259-266.

Table 3.1. Summary of leukocyte differentials of salamanders (*Ambystoma talpoideum*) in all treatments in this study. Shown are the average percentages (± 1 SD) of each cell type out of the total number of cells, as well as the average N:L ratio for each treatment group. Reference animals were sampled within 1 hour of capture, sham and corticosterone groups sampled 24 hours after capture and injection.

		Leukocyte type					
Salamander group	**N**	**Neutrophils**	**Lymphocytes**	**Eosinophils**	**Basophils**	**Monocytes**	**N:L**
Reference	11	21.3 (9.9)	40.5 (11.7)	32.0 (16.1)	4.9 (4.8)	1.3 (2.1)	0.56 (0.29)
Sham injected	8	44.8 (25.9)	17.7 (7.5)	27.5 (24.2)	5.8 (6.0)	4.1 (3.0)	2.17 (1.66)
Corticosterone injected	28	54.9 (19.7)	17.2 (19.8)	19.8 (18.9)	4.3 (3.7)	3.8 (3.1)	4.42 (2.67)

CHAPTER 3 FIGURES

3.1 (A) Paedogenic mole salamander, *Ambystoma talpoideum,* and leukocytes typical of this species after giemsa staining and at 1000X: (B) lymphocyte, (C) eosinophil with typical rounded, orange-staining granules, (D) basophil with diffuse, purple-staining granules, (E) monocyte, showing grey-blue cytoplasm with some vaculation, (F) normal neutrophil with three nuclear lobes and pink-staining cytoplasm, and (G) a neutrophil with foamy cytoplasm.

3.2.1 Distribution of neutrophil-lymphocyte ratios of all mole salamanders that received intraperitoneal corticosterone injections (n=28).

3.3 Effects of sham injection and corticosterone (cort) injection on absolute estimates of circulating neutrophil (A), lymphocyte (B) and eosinophil (C) abundance in mole salamanders. Shown are the log-transformed mean (± 1SE) abundance estimates for each cell (i.e. numbers of cells per 10 fields of view). Letters above the bars indicate statistically significant groups, based on Tukey's HSD tests.

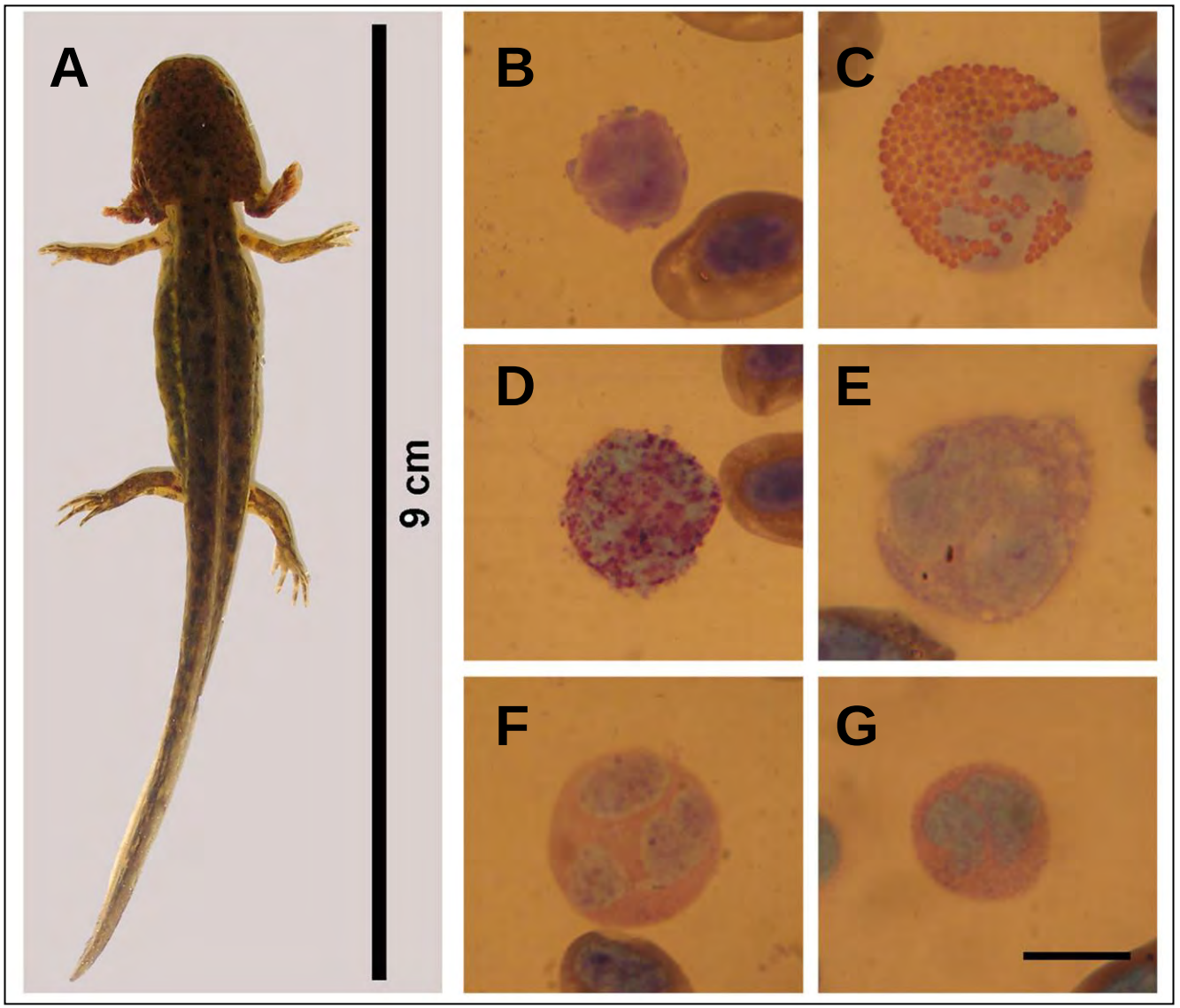

Figure 3.1

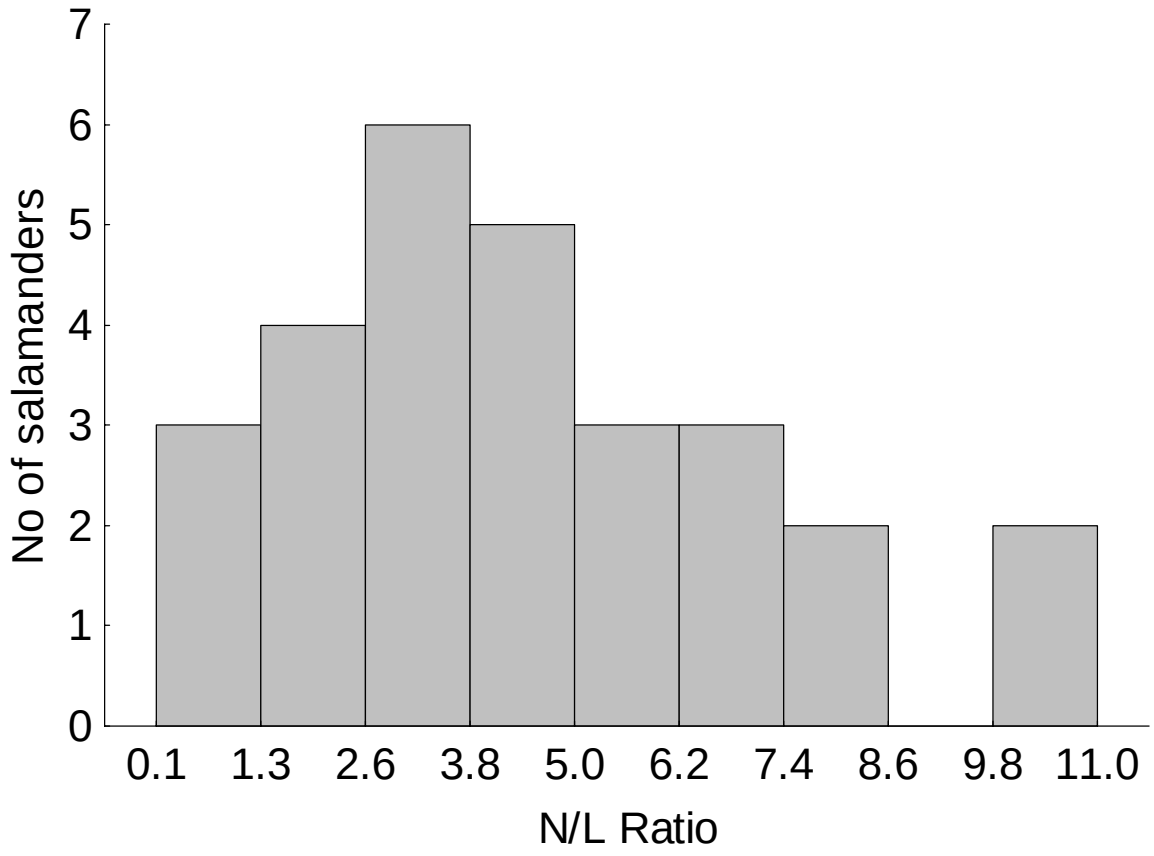
No of salamanders
0
1
2
3
4
5
6
7
0.1
1.3
2.6
3.8
5.0
6.2
7.4
8.6
9.8
11.0
N/L Ratio

Figure 3.2

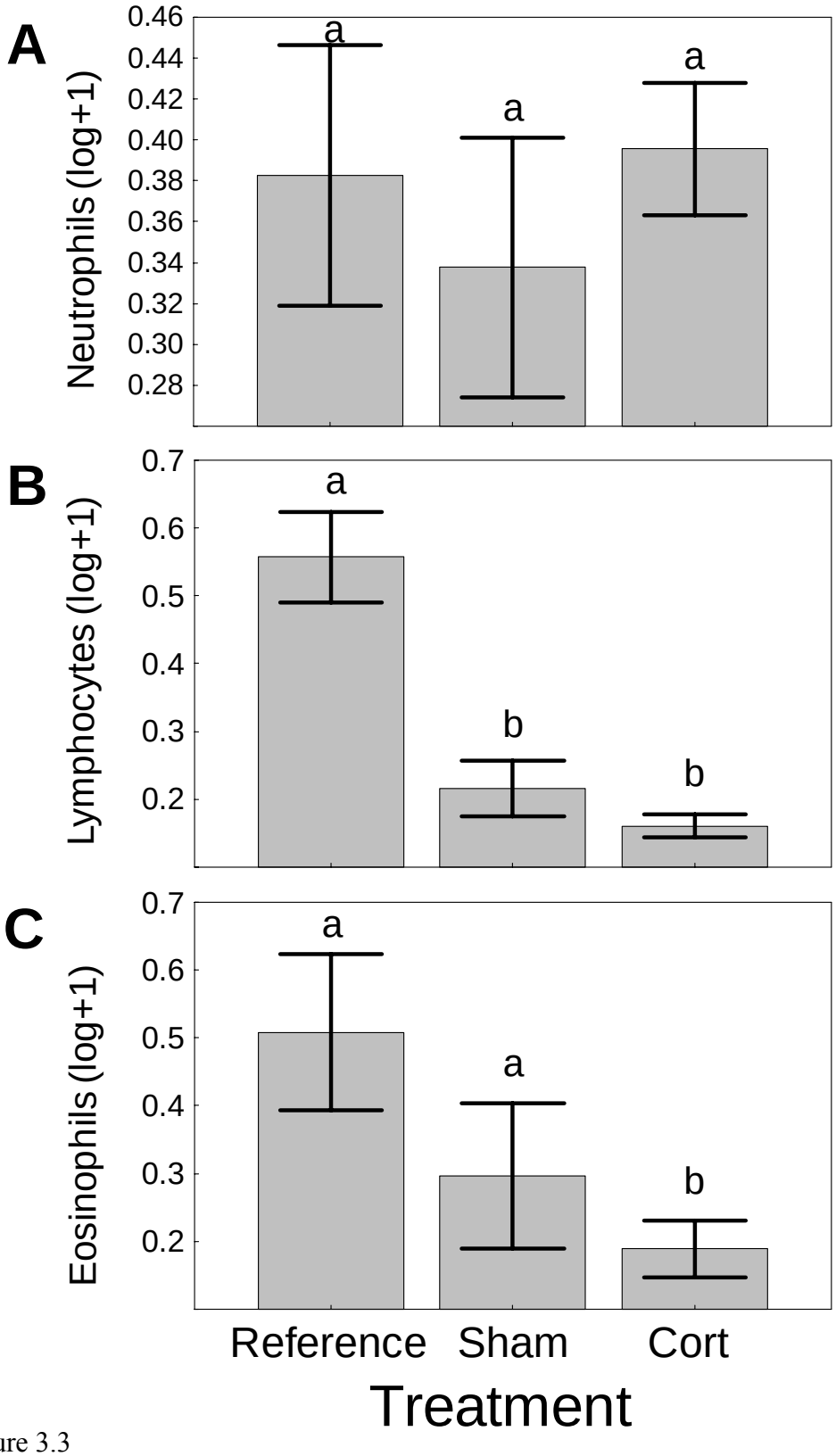
A
Neutrophils (log+1)
0.46
0.44
0.42
0.40
0.38
0.36
0.34
0.32
0.30
0.28
a
a
a
B
Lymphocytes (log+1)
0.7
0.6
0.5
0.4
0.3
0.2
a
b
b
C
Eosinophils (log+1)
0.7
0.6
0.5
0.4
0.3
0.2
a
a
b
Reference
Sham
Cort
Treatment

Figure 3.3

CHAPTER 4

COMPARISON OF HEMATOLOGICAL STRESS INDICATORS IN RECENTLY CAPTURED AND CAPTIVE PAEDOGENIC MOLE SALAMANDERS, *AMBYSTOMA TALPOIDEUM*[1]

[1]Davis, A.K., and Maerz, J.C. 2008. *Copeia* 2008(3): 613-617. Reprinted here with permission of publisher.

Abstract

Measuring stress in animals is an important component of many research studies and it has traditionally been performed via sampling levels of corticosterone in plasma. A secondary, "hematological" approach used most commonly by researchers of birds, mammals and other taxa involves evaluating leukocyte profiles from blood smears. Such research has shown that leukocytes have a characteristic response to stress, although in amphibians this phenomenon is not as well-studied. In general, stress can induce a rise in the ratio of neutrophils to lymphocytes. We evaluated the hematological response of paedogenic mole salamanders (*Ambystoma talpoideum*) to captivity stress, specifically focusing on this parameter, but also examining other white blood cell types. Individuals captured in the wild and held in captivity for 10 days before sampling had significantly more neutrophils, fewer lymphocytes and higher ratios of neutrophils to lymphocytes than those captured from the same locations and sampled within one hour. Captive individuals also had significantly higher numbers of eosinophils. These results are consistent with hematological research in birds and other taxa and highlight the utility of this approach for measuring stress in amphibians.

Introduction

The ability to identify physiological stress in animals is important in wildlife research, both in field and laboratory settings. One of the main physiological responses to stressful stimuli in vertebrates is an increase in the plasma levels of glucocorticoid hormones such as corticosterone (Moore and Jessop, 2003; Romero, 2004). This response is characteristic of most taxa including birds (Cockrem and Silverin, 2002), reptiles (Cash et al., 1997), mammals (Lopez-

Olvera et al., 2007) and amphibians (Mosconi et al., 2006), and researchers commonly assess plasma levels of these hormones to evaluate the degree of stress an animal is currently under. One drawback to this technique is that the potential for rapid increases in corticosterone (i.e. within minutes) following capture and handling (Romero and Romero, 2002) means that researchers must obtain blood samples from the specimen immediately after capture to obtain "baseline" hormone levels.

A secondary approach for assessing physiological stress is via the leukocyte component of the immune system, specifically the relative numbers of white blood cells in circulation. In general, release of stress hormones in vertebrates causes alterations of numbers of two of the five leukocyte types; that is an increase in neutrophils and a decrease in lymphocytes (Jain, 1986, 1993; Dhabhar et al., 1994). The reason for these alterations is not clear, but since it occurs predictably, detection of these alterations can be used by researchers to indirectly infer increases in stress hormones. Moreover, using this approach to measuring stress offers certain benefits to animal researchers, especially in field situations where it may be difficult to sample animals quickly after capture. First, the proliferation of leukocytes in circulation operates on a slower time scale than hormones such that hormone-induced changes in leukocyte numbers are not observed (in birds) within one hour after capture (Davis, 2005), therefore any possible stress of capture or handling is not reflected in initial ("baseline") samples. A secondary advantage is the relatively smaller amount of blood required for making a blood smear than for sampling corticosterone in plasma (AKD, pers. obs.).

There is convincing evidence that stress-induced hematological alterations (i.e., increases in neutrophils and decreases in lymphocytes) can be seen in nearly all vertebrates. The link between stress hormones and leukocytes has been well-documented in mammals (Jain, 1986,

1993; Dhabhar et al., 1994). In birds early work with poultry demonstrated how stress causes the number of heterophils (the neutrophil equivalent in birds and reptiles) to increase while the lymphocytes decrease (Gross and Siegel, 1983). The ratio of these cells has been increasingly used among ornithologists to document the effects of various stressful conditions including transport stress (Groombridge et al., 2004) and reproductive output (Moreno et al., 2002b). Research with Eastern Box Turtles (*Terrapene carolina carolina*) also used this approach to document stress (Case et al., 2005). Furthermore, a series of lab experiments beginning in the 1960s showed that amphibians appear to have the same response, in multiple stress-inducing conditions such as limb amputation (Bennett, 1986), temperature extremes (Bennett and Daigle, 1983), osmotic stress (Bennett and Johnson, 1973), photoperiod stress (Bennett and Reap, 1978) and direct administration of hydrocortisone (Bennett and Alspaugh, 1964; Bennett and Newell, 1965; Bennett and Harbottle, 1968). In all cases, the stressors caused increases in numbers of neutrophils and decreases in lymphocyte numbers in circulation. Unfortunately, a thorough review of more recent literature revealed that this early research has not been subsequently followed, nor has this "hematological" stress assay been used in recent studies of amphibians. The one exception is a recent study conducted by the authors of the current paper where neutrophil-lymphocyte ratios of breeding male and female Mole Salamanders (*Ambystoma talpoideum*) were used to assess sex-related differences in reproductive stress (Davis and Maerz, in press).

Short-term captivity is known to induce a hormonal stress response in wild animals (Cash et al., 1997; Mosconi et al., 2006). While this phenomenon is well-known, the effects of captivity-induced stress on leukocyte profiles have rarely been examined, and thus far only in birds (Ruiz et al., 2002). Similar research has not been conducted with amphibians, despite the

fact that many studies of frogs or salamanders involve captive husbandry. In this study we examined the hematological profile of a common amphibian species (Mole Salamander), captured in the wild and brought into captivity for 10 days to test the hypothesis that captivity induces a measurable hematological stress response, reflected specifically by increases in neutrophils, decreases in lymphocytes or increases in neutrophil-lymphocyte ratios. We also examined possible effects of stress on cell counts of the three other leukocyte types (eosinophils, basophils and monocytes), although since these cells are not known to be affected by stress (Thrall et al., 2004), we had no *a priori* reason for suspecting an effect of captivity on these cells.

Methods

Capturing salamanders.---We captured paedogenic Mole Salamanders on two days from three permanent ponds located in the Warnell School of Forestry's Whitehall Experimental Forest in northeast Georgia in March 2007. On the first sampling day (March 15) we dipnetted between five and six salamanders per pond (16 total), then placed them in plastic containers filled with pond water, and transported them to a location 10 km away where they were placed in a 40 L plastic container filled with aged well water. The container was placed in a semi-shaded spot outside and a small amount of leaf litter was added to provide refugia within the container. These individuals were left undisturbed until March 25 (10 days later), when they were hand-captured and transported to the lab for processing as described below. On the second sampling trip (March 20) we visited the same three ponds, captured 5-6 individuals per pond as before (16 total), but these individuals were immediately transported to the lab for processing. In this case, all individuals were sampled within one hour of capture, including the transport time.

Blood sampling and slide preparation.---In the lab each salamander was weighed then killed via overdose of MS-222. A blood sample was then obtained by siphoning a small sample of blood from the exposed heart region after decapitation with a microhematocrit tube following Davis and Maerz (in press). A drop of the blood was then used to make a standard blood smear. Smears were allowed to air-dry then were stained with Giemsa.

Leukocyte counting and data handling.---All slides were viewed with a standard light microscope under 1000X (by only one of the authors), and at least 100 leukocytes were counted, while keeping track of the number of fields of view examined following Davis and Maerz (in press) and Davis et al. (2004). During the counting, slides were moved in increments of approximately 1-2 mm (without the observer looking through the lens), in a standard zig-zag pattern across the blood smear, so that all parts of the smear were eventually sampled. While the observer did not view the slide during the incremental moves, if the field of view landed where there was limited coverage of erythrocytes (i.e., less than 15), that field was not used. We identified leukocytes as lymphocytes, neutrophils, eosinophils, basophils and monocytes following Thrall (2004). Thrombocytes were observed throughout the smears but are not considered leukocytes (Thrall et al., 2004) and therefore were not counted. When 100 leukocytes were counted, we calculated the number of leukocytes per field of view, based on the number of fields examined.

For each salamander we estimated the total number of leukocytes per 1000 red blood cells by dividing the number of leukocytes per field of view by the average number of erythrocytes per field of view (18 [standard deviation = 5], obtained from a subset of five fields of view from each of 10 individuals) and multiplying by 1000. We then calculated the proportion of all five leukocyte types, and estimated the relative numbers of each leukocyte type per 1000

erythrocytes for each salamander by multiplying the cell proportion by the total number of leukocytes per 1000 erythrocytes. Finally, we calculated the ratio of neutrophils to lymphocytes using the proportions of each cell type following Davis and Maerz (in press) and Davis et al. (2004). The ratios were arcsin squareroot transformed and numbers of leukocytes for each cell type were log-transformed prior to analyses to meet assumptions of normality.

Data analysis.---We examined the possible effect of captivity while simultaneously examining possible effects of body size using analysis-of-covariance with counts of each leukocyte type (i.e., estimates of cell number per 1000 erythrocytes), as well as neutrophil-lymphocyte ratios, included as dependent variables, and with treatment (recently captured or captive) and body mass included as independent variables. In all, five analyses were performed (one for each leukocyte type [except monocytes – see results], and one for neutrophil-lymphocyte ratios). We did not include a pond variable since all three ponds were of similar size and located within 50 m of each other and since *a priori* examination of possible differences in all five leukocyte parameters among ponds revealed no significant variation (one-way ANOVA, $df=2$, $p>0.05$ for all five parameters). All analyses-of-covariance were run once with an interaction term between the two independent variables included in the model, but because there was no significant effect of this term found in any analyses, it was removed from the model and the analyses repeated with main effects only. All tests were performed using Statistica 6.0.

Results

Body size.---The size of the salamanders (i.e., body mass) was not significantly related to the neutrophil-lymphocyte ratio, nor to counts of lymphocytes or neutrophils (Table 4.1).

However, body mass significantly affected counts of eosinophils, and evaluation of parameter estimates indicated this effect was positive such that larger salamanders had more eosinophils.

Effect of treatment.---The neutrophil-lymphocyte ratio was significantly higher in the captive-stressed individuals than that of the recently captured (Table 4.1). In terms of captivity effects on individual cell types, captive-stressed salamanders had significantly fewer lymphocytes and significantly more neutrophils and eosinophils (Table 4.2) than recently captured salamanders. There was no effect of captivity on numbers of basophils. Not enough monocytes were counted for statistical tests.

Discussion

The hematological approach we used here demonstrated that regardless of body size, captivity induced a general stress response in the paedogenic mole salamander. Specifically, captivity caused the estimated numbers of neutrophils to increase and numbers of lymphocytes to decrease, thus the ratio of neutrophils to lymphocytes of captive individuals was on average twice as high as that of recently captured individuals. These results are consistent with prior studies of other stress-inducing conditions on amphibian leukocyte parameters (Bennett and Reap, 1978; Bennett and Daigle, 1983; Bennett, 1986), although we point out that the stress-inducing stimulus involved in our study was considerably more benign than those previously studied in amphibians, which included limb amputation and direct injection of stress hormones. Indeed, in our study we did little more than capture wild salamanders and place them in a standard mesocosm-type container filled with water, and they were then left undisturbed for 10 days. The hematological parameters we observed after this 10 day period, compared to non-

captive individuals, indicated that even this relatively simple procedure induced a physiological stress response in the salamanders.

While it is clear that both sets of salamanders we assessed differed in hematological parameters (and we logically conclude that captivity caused this difference) we cannot completely rule out the possibility that the five-day time difference between sampling the first set (those later held captive for 10 days) and the second set, was the reason for the observed results, and not necessarily any effect of stress. We consider this possibility unlikely though, since prior collections of 10 non-breeding individuals made from one of these same ponds three months earlier revealed similar leukocyte profiles and a statistically similar average neutrophil-lymphocyte ratio to the recently captured individuals of this study (two-sample t-test, $t=1.69$, $df=24$, $p=0.103$). Thus we argue that if the hematological stress parameters did not change over a three month period in these ponds, it should not have changed over the five days examined here.

An aspect of this study that invokes further questions is why exactly the stress response was elicited in the captive salamanders. We envision two possible explanations that could be examined further in follow-up work. In the first, it may be that the stress of the initial capture and removal from the natural surroundings caused an acute increase in plasma stress hormone (i.e., corticosterone) levels, which then lead to the alterations in leukocyte parameters, and these alterations could have lasted for the duration of the experiment. Indeed, there is evidence that the production of leukocytes in amphibian tissues and migration into circulation requires days (as opposed to hours in mammals) to complete (Hightower, 1978). Alternatively, the stress of captivity (whether from crowding, suboptimal conditions, etc.) may have elicited a more chronic increase in stress hormones which then caused leukocyte alterations. Determining which of these

scenarios is of greater importance should help amphibian researchers pinpoint commonly-used procedures that are likely to cause stress.

While we had no *a priori* expectations for how captivity would affect other leukocyte parameters, we found that captivity induced an unexpected increase in numbers of eosinophils. Eosinophil numbers have not previously been reported to change with stress in amphibians, although there is evidence that eosinophils decrease in abundance following stressful stimuli in mammals (Jain, 1986). This cell is thought to be involved in parasitism defense since general increases occur in response to parasite infections (Thrall et al., 2004). Interestingly, this cell is also the most abundant leukocyte in paedogenic ambystomatid salamanders (Ussing and Rosenkilde, 1995; Davis and Maerz, in press). Following metamorphosis, the proportion of eosinophils declines to less than 10% of leukocytes (Ussing and Rosenkilde, 1995). However, the high proportions of eosinophils are likely not due to paedomorphism itself, since in the obligate paedogenic hellbender (*Cryptobranchus alleganiensis*) eosinophils amount to 4.5% of leukocytes in adults (Jerrett and Mays, 1973). Rosenkilde et al. (1995) suggested that the act of metamorphosis partly serves to clear the animal of parasites which explains the dramatic drop in numbers following (induced) metamorphosis, although since a thorough screening of parasites in ambystomatid salamanders has not yet been performed, we can not be sure of this idea.

Our study demonstrates the utility of using hematological techniques to assess stress in amphibians. Our results also have implications for a wide range of herpetological research, but specifically for projects involving captive husbandry of amphibians. In such cases, researchers must be aware that animals brought into captivity from wild sources become stressed, regardless of the experimental conditions being explored. Moreover, stressed animals may not behave or react in a manner typical of normal animals, although this point should be better studied in

herpetofauna. We can make some inferences from work with other taxa. In birds, juveniles with high heterophil-lymphocyte ratios grow more slowly to adulthood (Moreno et al., 2002a), and adults with high ratios survive less well from year to year than those with low ratios (Kilgas et al., 2006). Also in birds, individuals with high ratios have been shown to be more susceptible to diseases than those with low ratios (Al-Murrani et al., 2002). Finally, and perhaps more related to captive husbandry, the quality of the housing environment has been shown to be an important predictor of hematological stress parameters in Box Turtles (*Terrapene carolina*) in captivity (Case et al., 2005). These studies, as well as the present one, all highlight the importance of knowing the conditions under which animals become stressed in research projects, and more generally, the influence of stress on immune systems and life history patterns of all vertebrates, including herpetofauna.

Acknowledgements

During this project AKD was supported by funds from the D.B. Warnell School of Forestry and Natural Resources at the University of Georgia. Procedures in this study were approved by the University of Georgia Institutional Animal Care and Use Committee (AUP A2007-10014-0).

Literature Cited

Al-Murrani, W.K., I.K. Al-Rawi, and N.M. Raof. 2002. Genetic resistance to *Salmonella typhimurium* in two lines of chickens selected as resistant and sensitive on the basis of heterophil/lymphocyte ratio. British Poultry Science 43:501-507.

Bennett, M.F. 1986. Stress and changes in the blood of newts, *Notophthalmus viridescens*, during early regeneration. Journal of Comparative Physiology A 159:823-826.

Bennett, M.F., and J.K. Alspaugh. 1964. Some changes in the blood of frogs following administration of hydrocortisone. The Virginia Journal of Science 15:76-79.

Bennett, M.F., and K.R. Daigle. 1983. Temperature, stress and the distribution of leukocytes in red-spotted newts, *Notophthalmus viridescens*. Journal of Comparative Physiology A 153:81-83.

Bennett, M.F., C.A. Gaudio, A.O. Johnson, and J.H. Spisso. 1972. Changes in the blood of newts, *Notophthalmus viridescens*, following administration of hydrocortisone. Journal of Comparative Physiology A 80:233-237.

Bennett, M.F., and J.A. Harbottle. 1968. The effects of hydrocortisone on the blood of tadpoles and frogs, *Rana catesbeiana*. Biological Bulletin 135:92-95.

Bennett, M.F., and A.O. Johnson. 1973. Osmotic stress, ACTH and the white blood cell picture in newts, *Notophthalmus viridescens*. Journal of Comparative Physiology A 82:333-338.

Bennett, M.F., and N.C. Newell. 1965. A further study of the effects of hydrocortisone on the blood of frogs. The Virginia Journal of Science 16:128-130.

Bennett, M.F., and L.E. Reap. 1978. Photoperiod, stress and the distribution of leukocytes in the peripheral blood of *Notophthalmus viridescens*. Journal of Comparative Physiology A 125:205-207.

Case, B.C., G.A. Lewbart, and P.D. Doerr. 2005. The physiological and behavioural impacts of and preference for an enriched environment in the eastern box turtle (*Terrapene carolina carolina*). Applied Animal Behaviour Science 92:353-365.

Cash, W.B., R.L. Holberton, and S.S. Knight. 1997. Corticosterone secretion in response to capture and handling in free-living red-eared slider turtles. General and Comparative Endocrinology 108:427-433.

Cockrem, J.F., and B. Silverin. 2002. Sight of a predator can stimulate a corticosterone response in the Great Tit (*Parus major*). General and Comparative Endocrinology 125:248-255.

Davis, A.K. 2005. Effects of handling time and repeated sampling on avian white blood cell counts. Journal of Field Ornithology 76:334-338.

Davis, A.K., K.C. Cook, and S. Altizer. 2004. Leukocyte profiles of House Finches with and without mycoplasmal conjunctivitis, a recently emerged bacterial disease. Ecohealth 1:362-373.

Davis, A.K., and J.C. Maerz. In Press. Sex-related differences in hematological stress indices of breeding, paedogenic mole salamanders. Journal of Herpetology.

Dhabhar, F.S., A.H. Miller, M. Stein, B.S. Mcewen, and R.L. Spencer. 1994. Diurnal and acute stress-induced changes in distribution of peripheral blood leukocyte subpopulations. Brain Behavior and Immunity 8:66-79.

Groombridge, J.J., J.G. Massey, J.C. Brunch, T.R. Malcolm, C.N. Brosius, M.M. Okada, and B. Sparklin. 2004. Evaluating stress in a Hawaiian honeycreeper, *Paroreomyza montana*, following translocation. Journal of Field Ornithology 75:183-187.

Gross, W.B., and H.S. Siegel. 1983. Evaluation of the heterophil/lymphocyte ratio as a measure of stress in chickens. Avian Diseases 27:972-979.

Hightower, J.A. 1978. The cell cycle of the neutrophilic granulocyte in a Urodele amphibian. Copeia:86-92.

Jain, N.C. 1986. Schalm's Veterinary Hematology. Lea & Febiger, Philadelphia.

Jain, N.C. 1993. Essentials of Veterinary Hematology. Blackwell Publishing, Philadelphia.

Jerrett, D.P., and C.E. Mays. 1973. Comparative hematology of the hellbender, Cryptobranchus alleganiensis in Missouri. Copeia 1973:331-337.

Kilgas, P., V. Tilgar, and R. Mand. 2006. Hematological health state indices predict local survival in a small passerine bird, the great tit (*Parus major*). Physiological and Biochemical Zoology 79:565-572.

Lopez-Olvera, J.R., I. Marco, J. Montane, E. Casas-Diaz, and S. Lavin. 2007. Effects of acepromazine on the stress response in Southern chamois (*Rupicapra pyrenaica*) captured by means of drive-nets. Canadian Journal of Veterinary Research 71:41-51.

Moore, I.T., and T.S. Jessop. 2003. Stress, reproduction, and adrenocortical modulation in amphibians and reptiles. Hormones and Behavior 43:39-47.

Moreno, J., S. Merino, J. Martinez, J.J. Sanz, and E. Arriero. 2002a. Heterophil/lymphocyte ratios and heat-shock protein levels are related to growth in nestling birds. Ecoscience 9:434-439.

Moreno, J., P. Yorio, P. Garcia-Borboroglu, J. Potti, and S. Villar. 2002b. Health state and reproductive output in Magellanic penguins (*Spheniscus magellanicus*). Ethology Ecology and Evolution 14:19-28.

Mosconi, G., F. Palermo, M. Carotti, S. Kikuyama, K. Yamamoto, and A.M. Polzonetti-Magni. 2006. Neuroendocrine modulation of stress response in the anuran, *Rana esculenta*. Amphibia-Reptilia 27:401-408.

Romero, L.M. 2004. Physiological stress in ecology: lessons from biomedical research. Trends in Ecology & Evolution 19:249-255.

Romero, L.M., and R.C. Romero. 2002. Corticosterone responses in wild birds: the importance of rapid initial sampling. Condor 104:129-135.

Rosenkilde, P., I. Sorensen, and A.P. Ussing. 1995. Amphibian hematology - Metamorphosis-related changes in blood cells. Netherlands Journal of Zoology 45:213-215.

Ruiz, G., M. Rosenmann, F.F. Novoa, and P. Sabat. 2002. Hematological parameters and stress index in Rufous-collared Sparrows dwelling in urban environments. Condor 104:162-166.

Thrall, M.A., D.C. Baker, and E.D. Lassen. 2004. Veterinary Hematology and Clinical Chemistry. Iowa State University Press

Ussing, A.P., and P. Rosenkilde. 1995. Effect of induced metamorphosis on the immune system of the Axolotl, *Ambystoma mexicanum*. General and Comparative Endocrinology 97:308-319.

Table 4.1. Summary of results of ANCOVAs examining effects of body size (mass) and treatment (recently captured versus captive-stressed) on leukocyte parameters of paedogenic mole salamanders. Counts of leukocyte types (estimated numbers per 1000rbcs) were used in analyses of specific cell types. Not enough monocytes were counted to allow for statistical testing. In all tests, an interaction effect was initially included, but was non-significant ($p>0.05$) in all cases and therefore removed.

Dependent	**Independent**	**df**	**MS**	**F**	**p**
N-L ratio	Wt	1	0.00	0.10	0.749
	Treatment	1	0.52	28.97	0.000
	Error	29	0.02		
Lymphocytes	Wt	1	0.01	0.11	0.739
	Treatment	1	0.24	4.93	0.034
	Error	29	0.05		
Neutrophils	Wt	1	0.02	0.53	0.474
	Treatment	1	0.26	5.67	0.024
	Error	29	0.05		
Eosinophils	Wt	1	0.89	5.33	0.028
	Treatment	1	0.81	4.83	0.036
	Error	29	0.17		
Basophils	Wt	1	0.15	3.40	0.076
	Treatment	1	0.00	0.00	0.955
	Error	29	0.04		

Table 4.2. Summary of leukocyte profiles (as % of all white blood cells or estimated number per 1000 red blood cells) of recently captured (n=16) and captive-stressed (n=16) paedogenic mole salamanders. Shown are the means plus standard errors in parentheses. Asterisks indicate significant differences between treatments from ANCOVA results in Table 1.

	Recently Captured				**Captive-Stressed**			
	%		**per1000rbc**		**%**		**per1000rbc**	
Lymphocytes	39.0	(5.3)	23.2	(2.8)	21.5	(3.5)	13.4*	(1.1)
Neutrophils	5.7	(0.8)	3.8	(0.8)	8.6	(1.6)	5.2	(0.5)
Eosinophils	51.2	(5.8)	35.6	(6.2)	66.7	(5.4)	68.6*	(13.5)
Basophils	3.9	(0.7)	2.4	(0.5)	2.9	(0.5)	2.1	(0.4)
Monocytes	0.1	(0.1)	0.0	(0.0)	0.3	(0.1)	0.2	(0.1)
N-L Ratio			0.17	(0.03)			0.39**	(0.03)

**p<0.001, * p<0.05

CHAPTER 5

EFFECTS OF LARVAL DENSITY ON HEMATOLOGICAL STRESS INDICES IN SALAMANDERS[1]

[1]Davis, A.K., and Maerz, J.C. 2009. *Journal of Experimental Zoology Part A-Ecological Genetics and Physiology* 311A: 697-704. Reprinted here with permission of publisher.

Abstract

In animals with complex life cycles, the quality of juvenile environments is important in shaping the longer-term fitness of individuals. Larval density is a major factor governing quality of larval environments in amphibians, with high densities leading to reduced growth rates, smaller size at metamorphosis, and potentially long-lasting post-metamorphic effects. A little-studied effect of larval density is its impact on physiological stress of post-metamorphic individuals. We used a hematological approach, involving counts of specific white blood cells types (neutrophils and lymphocytes) that covary with corticosterone, to estimate stress levels in recently metamorphosed spotted salamanders (*Ambystoma maculatum*) that were reared in three different larval densities in outdoor mesocosms. In replicated treatments consisting of 12, 25 or 50 larvae, survival was, as expected, lowest and size at metamorphosis smallest in the highest density mesocosms. In addition, surviving salamanders from high density treatments had significantly higher neutrophil to lymphocyte ratios, indicative of high levels of stress hormones (corticosterone). This trend was not a result of density-related differences in body condition as these did not vary with density. Further, estimated stress levels were similar regardless of whether the salamanders metamorphosed early or late, suggesting that the density effect on stress is long-lasting even once realized density has been reduced through mortality or early metamorphosis. These results may be important in understanding amphibian population dynamics, since research on other vertebrate taxa demonstrates that high hematological stress indicators lead to reduced growth, survival, and increased disease susceptibility in vertebrate animals.

Introduction

In animal species with complex life cycles, it is important for zoologists to consider how the fitness of any individual can be greatly influenced by its experiences in earlier life stages. Amphibians are a prime example, since many species have aquatic larval stages which eventually metamorphose into morphologically distinct terrestrial forms, and numerous examples show how larval environments can influence the performance of post-metamorphic individuals (e.g. Beck and Congdon, 2000; Gervasi and Foufopoulos, 2008; Scott, 1994; Scott et al., 2007; Semlitsch et al., 1988; Werner, 1986). One of the most important factors that determines the quality of larval environments in amphibians is the density of individuals (e.g. Loman, 2004; Petranka, 1989; Semlitsch and Reichling, 1989; Wilbur, 1976), with high larval densities invariably leading to a number of detrimental effects, such as reductions in larval growth or size at metamorphosis (Altwegg and Reyer, 2003; Collins, 1979; Semlitsch and Caldwell, 1982; Travis, 1984). Aside from these traditional measures of larval fitness, there is also growing evidence that high larval density and/or food limitation also leads to increases in physiological stress of amphibian larvae (Crespi and Denver, 2005; Glennemeier and Denver, 2002b; Hu et al., 2008).

Physiological stress in amphibians, as with all vertebrates, is usually inferred when increases in glucocorticoid hormones are detected (reviewed in Wingfield and Romero, 2001). These hormones (corticosterone in amphibians, birds and reptiles) are released into the bloodstream when animals perceive harmful stimuli, where they orchestrate a number of physiological changes thought to promote survival. If the stimulus is short-term or ‘acute’ in nature, such as being chased by a predator, a temporary increase in corticosterone occurs, followed by a return to baseline levels within hours after escape (Langkilde and Shine, 2006).

Alternatively, ‘chronic’ stressors such as high larval density in amphibians (which lasts for the duration of the larval period and which the larvae cannot necessarily escape from), can lead to chronically elevated baseline levels of glucocorticoid hormones in the larvae (Glennemeier and Denver, 2002b). Chronic stress can be detrimental to animals if occurring over long time periods, with known effects including suppression of immune activity (Kiank et al., 2006; Martin et al., 2005) and even disruption of the aforementioned acute stress response (Hull et al., 2007).

Stress-related increases in corticosterone have an additional effect on circulating leukocyte populations that is less well-known, but which conveniently provides researchers with a way to indirectly assess chronic stress in vertebrates (reviewed in Davis et al., 2008). In all vertebrates, there are five different types of white blood cells: neutrophils, lymphocytes, eosinophils, basophils and monocytes (Jain, 1986), and stress-induced increases in glucocorticoid hormones cause characteristic alterations in the circulating numbers of two of these cell types. Specifically, increases in these hormones invariably lead to increases in the number of neutrophils (or heterophils, the avian and reptilian equivalent) and decreases in lymphocytes in the circulating blood (Bennett, 1986; Dhabhar et al., 1996; Gross and Siegel, 1983). The reason for these alterations in circulating cell numbers is thought to be so that the cells are routed, or ‘redistributed’, to where they would be most needed during stressful events (Dhabhar et al., 1996). Specifically, glucocorticoid hormones cause neutrophils, which are phagocytic and attack foreign substances, to be released into the bloodstream from reserve banks in tissues or on capillary walls, while they cause lymphocytes to emigrate from the blood into tissues and simultaneously be retained from recirculation within the hemopoetic tissue (Dhabhar et al., 1995). Other cell types appear to be less affected by stress hormones (Davis et al., 2008). Thus for the researcher, the ratio of the numbers of neutrophils and lymphocytes detected from

standard blood smears (the neutrophil-lymphocyte or 'N/L' ratio) can be effectively used to infer levels of stress hormones, and in effect to estimate physiological stress (i.e. baseline levels) in animals in a variety of natural or experimental settings (reviewed in Davis et al., 2008). Indeed, recent work by the authors shows this hematological approach is useful for assessing chronic stress in salamanders (Davis and Maerz, 2008a; Davis and Maerz, 2008b).

Unlike other vertebrates, amphibians must also contend with the stress of metamorphosis in the transition from larval to adult form. During this transition, corticosterone is produced and is involved in the reorganization of the amphibian immune system (Rollins-Smith et al., 1997). Indeed, levels of this hormone show a general increase during metamorphic climax (Glennemeier and Denver, 2002a; Krain and Denver, 2004). Curiously though, during this time there is no corresponding increase in circulating neutrophil leukocytes as is seen during typical stress responses (Davis, 2009; Rosenkilde et al., 1995), perhaps because of the changes occurring in the immune system during the metamorphosis, or perhaps the metamorphosis-related stress differs from the typical stress response in some manner. Regardless of the reason, for the amphibian researcher this result means that neutrophil-lymphocyte ratios of amphibians taken during the metamorphic period would not be unduly influenced by the 'stress' of metamorphosis.

The current study is aimed at elucidating the relationships between larval density, a known stressor, and physiological stress levels of post-metamorphic amphibians. Specifically, given the known effect of density on the quality of amphibian larval environments and stress of larvae, we questioned if individuals survive this environment, do they then enter the terrestrial world with elevated stress (i.e. after they finish metamorphosis)? Thus, our goal in this study was to determine if larval density affects 'baseline' stress levels of recently metamorphosed spotted salamanders (*Ambystoma maculatum*) as revealed by hematological stress indices (i.e.

neutrophil-lymphocyte ratios). We reared salamander larvae at three densities to create environments of varying quality which we compared neutrophil-lymphocyte ratios and traditional measures of larval performance across: survival and time to metamorphosis, mass and body condition.

Methods

Rearing Larvae – Fourteen egg masses of *A. maculatum* were gathered from natural, ephemeral ponds near Athens, GA (33.88° latitude, -83.36° longitude) in February 2006 and housed in separate containers until hatching. Within 3 days after hatching, larvae were haphazardly assigned to one of 3 groups: three replicate groups of 12 larvae (low density treatment), three groups of 25 (medium density), and three groups of 50 (high density), for a total of 261 larvae. Nine non-permeable, plastic mesocosms (i.e. 'cattle tanks', with 1000 liter capacity) were submerged halfway in an impounded, permanent pond in the Whitehall Experimental Forest near Athens, GA. The pond was surrounded by mixed hardwood and softwood trees, and contained no fish. The mesocosms were each filled with approximately 650 liters of filtered pond water and approximately 2 liters of leaf litter was placed in the bottom of each. With this arrangement, the conditions in each mesocosm (i.e. diurnal and monthly water and air temperatures) therefore mimicked those of the pond, except for the absence of aquatic predators (i.e. turtles, bullfrogs, etc.) and other amphibian species. Moreover, mesocosms were not covered so that natural colonization or oviposition by aerial insects would take place and serve as a larval food resource (mesocosms were not supplemented with food during the

experiment). This also made it possible for aquatic insect predators to colonize the mesocosms (i.e. dragonfly larvae), though we did not observe any during the experiment.

Each mesocosm was randomly assigned to one of the 3 treatments (i.e. so that the treatments were interspersed within the mesocosm array) and on March 29, larvae were added. Since each mesocosm contained 650L of water, the realized larval density for each mesocosm was therefore approximately 0.02 larvae/L, 0.04 larvae/L and 0.08larvae/L for the low, medium and high densities, respectively. The realized low density used here is consistent with 'low' densities used in other work with larval salamanders (Metts et al., 2005), although the high density we used is twice as high as in that study. However, Figiel and Semlitsch (1990) report natural ranges for *A. maculatum* to be from 0.0002 to 0.08 larvae/L, and the high density used in the current study is consistent with the upper end of this range.

After adding larvae, each mesocosm was checked weekly during the next two months. Every two weeks, sets of larvae were haphazardly dipnetted in each mesocosm to monitor larval development (larvae were visually inspected and immediately released back into mesocosms). When we began to find larvae nearing metamorphosis, we switched from weekly to daily monitoring, where we visually checked each mesocosm for individuals that had *finished* metamorphosis (i.e. their gills had been resorbed). Metamorphosed individuals were dipnetted from the mesocosm, placed in a plastic container with pond water, and transported immediately to the lab. It is important to point out here that the hematological approach to estimating stress is not as time-sensitive as direct corticosterone sampling (Davis et al., 2008), where animals must be sampled within minutes of capture (Romero and Reed, 2005; Romero and Romero, 2002). In fact, in amphibians the time for the hematological effect of corticosterone to occur is on the order of hours to days (Bennett et al., 1972; Bennett and Newell, 1965), which means that any

potential effects of capture, handling, or transport are minimal if blood is obtained the same day of capture.

Processing Adults – Later in the day of capture, each individual was photographed from above with a digital camera, weighed, then euthanized by overdose of MS-222. Immediately after death, salamanders were decapitated and a heparinized microcapillary tube was used to siphon blood from the exposed heart region following previously established procedures (Davis and Maerz, 2008a; Davis and Maerz, 2008b). A drop of blood was placed on a clean microscope slide, and a second slide was used to smear the blood on the first slide. All slides were air-dried, then stained with giemsa. We were only able to obtain enough blood for a readable smear from 30 individuals, although there were enough individuals sampled in each mesocosm to obtain mesocosm-averages (the unit of replication for analyses, below) for blood parameters. Further, the individuals that were not sampled were evenly divided across treatments. Later, body length measurements of all salamanders were obtained from their digital photos using image analysis software following Davis and Maerz (2007). From these data and the body mass data, we created a body condition score for each salamander by retaining the residuals of a linear regression of the cubed-root of mass on body length.

Leukocyte Counting – Counting procedures generally followed Davis and Maerz (2008a; 2008b). All slides were viewed using a light microscope at 1000X magnification and the numbers of all white blood cell types were recorded until at least 100 cells were counted or 150 fields of view were reached. Fields of view with low numbers of red blood cells were not included. White blood cell types that we identified included neutrophils, lymphocytes, eosinophils, basophils and monocytes, following Thrall (2004), Turner (1988) and Hadji-Azimi et al. (1987), although the focus here was on neutrophils and lymphocytes. To ensure accurate

counts, each slide was read twice (by AKD), blindly, and in non-consecutive order, and the average of each leukocyte number was used thereafter. The proportions of each leukocyte type were calculated based on the numbers of each type counted and the total number of all types. We calculated the neutrophil-lymphocyte ratio based on their proportions and used the log (+1) of this variable in the analyses.

Data Analysis – The effect of larval density on all parameters was examined using multiple regression where the mesocosms were the replicates (experimental units), larval density was the single explanatory variable (treated as a continuous variable), and the mean proportion surviving (arcsine square-root transformed), mean development time, mean mass, mean body condition score, and mean N/L ratio (log +1) of surviving salamanders from each mesocosm were the multiple dependent variables. Levene's tests showed all variables were homogeneous across treatments ($p<0.05$ for all). This statistical approach, where the mesocosm mean is the unit of replication, is highly appropriate to address our experimental objectives, since variations in number of salamanders that survive in each mesocosm (which we expected) would not affect the power of the analysis (since the number of mesocosms remained the same). Indeed, only the sensitivity of the mesocosm mean would be affected by variation in the numbers of salamanders sampled per mesocosm. Analyses were conducted using the Statistica 6.1 software package (Statistica, 2003).

Results

A total of 52 salamanders survived the larval treatments and were measured in this experiment, with 18, 21 and 13 individuals from low medium and high densities, respectively.

Counts of leukocytes from all salamanders for which blood samples were obtained (N=30) are presented in Table 5.1, grouped according to initial larval density. There were 11 individuals sampled in the low density treatment, 11 in the medium and 8 in the high density treatment. While these parameters were not statistically examined (the experimental unit for analysis was the mesocosm mean, below), they do show how the distributions of cell types within individuals changed with larval density. In general, and considering all individuals, lymphocytes, neutrophils and eosinophils were counted most often, and very few monocytes were seen, which is consistent with prior research on related *Ambystoma* species (Davis and Durso, 2009; Davis and Maerz, 2008a; Davis and Maerz, 2008b). With regard to density, the average percentage of lymphocytes declined with increasing density, from 32% to 24% and finally 18%, in the low, medium and high densities, respectively, while the percentage of neutrophils increased from 18% in low densities to 23% in the medium density and 50% of all leukocytes in high densities.

Regression analysis revealed that the overall effect of larval density created significant variation in the parameters evaluated ($F_{5,3}$=15.80, p=0.023). Consistent with the percentages reported above, there was a significant positive relationship between larval density and mesocosm-mean neutrophil-lymphocyte ratios ($F_{1,7}$=29.21, p=0.001; Fig. 5.1A). This relationship was not driven by differences in body condition across densities, as this variable was not significant ($F_{1,7}$=0.13, p=0.730; Fig. 5.1B). Nor was the effect of larval density on N/L ratios was not influenced by development time; using data from all individual salamanders, there was no overall relationship between larval duration and N/L ratios (Pearson correlation, r=0.22, p=0.141; Fig. 5.2), nor was there a relationship when each density was considered separately (p>0.2 for all). Thus, salamanders in the low density mesocosms emerged with low N/L ratios regardless of whether they metamorphosed early or late in the experiment, and vice versa for the

high density; salamanders in high larval densities emerged with high ratios indices regardless of their larval duration.

There were effects of rearing density on other, conventional, parameters as well, which support the idea that increasing density is stressful. There was a significant negative relationship between larval density and survival ($F_{1,7}$=37.40, $p<0.001$; Table 5.2), with the highest survival in the low density treatments (averaging 50% across all 3 replicate mesocosms), 28% in the medium density, and 8.7% in the high density treatments. In addition, weights of surviving salamanders decreased with increasing larval densities ($F_{1,7}$=43.05, $p<0.001$); salamanders from the low density treatments weighed an average of 701.9mg, those from the medium density treatments weighed 513.0mg, and high density individuals weighed 324.5mg on average (Table 5.2). There was no significant relationship between larval density and development time ($F_{1,7}$=2.46, $p=0.161$).

Discussion

In this experiment, our three larval densities effectively created rearing environments that varied in their quality as shown by two of the traditional measures of larval performance: salamanders reared in the highest larval densities had the poorest survival and lowest masses at metamorphosis compared with the lowest densities, which is consistent with many prior studies (e.g. Altwegg and Reyer, 2003; Collins, 1979; Loman, 2004; Petranka, 1989; Semlitsch and Caldwell, 1982; Werner, 1986; Wilbur, 1976). Importantly, we show here for the first time that this variation in environmental quality also affects the stress levels of recently metamorphosed salamanders that survive these ‘stressful’ conditions, as determined from neutrophil-lymphocyte

ratios, which are known to reflect stress hormone levels in amphibians (Bennett, 1986; Bennett et al., 1972; Bennett and Harbottle, 1968; Davis and Maerz, 2008a; Davis and Maerz, 2008b), birds (Gross and Siegel, 1983) and other animals (reviewed in Davis et al., 2008). In the highest larval density, those individuals that successfully survived had the highest proportion of neutrophils, the lowest proportion of lymphocytes, and the highest N/L ratios (i.e. highest stress levels). Further, it did not matter if they metamorphosed early or late in the experiment, the degree of stress was the same after they emerged (Fig. 5.2).

The lack of an effect of larval duration on stress levels of post-metamorphic salamanders deserves further comment, since it is somewhat counterintuitive to conventional ideas of density effects. Specifically, the effects of high densities on individual fitness are thought to be more pronounced early in development when many larvae are present, since as time goes on and larvae metamorphose (or die from other factors), fewer individuals remain and competition for resources is lessened, leading to 'density-mediated compensation' in growth or survival (Rohr et al., 2006). Thus, one might initially expect later-emerging salamanders to have lower stress than early emerging individuals. Since our results do not support this idea, we interpret this to mean that either some other factor besides food resources or competition influences stress levels in amphibians, or that the effect of the *initial* density on amphibian stress levels is long-lasting among individuals.

The variation in stress indices we observed among densities did not appear to be caused by variation in nutritional condition of salamanders, since surviving individuals did not vary significantly in body condition across larval densities (Fig. 5.1B). In high density conditions (especially early in development) there is considerable competition for food resources, which usually leads to reductions in body size (e.g. Petranka, 1989; Steinwascher, 1979; Travis, 1984;

Wilbur, 1976), which we did see, but it is also possible that this increased competition leads to increased aggression among larvae (Faragher and Jaeger, 1998; Reques and Tejedo, 1996; Semlitsch and Reichling, 1989) and therefore higher stress levels. This idea has certainly been shown before in leopard frog larvae, with higher densities and/or reduced food availability leading to higher levels of corticosterone among individuals (Crespi and Denver, 2005; Glennemeier and Denver, 2002b). Interestingly though, Rot-Nikcevic et al (2006) also demonstrated that amphibian larvae can become stressed even if they simply perceive high densities (i.e. in that case by the sight of larvae reflected in mirrors), when in fact the real density is not high. It may well be then that it is not physical competition *per se* that leads to stress in individuals, but merely the perception of it.

While we do not know how long the elevated stress in salamanders lasts after they leave the aquatic environment, we can infer from studies of other taxa what the consequences would be if it does persist. High ratios of neutrophils (or heterophils in birds) to lymphocytes is often correlated with reduced growth (Moreno et al., 2002), survival (Kilgas et al., 2006), reproductive success (Al-Murrani et al., 2006), and increased susceptibility to infections (Al-Murrani et al., 2002). In amphibians, high stress hormone levels in adults lead to reductions in foraging success (Watson et al., 2004) and increased susceptibility to trematode infections in larvae (Belden and Kiesecker, 2005). Thus if the high hematological stress parameters we observed in recently metamorphosed salamanders persist, some of these consequences could be realized in the long-term. Interestingly, evidence that low-quality larval environments leads to reduced survival and reproductive success in adult salamanders (Scott, 1994) is consistent with effects of chronic stress. Moreover, recent work with anurans demonstrated that experimental exposure of tadpoles to corticosterone lead to juvenile frogs with elevated corticosterone 2 months later (Hu et al.,

2008). While these studies all point to the potential for stress to be long-lasting in amphibians, longer-term studies of stress levels of marked individuals would be needed to more clearly elucidate the temporal persistence of stress induced from larval environments.

Finally, besides the question of temporal persistence, our results engender further questions regarding effects of other low-quality larval environments such as wetlands contaminated with agricultural or industrial chemicals. Indeed, while the effects of such chemicals on amphibians are currently being considered (e.g. Forson and Storfer, 2006; Rohr and Crumrine, 2005), the possibility that such larval conditions could lead to stressed metamorphic individuals has not yet been addressed. Given the results from the current study, we might expect this to be true. In any case, our results at the very least show that physiological stress can be considered one more example of the legacy that post-metamorphic amphibians carry over from their larval environments.

Acknowledgements

We thank Andrew Grosse and Leah Connell for help with mesocosm monitoring. We also are grateful to Sonia Altizer for helpful statistical advice and to Brian Todd and several anonymous reviewers for providing comments on earlier drafts of the manuscript. AKD was supported by funding from the D.B. Warnell School of Forestry and Natural Resources at the University of Georgia.

Literature cited

Al-Murrani, W. K., I. K. Al-Rawi, and N. M. Raof. 2002. Genetic resistance to *Salmonella typhimurium* in two lines of chickens selected as resistant and sensitive on the basis of heterophil/lymphocyte ratio. British Poultry Science 43: 501-507.

Al-Murrani, W. K., A. J. Al-Rawi, M. F. Al-Hadithi, and B. Al-Tikriti. 2006. Association between heterophil/lymphocyte ratio, a marker of 'resistance' to stress, and some production and fitness traits in chickens. British Poultry Science 47: 443-448.

Altwegg, R., and H. U. Reyer. 2003. Patterns of natural selection on size at metamorphosis in water frogs. Evolution 57: 872-882.

Beck, C. W., and J. D. Congdon. 2000. Effects of age and size at metamorphosis on performance and metabolic rates of Southern Toad, *Bufo terrestris*, metamorphs. Functional Ecology 14: 32-38.

Belden, L. K., and J. M. Kiesecker. 2005. Glucocorticosteroid hormone treatment of larval treefrogs increases infection by *Alaria* sp trematode cercariae. Journal of Parasitology 91: 686-688.

Bennett, M. F. 1986. Stress and changes in the blood of newts, *Notophthalmus viridescens*, during early regeneration. Journal of Comparative Physiology A 159: 823-826.

Bennett, M. F., and N. C. Newell. 1965. A further study of the effects of hydrocortisone on the blood of frogs. The Virginia Journal of Science 16: 128-130.

Bennett, M. F., and J. A. Harbottle. 1968. The effects of hydrocortisone on the blood of tadpoles and frogs, *Rana catesbeiana*. Biological Bulletin 135: 92-95.

Bennett, M. F., C. A. Gaudio, A. O. Johnson, and J. H. Spisso. 1972. Changes in the blood of newts, *Notophthalmus viridescens*, following administration of hydrocortisone. Journal of Comparative Physiology A 80: 233-237.

Collins, J. P. 1979. Intra-population variation in the body size at metamorphosis and timing of metamorphosis in the bullfrog, *Rana catesbeiana*. Ecology 60: 738-749.

Crespi, E. J., and R. J. Denver. 2005. Roles of stress hormones in food intake regulation in anuran amphibians throughout the life cycle. Comparative Biochemistry and Physiology A: 381-390.

Davis, A. K. 2009. Metamorphosis-related changes in leukocyte profiles of larval bullfrogs (*Rana catesbeiana*). Comparative Clinical Pathology 18: 181-186.

Davis, A. K., and J. C. Maerz. 2007. Spot symmetry predicts body condition in spotted salamanders, *Ambystoma maculatum*. Applied Herpetology 4: 195-205.

Davis, A. K., and J. C. Maerz. 2008a. Sex-related differences in hematological stress indices of breeding, paedomorphic mole salamanders. Journal of Herpetology 42: 197-201.

Davis, A. K., and J. C. Maerz. 2008b. Comparison of hematological stress indicators in recently captured and captive paedomorphic mole salamanders, *Ambystoma talpoideum*. Copeia 2008: 613-617.

Davis, A. K., and A. M. Durso. In Press. Leukocyte differentials of northern cricket frogs (*Acris c. crepitans*) with a compilation of published values from other amphibians. Herpetologica.

Davis, A. K., D. L. Maney, and J. C. Maerz. 2008. The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. Functional Ecology 22: 760-772.

Dhabhar, F. S., A. H. Miller, B. S. McEwen, and R. L. Spencer. 1995. Effects of stress on immune cell distribution - Dynamics and hormonal mechanisms. Journal of Immunology 154: 5511-5527.

Dhabhar, F. S., A. H. Miller, B. S. McEwen, and R. L. Spencer. 1996. Stress-induced changes in blood leukocyte distribution - Role of adrenal steroid hormones. Journal of Immunology 157: 1638-1644.

Faragher, S. G., and R. G. Jaeger. 1998. Tadpole bullies: examining mechanisms of competition in a community of larval anurans. Canadian Journal of Zoology 76: 144-153.

Figiel, C. R., and R. D. Semlitsch. 1990. Population variation in survival and metamorphosis of larval salamanders (*Ambystoma maculatum*) in the presence and absence of fish predation. Copeia: 818-826.

Forson, D., and A. Storfer. 2006. Atrazine increases ranavirus susceptibility in the tiger salamander, *Ambystoma tigrinum*. Ecological Applications 16: 2325-2332.

Gervasi, S. S., and J. Foufopoulos. 2008. Costs of plasticity: responses to desiccation decrease post-metamorphic immune function in a pond-breeding amphibian. Functional Ecology 22: 100-108.

Glennemeier, K. A., and R. J. Denver. 2002a. Role for corticoids in mediating the response of *Rana pipiens* tadpoles to intraspecific competition. Journal of Experimental Zoology 292: 32-40.

Glennemeier, K. A., and R. J. Denver. 2002b. Developmental changes in interrenal responsiveness in anuran amphibians. Integrative and Comparative Biology 42: 565-573.

Gross, W. B., and H. S. Siegel. 1983. Evaluation of the heterophil/lymphocyte ratio as a measure of stress in chickens. Avian Diseases 27: 972-979.

Hadji-Azimi, I., V. Coosemans, and C. Canicatti. 1987. Atlas of adult *Xenopus laevis laevis* hematology. Developmental and Comparative Immunology 11: 807-874.

Hu, F., E. J. Crespi, and R. J. Denver. 2008. Programming neuroendocrine stress axis activity by exposure to glucocorticoids during postembryonic development of the frog, *Xenopus laevis*. Endocrinology 149: 5470-5481.

Hull, K. L., J. F. Cockrem, J. P. Bridges, E. J. Candy, and C. M. Davidson. 2007. Effects of corticosterone treatment on growth, development, and the corticosterone response to handling in young Japanese quail. Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology 148: 531-543.

Jain, N. C. 1986. Schalm's Veterinary Hematology. Lea & Febiger, Philadelphia.

Kiank, C., B. Holtfreter, A. Starke, A. Mundt, C. Wilke, and C. Schutt. 2006. Stress susceptibility predicts the severity of immune depression and the failure to combat bacterial infections in chronically stressed mice. Brain Behavior and Immunity 20: 359-368.

Kilgas, P., V. Tilgar, and R. Mand. 2006. Hematological health state indices predict local survival in a small passerine bird, the great tit (*Parus major*). Physiological and Biochemical Zoology 79: 565-572.

Krain, L. P., and R. J. Denver. 2004. Developmental expression and hormonal regulation of glucocorticoid and thyroid hormone receptors during metamorphosis in *Xenopus laevis*. Journal of Endocrinology 181: 91-104.

Langkilde, T., and R. Shine. 2006. How much stress do researchers inflict on their study animals? A case study using a scincid lizard, *Eulamprus heatwolei*. Journal of Experimental Biology 209: 1035-1043.

Loman, J. 2004. Density regulation in tadpoles of *Rana temporaria*: a full pond field experiment. Ecology 85: 1611-1618.

Martin, L. B., J. Gilliam, P. Han, K. Lee, and M. Wikelski. 2005. Corticosterone suppresses cutaneous immune function in temperate but not tropical House Sparrows, *Passer domesticus*. General and Comparative Endocrinology 140: 126-135.

Metts, B. S., W. A. Hopkins, and J. P. Nestor. 2005. Interaction of an insecticide with larval density in pond-breeding salamanders (Ambystoma). Freshwater Biology 50: 685-696.

Moreno, J., S. Merino, J. Martinez, J. J. Sanz, and E. Arriero. 2002. Heterophil/lymphocyte ratios and heat-shock protein levels are related to growth in nestling birds. Ecoscience 9: 434-439.

Petranka, J. W. 1989. Density-dependent growth and survival of larval *Ambystoma*: evidence from whole-pond manipulations. Ecology 70: 1752-1767.

Reques, R., and M. Tejedo. 1996. Intraspecific aggressive behaviour in fire salamander larvae (*Salamandra salamandra*): The effects of density and body size. Herpetological Journal 6: 15-19.

Rohr, J. R., and P. W. Crumrine. 2005. Effects of an herbicide and an insecticide on pond community structure and processes. Ecological Applications 15: 1135-1147.

Rohr, J. R., T. Sager, T. M. Sesterhenn, and B. D. Palmer. 2006. Exposure, postexposure, and density-mediated effects of atrazine on amphibians: Breaking down net effects into their parts. Environmental Health Perspectives 114: 46-50.

Rollins-Smith, L. A., K. S. Barker, and A. T. Davis. 1997. Involvement of glucocorticoids in the reorganization of the amphibian immune system at metamorphosis. Developmental Immunology 5: 145-152.

Romero, L. M., and R. C. Romero. 2002. Corticosterone responses in wild birds: the importance of rapid initial sampling. Condor 104: 129-135.

Romero, L. M., and J. M. Reed. 2005. Collecting baseline corticosterone samples in the field: is under 3 min good enough? Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology 140: 73-79.

Rosenkilde, P., I. Sorensen, and A. P. Ussing. 1995. Amphibian hematology: Metamorphosis-related changes in blood cells. Netherlands Journal of Zoology 45: 213-215.

Rot-Nikcevic, I., C. N. Taylor, and R. J. Wassersug. 2006. The role of images of conspecifics as visual cues in the development and behavior of larval anurans. Behavioral Ecology and Sociobiology 60: 19-25.

Scott, D. E. 1994. The effect of larval density on adult demographic traits in *Ambystoma opacum*. Ecology 75: 1383-1396.

Scott, D. E., E. D. Casey, M. F. Donovan, and T. K. Lynch. 2007. Amphibian lipid levels at metamorphosis correlate to post-metamorphic terrestrial survival. Oecologia 153: 521-532.

Semlitsch, R. D., and J. P. Caldwell. 1982. Effects of density on growth, metamorphosis, and survivorship in tadpoles of *Scaphiopus holbrooki*. Ecology 63: 905-911.

Semlitsch, R. D., and S. B. Reichling. 1989. Density-dependent injury in larval salamanders. Oecologia 81: 100-103.

Semlitsch, R. D., D. E. Scott, and J. H. K. Pechmann. 1988. Time and size at metamorphosis related to adult fitness in *Ambystoma talpoideum*. Ecology 69: 184-192.

Statistica. 2003. Statistica version 6.1, Statsoft Inc.

Steinwascher, K. 1979. Competitive interactions among tadpoles: responses to resource level. Ecology 60: 1172-1183.

Thrall, M. A. 2004. Hematology of amphibians. *In* M. A. Thrall, D. C. Baker and E. D. Lassen [eds.], Veterinary Hematology and Clinical Chemistry: Text and Clinical Case Presentations. Lippincott Williams & Wilkins, Philadelphia, PA.

Travis, J. 1984. Anuran size at metamorphosis - experimental test of a model based on intraspecific competition. Ecology 65: 1155-1160.

Turner, R. J. 1988. Amphibians, pp. 129-209. *In* A. F. Rawley and N. A. Ratcliffe [eds.], Vertebrate blood cells. Cambridge University Press, Cambridge.

Watson, R. T., A. Mathis, and R. Thompson. 2004. Influence of physical stress, distress cues, and predator kaiomones on the foraging behavior of Ozark zigzag salamanders, *Plethodon angusiclavius*. Behavioural Processes 65: 201-209.

Werner, E. E. 1986. Amphibian metamorphosis: growth rate, predation risk, and the optimal size at transformation. American Naturalist 128: 319-341.

Wilbur, H. M. 1976. Density-dependent aspects of metamorphosis in *Ambystoma* and *Rana sylvatica*. Ecology 57: 1289-1296.

Wingfield, J. C., and L. M. Romero. 2001. Adrenocortical responses to stress and their modulation in free-living vertebrates, pp. 211-234. *In* B. S. McEwen and H. M. Goodman [eds.], Handbook of physiology, section 7: The endocrine system, Vol. IV: Coping with the environment: neural and endocrine mechanisms. Oxford University Press, New York.

Table 5.1. Summary of leukocyte profiles of post-metamorphic spotted salamanders from all three densities. Shown are the average percentage (SE in parentheses) of each leukocyte type for all salamanders for which blood samples were obtained (N=30) in each density.

Density	N	Lymphocytes	Neutrophils	Eosinophils	Basophils	Monocytes
Low	11	31.7 (4.3)	18.1 (5.4)	25.5 (4.4)	24.2 (5.1)	0.6 (0.2)
Medium	11	23.9 (2.7)	22.7 (4.3)	33.0 (6.0)	19.6 (3.2)	0.9 (0.4)
High	8	17.8 (2.5)	49.9 (4.1)	22.8 (5.5)	9.3 (2.4)	0.2 (0.2)
All	30	25.1 (2.2)	28.2 (3.6)	27.5 (3.1)	18.5 (2.5)	0.6 (0.2)

Table 5.2. Summary of larval survival, duration and weights of surviving salamanders from all densities in this experiment. Values shown are the grand means of each mesocosm mean, with standard errors in parentheses. Each mesocosm contained 650 liters of water.

Initial Density	N (mesocosms)	Survival		Larval Duration (d)		Salamander Mass (mg)	
12	3	50.0 %	(4.8 %)	71.3	(4.1)	701.9	(63.7)
25	3	28.0 %	(6.9 %)	89.1	(4.4)	513.0	(17.9)
50	3	8.7 %	(1.8 %)	86.2	(4.8)	324.5	(8.7)

CHAPTER 5 FIGURES

5.1 Relationship between initial larval density and (A) mean neutrophil-lymphocyte ratios (log-transformed) and (B) mean body condition scores (residuals from regression of cubed-root of mass on body length) of surviving salamanders, using the mesocosm mean as the unit of replication.

5.2 Neutrophil-lymphocyte ratios (log-transformed) of all salamanders in relation to their larval development time. There was no overall relationship between larval duration and N/L ratios (Pearson correlation, $r=0.22$, $p=0.141$), nor was there a relationship when each density was considered separately ($p>0.2$ for all).

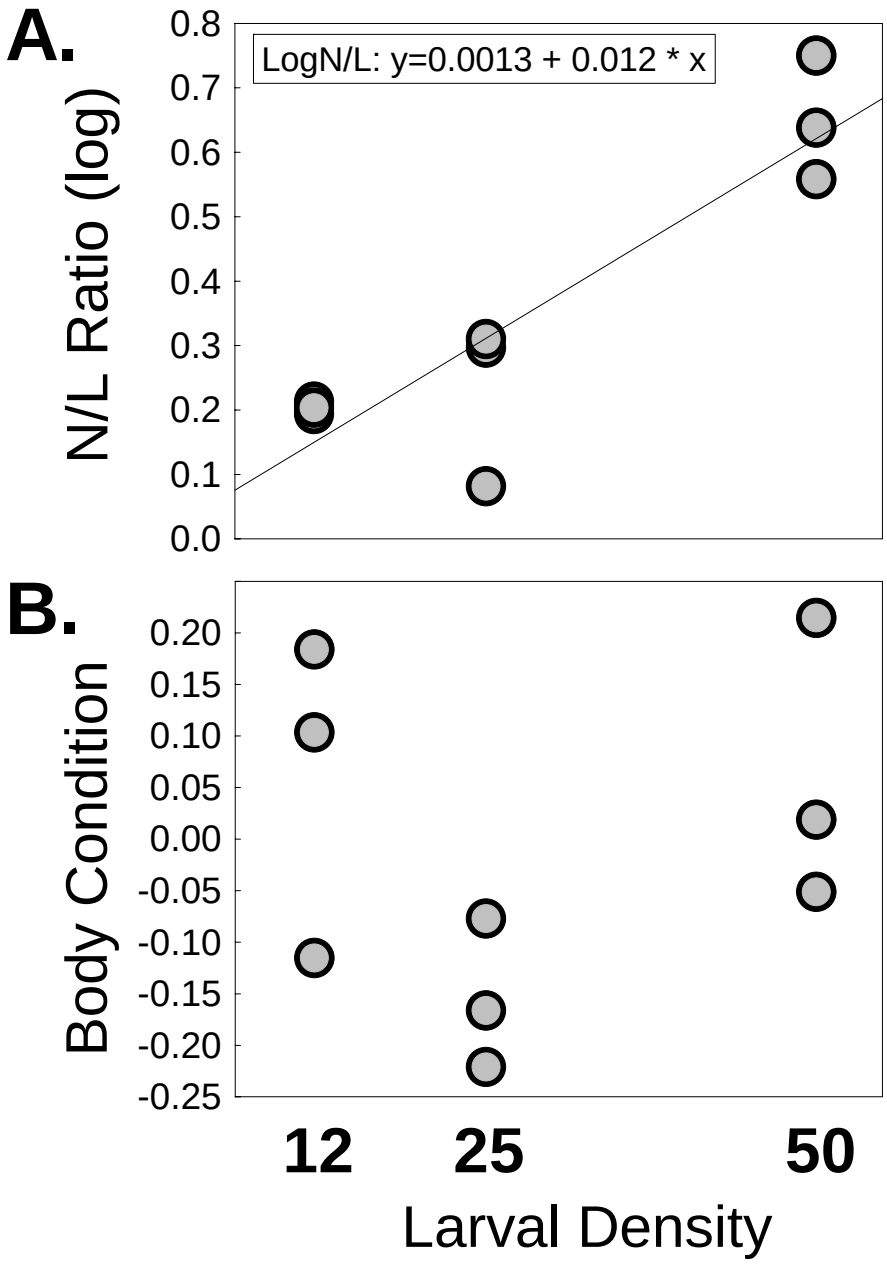
A.
LogN/L: y=0.0013 + 0.012 * x
N/L Ratio (log)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
B.
Body Condition
0.20
0.15
0.10
0.05
0.00
-0.05
-0.10
-0.15
-0.20
-0.25
12
25
50
Larval Density

Figure 5.1

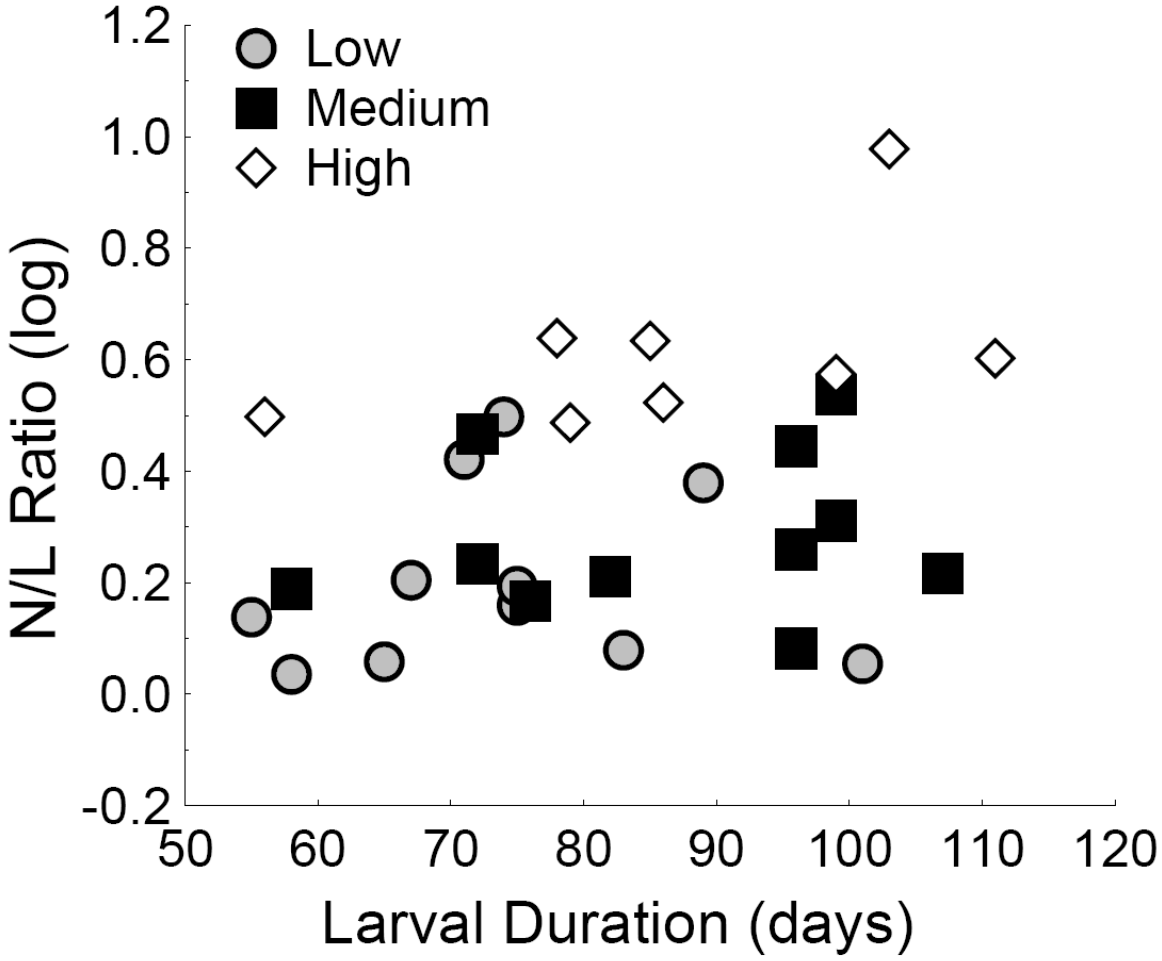
Low
Medium
High
N/L Ratio (log)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
50
60
70
80
90
100
110
120
Larval Duration (days)

Figure 5.2

CHAPTER 6

EFFECTS OF CHYTRIDIOMYCOSIS ON CIRCULATING WHITE BLOOD CELL DISTRIBUTIONS OF BULLFROG LARVAE (*RANA CATESBEIANA*)[1]

---

[1]Davis, A.K., Keel, M.K., Ferreira, A., and Maerz, J.C. 2010. *Comparative Clinical Pathology* 19: 49-55. Reprinted here with permission of publisher.

Abstract

Bullfrogs (*Rana catesbeiana*) are widely-believed to be non-clinical carriers of *Batrachochytrium dendrobatidis* (Bd), the fungal pathogen that invades keratinized tissues of amphibians and causes the disease, chytridiomycosis. Although most research on this disease focuses on adults, larval anurans are also susceptible to infections in their keratinized mouthparts, and this allows for visual diagnosis of the disease via the degree of mouthpart depigmentation. When an unplanned outbreak of chytridiomycosis occurred in a set of captive bullfrog tadpoles in our lab we conducted the current investigation into its effects on the non-specific immune system (i.e. the leukocyte populations) of the tadpoles. We compared leukocyte counts from blood smears of 27 tadpoles that had contracted the disease (evidenced by severe mouthpart depigmentation and confirmed by histology) to those of 21 tadpoles that had little depigmentation (i.e. with little evidence of the disease). Tadpoles with severe depigmentation had significantly more neutrophils and less eosinophils than those with little depigmentation, while numbers of lymphocytes, basophils and monocytes were not statistically different. That there was any effect at all on circulating leukocyte numbers is surprising since leukocytes are usually not seen migrating to sites of infection in tissue sections of amphibians infected with Bd, and since most research points to this disease having little outward effect on bullfrogs. Since monocyte numbers were unchanged, the leukocyte alterations were likely not due to a simple inflammation response. It is possible that Bd infections elicit increases in glucocorticoid hormones, which can cause increased numbers of circulating neutrophils and lower numbers of eosinophils, although this is often accompanied by a reduction in lymphocyte numbers, which we did not see. Further research is warranted to clarify if this effect is limited to this species.

Introduction

Of the myriad of diseases affecting animal populations world-wide, none may be as well-known as the amphibian disease, chytridiomycosis, which is caused by infection with the fungal pathogen, *Batrachochytrium dendrobatidis* (Bd) and is widely-believed to be responsible for massive population declines in tropical regions of the world (reviewed in Lips et al. 2005). Moreover, there is now evidence that temperate populations are also susceptible to this pathogen (Fellers et al. 2001, Muths et al. 2003, Green and Dodd 2007, Schlaepfer et al. 2007). However, it has also recently become clear that not all amphibian species are affected by the pathogen (Kriger and Hero 2006, Longcore et al. 2007), and that antimicrobial peptides in the skin of some species can inhibit the growth of Bd (Rollins-Smith and Conlon 2005, Woodhams et al. 2007). One well-known example of a species that appears unaffected by the disease is the American bullfrog (*Rana catesbeiana*), which has native and introduced populations world-wide. In fact, numerous researchers have speculated that bullfrogs may be non-clinical carriers, or 'resevoirs' of Bd, since the disease appears to have little effect on adults (Daszak et al. 2004, Hanselmann et al. 2004, Garner et al. 2006, Green and Dodd 2007). Furthermore, it is also thought that Bd does not negatively affect larval bullfrogs, since high numbers of infected larvae can be found in ponds with apparently stable populations (Peterson et al. 2007). In other species, the effects of Bd on anuran larvae have varied. Smith et al (2007) found no evidence of decreased growth in *Heleophryne natalensis* and *Strongylopus hymenopus* larvae infected with Bd. However, Parris and Cornelius (2004) showed that infections in larval stages cause increases in developmental instability of *Bufo fowleri* and *Hyla chrysoselis* metamorphs. Collectively though, most evidence points to few directly observable effects of Bd infections in anuran larvae, and especially

bullfrogs. Missing from this body of work though, are investigations into the range of possible physiological effects of Bd on anuran larvae.

One important physiological component that should be considered with infections is the effect on animals' immune systems. In particular, the non-specific immune system (i.e. the leukocyte, or white blood cell population) of any animal is one of the primary lines of defense against invading pathogens, and is made up of 5 different types of white blood cells, which each perform different tasks in the immune process (Jain 1986, 1993). Thus, by assessing the relative numbers of each cell type currently in circulation (i.e. the leukocyte profile of an animal), it is possible to determine if this component of the immune system has been activated in study subjects. For example, in most animals, infections cause increases in circulating numbers of neutrophils (or heterophils in birds and reptiles), which are the primary phagocytic cells that attack (i.e. engulf) foreign particles and organisms (Turner 1988, Jain 1993, Campbell 1995, Davis et al. 2004). Similarly, monocytes are also phagocytic and can increase in circulation during infections (Latimer et al. 1988, Davis et al. 2004, Thrall et al. 2006). Eosinophils are involved in modulation of the immune response by secretions of chemical substances that promote phagocytosis (Maxwell 1987, Rothenberg and Hogan 2006), though are not typically found in higher numbers in circulation during infections. Finally, infections can also lead to a general stress response, marked by increases in stress hormones (Lindström et al. 2005), and this hormonal increase can in turn lead to reductions in circulating numbers of lymphocytes, increases in numbers of neutrophils, and even reductions in eosinophils in some animals (reviewed in Davis et al. 2008a).

The effects of Bd on leukocyte populations has rarely been examined in amphibians, perhaps because histological sections of infected skin usually do not show leukocytes migrating

to sites of tissue infection (Berger et al. 2005a), and it is therefore assumed that the leukocyte response is minimal (Densmore and Green 2007). In the only study to examine this topic thus far, Woodhams et al. (2007) examined circulating numbers of leukocytes in post-metamorphic *Litoria chloris* and did find an effect of chytrid infection, but surprisingly, it was a reduced number of neutrophils (and also eosinophils) in infected individuals, which is not consistent with the classical immune response, *per se*, to a pathogen infection (typified by increased numbers of phagocytic cells).

While conducting a laboratory experiment involving late-stage bullfrog tadpoles in the summer of 2007, an outbreak of chytridiomycosis occurred in our captive population. While this unplanned outbreak disrupted our original plans for the tadpoles, it fortuitously allowed us to conduct an investigation into the effects of this disease on the leukocyte populations of bullfrog tadpoles, and we present the results of this investigation here. We compared leukocyte populations of tadpoles that had contracted the disease (based on visual assessment of mouthpart depigmentation) to those that had not yet become infected. While we examined all white blood cell types, we were particularly interested in numbers of neutrophils and monocytes, which when elevated is a sign of immune activation. We also looked for evidence of increases in stress hormones in the depigmented group, which would be primarily indicated by low numbers of lymphocytes combined with high numbers of neutrophils (Davis and Maerz 2008b, a, Davis et al. 2008a).

Methods

Lab setup. – As part of the initial experiment we hand captured late-stage bullfrog tadpoles from a local pond in Clarke County, GA in the spring of 2007 and brought them into captivity, where they were housed in 38 L aquaria in a temperature-controlled room set to 23°C. Thereafter they were fed ReptoMin turtle sticks *ad libitum*, and their water was changed weekly. At the same time, bullfrog tadpoles from a separate site, where there were known cases of chytridiomycosis, had also been captured and housed separately in the same room. At some point, water or equipment from these groups was unintentionally mixed, and the tadpoles in the initial set became exposed to the disease. When we discovered the outbreak in July 2007, we ceased the initial experiment and removed the tadpoles (n=55) to conduct the present study.

Processing tadpoles. – Tadpoles were anesthetized via immersion in a solution of 5% MS-222, then they were blotted dry, weighed, and their developmental stage was recorded following Gosner (1960). Next we examined the tadpoles for evidence of chytridiomycosis. Since Bd only attacks keratinized tissue of amphibians, the darkly-pigmented keratinized mouthparts of tadpoles are where the infection is typically found, with infections usually leading to depigmentation of the keratinized toothrows around the oral disc (Fig. 6.1). Because of this, many investigators have relied on the degree of tadpole mouthpart depigmentation as a proxy for infection with Bd (Fellers et al. 2001, Knapp and Morgan 2006, Symonds et al. 2007). We also used this approach and visually assessed the degree of mouthpart depigmentation based on a scoring system we devised that evaluated all keratinzed parts of the mouth (i.e. toothrows and beak). In this system an observer (AKD) separately scored the beak, upper, and all three lower toothrows of each tadpole on a 0 to 3 scale based on how much pigmentation was lost (3 being nearly all depigmented). Then the 5 separate scores were summed for each individual so that

each tadpole was assigned a single number that varied between 0 and 15, with 15 being the most depigmented. To be conservative, we then collapsed these into 5 'mouth score' groups: scores 0-2, 3-5, 6-8, 9-11, and 12-15, were groups 1 through 5, respectively. Finally, a blood sample was obtained from each tadpole via heart puncture and a standard blood film made on a clean microscope slide. Slides were air-dried and later stained with giemsa.

Histological examination.- As a check of the visual scoring method for diagnosing chytridiomycosis and to further elucidate the infection in tissues, a random subset (n=18) of tadpoles were preserved in formalin after processing and prepared for histological examination of keratinized mouthparts. To prepare slides, 5-µm-thick sections of paraffin-embedded tissues were rehydrated through graded alcohol and stained with hematoxylin and eosin (H&E). These tissue sections of mouthparts were examined by one observer (KK) and the degree of lesions was subjectively scored on a 0-3 scale, based on severity of erosions and loss of denticles.

Comparison of both scoring schemes from the subset of 18 tadpoles showed that tadpoles with visual mouthpart scores of 3 or higher were always assigned the maximum lesion score histologically (Fig. 6.2), so we therefore considered all individuals from the larger tadpole set with a 3 or more to be infected with chytridiomycosis (n=27). Those with a visual score of 2 in the subset (n=7) appeared to have variable histological scores, so we did not include any individuals with this visual score in our investigation. In general, tadpoles with the lowest visual scores (category 1, n=21) also had low histological lesion scores (average of 1 for histological scoring), though of the 9 tadpoles in this subset, 2 had evidence of light to moderate lesions in tissue sections (i.e. lesion scores 1 and 2) and 2 tadpoles showed heavy lesions (score 3). Thus, even though we observed very little depigmentation in the mouthparts of these two individuals, there was clear evidence of Bd infections from the tissue sections. This means that in our visual

scoring scheme for the entire set of tadpoles, we can not rule out the possibility that those with no mouthpart depigmentation (i.e. score 1) had active infections. However, since the majority of individuals in this category appeared free of infection, comparisons of the *mean* leukocyte parameters of tadpoles in this category versus the unquestionably infected tadpoles (score 3 or higher) are still valuable to help elucidate the effects of this disease.

Reading blood smears.- All slides were viewed with a standard light microscope under 1000X oil immersion, and leukocytes were counted (by AKD) following established procedures in our lab (Davis et al. 2008b, Davis and Maerz 2008b, a, Davis 2009). Leukocytes were identified as neutrophils, lymphocytes, eosinophils, basophils and monocytes, following Thrall et al. (2004), Hadji-Azimi et al. (1987) and Turner (1988). All leukocytes were counted until at least 100 leukocytes had been recorded, or when 150 fields of view had been examined. Only fields of view with even distributions of red blood cells were used. Fields of view in this study had an average of 30 red blood cells (+- 7, based on examination of 60 fields). The number of each cell type counted was then transformed into the number counted per 10 fields of view for analyses (which is equivalent to ~300 red blood cells).

Data analysis.- For each of the 5 cells types we log-transformed the number of cells per 10 fields of view to approximate normal distributions. To examine the effect of Bd infection on numbers of each cell type we used a MANCOVA design, where the log-transformed abundance of the cell type were the (5) response variables and infection was the fixed dichotomous independent variable (i.e. mouthparts severely depigmented vs little depigmentation). Since anuran white blood cell populations have recently been found to vary naturally throughout tadpole development (Davis 2009), Gosner stage was included as a continuous covariate. The analysis was conducted using Statistica 6.1 software (Statistica 2003).

Results

Histological Observations.- Microscopic examination of the subset of tadpoles with active infections in their mouthparts revealed erosion of the keratin layers and attenuation or complete loss of denticles (Fig. 6.3). Fungal elements were only present in keratin and areas lacking keratin did not have any active infection. Dermal infiltrates of lymphocytes were not correlated with the presence of Bd and were present at a low level in all tadpoles regardless of infection.

General Comparisons.- The mean Gosner stage of the 21 tadpoles with little mouthpart depigmentation was 34, which was not significantly different from the average stage of the 27 tadpoles with severe mouthpart depigmentation (two-sample t-test, t=0.988, df=46, p=0.328). There was also no difference in average mass of both groups (7.3g; two-sample t-test, t=1.67, df=46, p=0.100).

Leukocyte differentials.- The relative numbers of each leukocyte type for both groups of tadpoles are shown in Table 6.1. In general, tadpoles with severely depigmented mouthparts had relatively more abundant neutrophils than those with little depigmentation (16% versus 8% of all white blood cells). It is also interesting to compare the leukocyte profiles of the current study to those reported previously for larval (Davis 2009) and adult bullfrogs (Cathers et al. 1997). In general, there was concordance with most cells types (Table 6.1), though the relative number of monocytes in all tadpoles in this study (mean = 6.9%) appeared to be greater than normal (<1%).

Statistical Results.- The overall MANCOVA that considered the effects of infection on counts of all white blood cell types simultaneously showed the expected effect of Gosner stage ($F_{5,41}$=4.97, p=0.001), which is addressed in detail elsewhere (Davis 2009), but more importantly, a significant overall effect of Bd infection on leukocyte numbers ($F_{5,41}$=2.47,

p=0.048). To understand which cell types were most affected by Bd infection, the effects of infection on the abundance of individual cell types are presented graphically (Fig. 6.4), and we report results from follow-up models that considered individual cell types (with the same independent variables included). Lymphocyte numbers were unaffected by chytridiomycosis ($F_{1,45}$=0.013, p=0.908). The abundance of circulating neutrophils, however, significantly increased in the severely depigmented tadpoles ($F_{1,45}$=6.38, p=0.015; Fig. 6.4). In contrast, the numbers of circulating eosinophils tended to decrease with infection ($F_{1,45}$=4.36, p=0.042; Fig. 6.4). Finally, there was no difference in numbers of basophils ($F_{1,45}$=0.012, p=0.734) or monocytes ($F_{1,45}$=1.08, p=0.304) between infection groups (Fig. 6.4).

Discussion

The data from this study indicate that *B. dendrobatidis* infection in bullfrog larvae has an effect on their non-specific immune system (i.e. the numbers of circulating white blood cells). This effect is at least partly consistent with the reaction typical of most infections, which begins with an increase in phagocytic neutrophils (Stockham and Scott 2002, Thrall et al. 2006). These cells act to rid the blood of foreign bodies, including inert particles and microbial cells (Turner 1988). However, it is not clear what these cells are targeting in the chytrid infection, since most histological investigations, including our own (Fig. 6.3), do not show neutrophils (or any other leukocyte) migrating to the site of zoospore infection. To answer this question it is helpful to understand the normal course of chytridiomycosis infection in amphibian skin. Infections are typified by the presence of zoosporangia which live inside cells of the keratinized epidermal tissue of the host (mouthparts for anuran tadpoles, skin in adults) (Berger et al. 2005b). The

zoosporangia initially infect cells a few layers deep but eventually these cells move outward to the skin surface. Multiple zoospores develop within zoosporangia and when mature are released through discharge tubes which open to the surface of the animal. Importantly, this process has been shown to result in a degree of host tissue damage; in heavily infected animals, many epidermal cells become swollen, or necrotic, often with degenerate nuclei (Berger et al. 2005b). While it has not been specifically examined thus far, this type of tissue damage is sure to lead to cellular debris. It is therefore possible that the increase in numbers of circulating phagocytic neutrophils we observed in infected bullfrog tadpoles is a response to this tissue damage and cellular debris, not necessarily to the foci of infection *per se*. Berger et al. (2005b) also report that chytrid infections can lead to opportunistic entry of bacteria through the zoosporangia discharge tubes. These too would likely be targeted by neutrophils (Wright 2001).

There is an alternative explanation for the rise in neutrophil abundance, and which might also explain the decrease in eosinophils. Infections in animals can lead to chronically elevated stress hormones (Lindström et al. 2005), either as a direct result of the infection, or perhaps in this case, by the reduced capacity of the tadpoles to consume food (since infections damage tadpole mouthparts). Stress hormones are well-known to cause alterations in the normal distributions of white blood cells, and the primary cell affected is the neutrophil, which increases when hormones increase (reviewed in Davis et al. 2008a). In most, but not all cases, this increase is accompanied by a decrease in numbers of lymphocytes. These opposing effects are thought to 'redistribute' cells to where they would best be served in stressful situations (neutrophils in circulation, lymphocytes migrate into tissues) (Dhabhar et al. 1996). In some animals, stress can also cause a reduction in the numbers of circulating eosinophils (Jain 1986, Davis et al. 2008a), which was indeed seen here. Importantly, eosinophils are the primary cell responsible for

protections against metazoan parasites, and within amphibians, investigators have shown that experimental reductions in eosinophil numbers by administration of corticosterone leads to increased susceptibility to other biologically important pathogens such as trematodes (Belden and Kiesecker 2005). Interestingly, in the only other investigation of the effects of chytridiomycosis on amphibian white blood cells (adults in this case), a reduction in eosinophils was also found (Woodhams et al. 2007). Thus, given the proven role of eosinophils in defending against metazoan parasites, the combined evidence of this and the Woodhams et al. study strongly suggest that Bd infections could lead to increased host susceptibility to metazoan parasites.

In contrast to our study, Woodhams et al. (2007) observed reductions in neutrophil counts, while here, there was a general increase. These opposing results could be because larvae were examined here as opposed to later life stages in the Woodhams et al. study, or because different anuran species were examined in the two studies. In fact, this last point brings up an interesting question in itself; since chytridiomycosis does not appear to kill bullfrog tadpoles, could the immune response we observed be one of the reasons why? Comparisons of infected larvae of other amphibian species (such as those known to be susceptible to Bd) would no doubt clarify this issue.

In summary, comparison of leukocyte populations in bullfrog tadpoles with and without evidence of chytridiomycosis suggests the disease causes an alteration in the circulating complement of leukocytes in this species. This result is surprising, both because of the fact that Bd zoospores are not attacked by leukocytes, and given the otherwise benign effects chytridiomycosis has on this species. Further study is warranted to determine if this effect is limited to bullfrogs and if this response is what makes them resistant to this disease.

Acknowledgements

We thank the members of the herpetology lab for assistance with lab procedures. Kerry Holcomb and Andrew Grosse helped with tadpole collection. Statistical advice was provided by Sonia Altizer. AKD was supported by the Warnell School of Forestry and Natural Resources, at the University of Georgia. All procedures in this study were approved by the University of Georgia Animal Care and Use Committee.

Literature cited

Belden, L. K., and J. M. Kiesecker. 2005. Glucocorticosteroid hormone treatment of larval treefrogs increases infection by *Alaria* sp trematode cercariae. Journal of Parasitology 91: 686-688.

Berger, L., R. Speare, and L. F. Skerratt. 2005a. Distribution of *Batrachochytrium dendrobatidis* and pathology in the skin of green tree frogs *Litoria caerulea* with severe chytridiomycosis. Diseases of Aquatic Organisms 68: 65-70.

Berger, L., A. D. Hyatt, R. Speare, and J. E. Longcore. 2005b. Life cycle stages of the amphibian chytrid *Batrachochytrium dendrobatidis*. Diseases of Aquatic Organisms 68: 51-63.

Campbell, T. W. 1995. Avian Hematology and Cytology. Iowa State University Press, Ames.

Cathers, T., G. A. Lewbart, M. Correa, and J. B. Stevens. 1997. Serum chemistry and hematology values for anesthetized American bullfrogs (*Rana catesbeiana*). Journal of Zoo and Wildlife Medicine 28: 171-174.

Daszak, P., A. Strieby, A. A. Cunningham, J. E. Longcore, C. C. Brown, and D. Porter. 2004. Experimental evidence that the bullfrog (*Rana catesbeiana*) is a potential carrier of chytridiomycosis, an emerging fungal disease of amphibians. Herpetological Journal 14: 201-207.

Davis, A. K. 2009. Metamorphosis-related changes in leukocyte profiles of larval bullfrogs (*Rana catesbeiana*). Comparative Clinical Pathology 18: 181-186.

Davis, A. K., and J. C. Maerz. 2008a. Comparison of hematological stress indicators in recently captured and captive paedomorphic mole salamanders, *Ambystoma talpoideum*. Copeia 2008: 613-617.

Davis, A. K., and J. C. Maerz. 2008b. Sex-related differences in hematological stress indices of breeding, paedomorphic mole salamanders. Journal of Herpetology 42: 197-201.

Davis, A. K., K. C. Cook, and S. Altizer. 2004. Leukocyte profiles of House Finches with and without mycoplasmal conjunctivitis, a recently emerged bacterial disease. Ecohealth 1: 362-373.

Davis, A. K., D. L. Maney, and J. C. Maerz. 2008a. The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. Functional Ecology 22: 760-772.

Davis, A. K., N. E. Diggs, P. P. Marra, and R. J. Cooper. 2008b. Hematological stress indices show no effect of radio-transmitters on wintering Hermit Thrushes. Journal of Field Ornithology 79: 293-297.

Densmore, C. L., and D. E. Green. 2007. Diseases of amphibians. Institute for Laboratory Animal Research Journal 48: 235-254.

Dhabhar, F. S., A. H. Miller, B. S. McEwen, and R. L. Spencer. 1996. Stress-induced changes in blood leukocyte distribution - Role of adrenal steroid hormones. Journal of Immunology 157: 1638-1644.

Fellers, G. M., D. E. Green, and J. E. Longcore. 2001. Oral chytridiomycosis in the mountain yellow-legged frog (*Rana muscosa*). Copeia: 945-953.

Garner, T. W. J., M. W. Perkins, P. Govindarajulu, D. Seglie, S. Walker, A. A. Cunningham, and M. C. Fisher. 2006. The emerging amphibian pathogen *Batrachochytrium dendrobatidis* globally infects introduced populations of the North American bullfrog, *Rana catesbeiana*. Biology Letters 2: 455-459.

Gosner, K. L. 1960. A simplified table for staging anuran embryos and larvae. Herpetologica 16: 183-190.

Green, D. E., and C. K. Dodd. 2007. Presence of amphibian chytrid fungus, *Batrachochytrium dendrobatidis*, and other amphibian pathogens at warm-water fish hatcheries in southeastern North America. Herpetological Conservation and Biology 2: 43-47.

Hadji-Azimi, I., V. Coosemans, and C. Canicatti. 1987. Atlas of adult *Xenopus laevis laevis* hematology. Developmental and Comparative Immunology 11: 807-874.

Hanselmann, R., A. Rodriguez, M. Lampo, L. Fajardo-Ramos, A. A. Aguirre, A. M. Kilpatrick, J. P. Rodriguez, and P. Daszak. 2004. Presence of an emerging pathogen of amphibians in introduced bullfrogs *Rana catesbeiana* in Venezuela. Biological Conservation 120: 115-119.

Jain, N. C. 1986. Schalm's Veterinary Hematology. Lea & Febiger, Philadelphia.

Jain, N. C. 1993. Essentials of Veterinary Hematology. Blackwell Publishing, Philadelphia.

Knapp, R. A., and J. A. T. Morgan. 2006. Tadpole mouthpart depigmentation as an accurate indicator of chytridiomycosis, an emerging disease of amphibians. Copeia: 188-197.

Kriger, K. M., and J. M. Hero. 2006. Survivorship in wild frogs infected with chytridiomycosis. Ecohealth 3: 171-177.

Latimer, K. S., K. N. Tang, M. A. Goodwin, W. L. Steffens, and J. Brown. 1988. Leukocyte changes associated with acute inflammation in chickens. Avian Diseases 32: 760-772.

Lindström, K., D. Hawley, A. K. Davis, and M. Wikelski. 2005. Stress responses and disease in three wintering house finch (*Carpodacus mexicanus*) populations along a latitudinal gradient. General and Comparative Endocrinology 143: 231-239.

Lips, K. R., P. A. Burrowes, J. R. Mendelson, and G. Parra-Olea. 2005. Amphibian population declines in Latin America: A synthesis. Biotropica 37: 222-226.

Longcore, J. R., J. E. Longcore, A. P. Pessier, and W. A. Halteman. 2007. Chytridiomycosis widespread in anurans of northeastern United States. Journal of Wildlife Management 71: 435-444.

Maxwell, M. H. 1987. The avian eosinophil: a review. Worlds Poultry Science Journal 43: 190-207.

Muths, E., P. S. Corn, A. P. Pessier, and D. E. Green. 2003. Evidence for disease-related amphibian decline in Colorado. Biological Conservation 110: 357-365.

Parris, M. J., and T. O. Cornelius. 2004. Fungal pathogen causes competitive and developmental stress in larval amphibian communities. Ecology 85: 3385-3395.

Peterson, J. D., M. B. Wood, W. A. Hopkins, J. M. Unrine, and M. T. Mendonca. 2007. Prevalence of batrachochytrium dendrobatisis in American bullfrog and southern leopard

frog larvae from wetlands on the Savannah river site, South Carolina. Journal of Wildlife Diseases 43: 450-460.

Rollins-Smith, L. A., and J. M. Conlon. 2005. Antimicrobial peptide defenses against chytridiomycosis, an emerging infectious disease of amphibian populations. Developmental and Comparative Immunology 29: 589-598.

Rothenberg, M. E., and S. P. Hogan. 2006. The eosinophil. Annual Review of Immunology 24: 147-174.

Schlaepfer, M. A., M. J. Sredl, P. C. Rosen, and M. J. Ryan. 2007. High prevalence of *Batrachochytrium dendrobatidis* in wild populations of lowland leopard frogs *Rana yavapaiensis* in Arizona. Ecohealth 4: 421-427.

Smith, K. G., C. Weldon, W. Conradie, and L. H. du Preez. 2007. Relationships among size, development, and *Batrachochytrium dendrobatidis* infection in African tadpoles. Diseases of Aquatic Organisms 74: 159-164.

Statistica. 2003. Statistica version 6.1, Statsoft Inc.

Stockham, S. L., and M. A. Scott. 2002. Fundamentals of veterinary clinical pathology. Blackwell Publishing, Ames, Iowa USA.

Symonds, E. P., H. B. Hines, P. S. Bird, J. M. Morton, and P. C. Mills. 2007. Surveillance for *Batrachochytrium dendrobatidis* using *Mixophyes* (Anura: Myobatrachidae) larvae. Journal of Wildlife Diseases 43: 48-60.

Thrall, M. A. 2004. Hematology of amphibians. *In* M. A. Thrall, D. C. Baker and E. D. Lassen [eds.], Veterinary Hematology and Clinical Chemistry: Text and Clinical Case Presentations. Lippincott Williams & Wilkins, Philadelphia, PA.

Thrall, M. A., D. C. Baker, E. D. Lassen, T. W. Campbell, D. DeNicola, A. Rebar, M. J. Fettman, and G. Weiser. 2006. Veterinary hematology and clinical chemistry. Blackwell Publishing, Ames, Iowa USA.

Turner, R. J. 1988. Amphibians, pp. 129-209. *In* A. F. Rawley and N. A. Ratcliffe [eds.], Vertebrate blood cells. Cambridge University Press, Cambridge.

Woodhams, D. C., K. Ardipradja, R. A. Alford, G. Marantelli, L. K. Reinert, and L. A. Rollins-Smith. 2007. Resistance to chytridiomycosis varies among amphibian species and is correlated with skin peptide defenses. Animal Conservation 10: 409–417.

Wright, K. M. 2001. Amphibian hematology, pp. 129-146. *In* K. M. Wright and B. R. Whitaker [eds.], Amphibian medicine and captive husbandry. Krieger Publishing Company, Malabar, FL.

Table 6.1. Relative numbers (percentages of all cells) of each white blood cell type in bullfrog tadpoles with and without evidence of chytridiomycosis (i.e. mouthpart depigmentation). Means shown with standard deviations in parentheses.

Mouthparts	**% Lymphocytes**	**% Neutrophils**	**% Eosinophils**	**% Basophils**	**% Monocytes**
Little depigmentation (n=21)	68.7 (11.6)	8.2 (7.1)	2.5 (1.8)	4.1 (2.6)	8.2 (5.5)
Severe depigmentation (n=27)	57.4 (18.3)	16.3 (10.2)	1.3 (1.2)	2.9 (2.9)	5.9 (4.9)
All	62.3 (16.6)	12.7 (9.8)	1.8 (1.6)	3.4 (2.8)	6.9 (5.2)
Larval Reference Values*	73.0 (12.3)	20.8 (12.8)	3.6 (3.4)	2.3 (2.1)	0.3 (0.9)
Adult Reference Values**	62.9 (15.0)	22.0 (15.2)	8.9 (6.1)	2.5 (2.9)	0.6 (1.0)

* Data from Davis (2009) (Gosner stage 26-39)

** Data from Cathers et al. (1997).

CHAPTER 6 FIGURES

6.1 Bullfrog tadpole with mouthparts enlarged. This specimen has little depigmentation in its toothrows and beak.

6.2 Comparison of histological scoring of chytridiomycosis and visual assessment of mouthparts from 18 tadpoles (see methods). Mean histological scores (which ranged from 0-3) shown with standard deviations. High visual scores indicate severe mouthpart depigmentation.

6.3 Tissue section of mouthparts of a bullfrog larva infected with *Batrachochytrium dendrobatidis*. Note the numerous empty zoosporangia (arrows).

6.4 Average number of circulating white blood cells of each type in tadpoles with little mouthpart depigmentation (assumed to be not infected) and with severe depigmentation (indicating chytridiomycosis infection). Whiskers represent 95% confidence intervals.

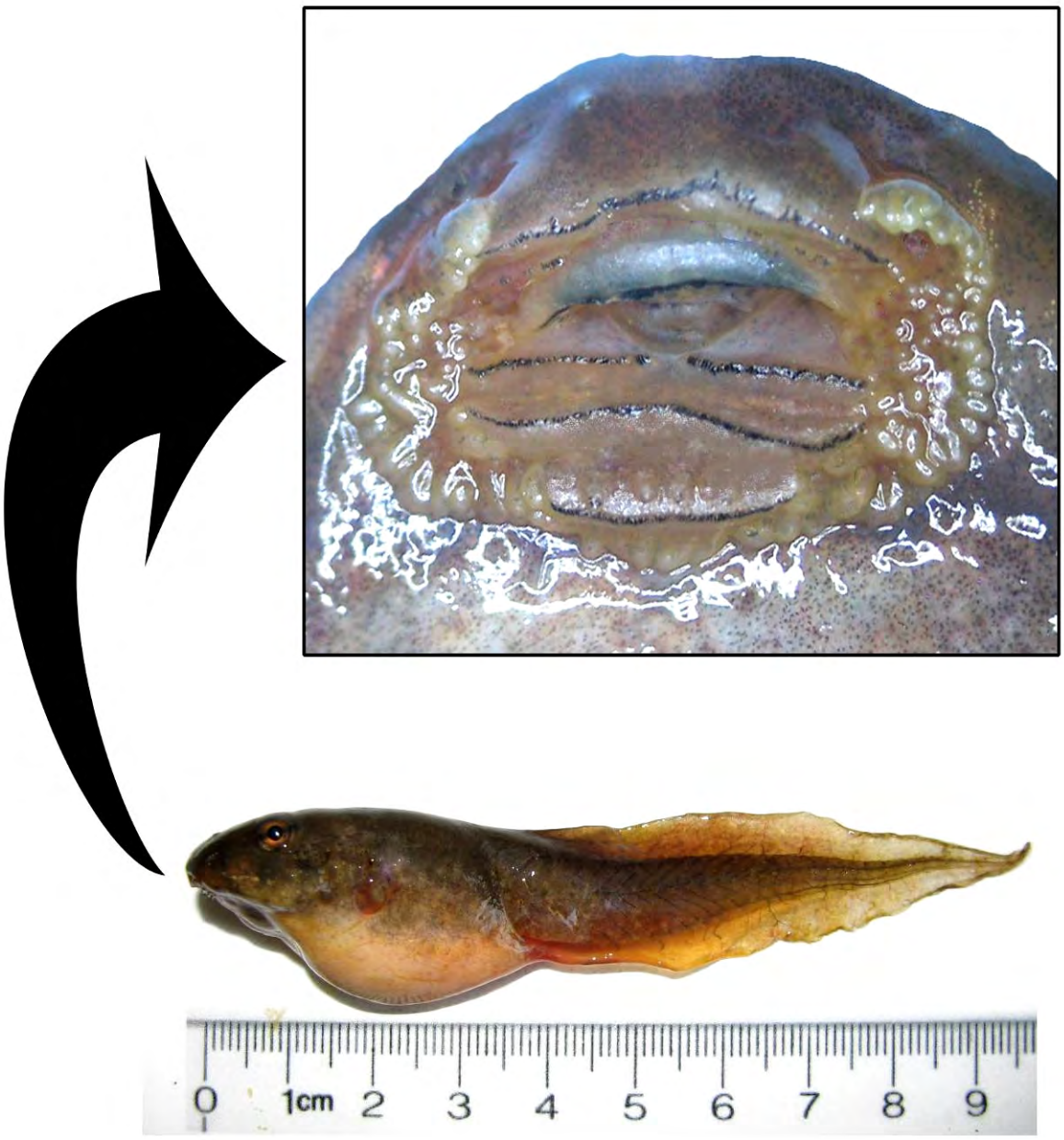

Figure 6.1

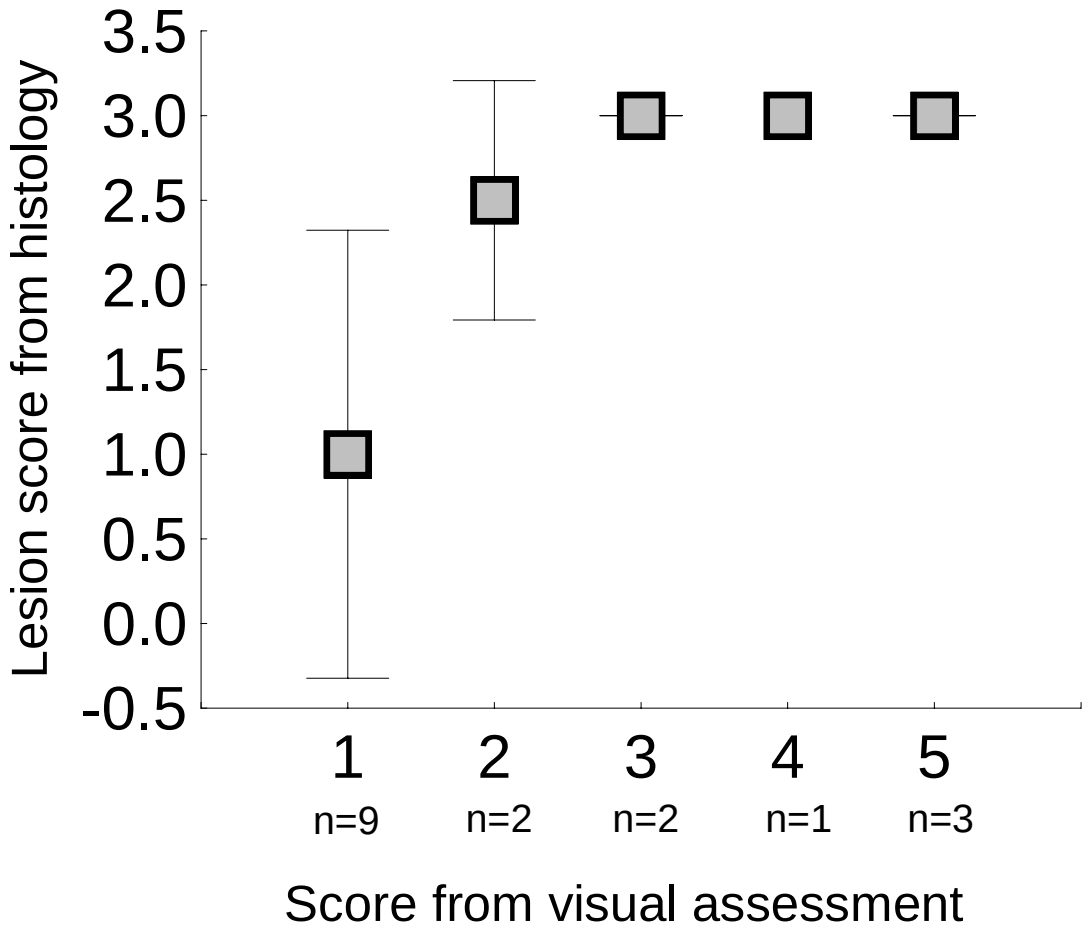
Lesion score from histology
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
1
2
3
4
5
n=9
n=2
n=2
n=1
n=3
Score from visual assessment

Figure 6.2

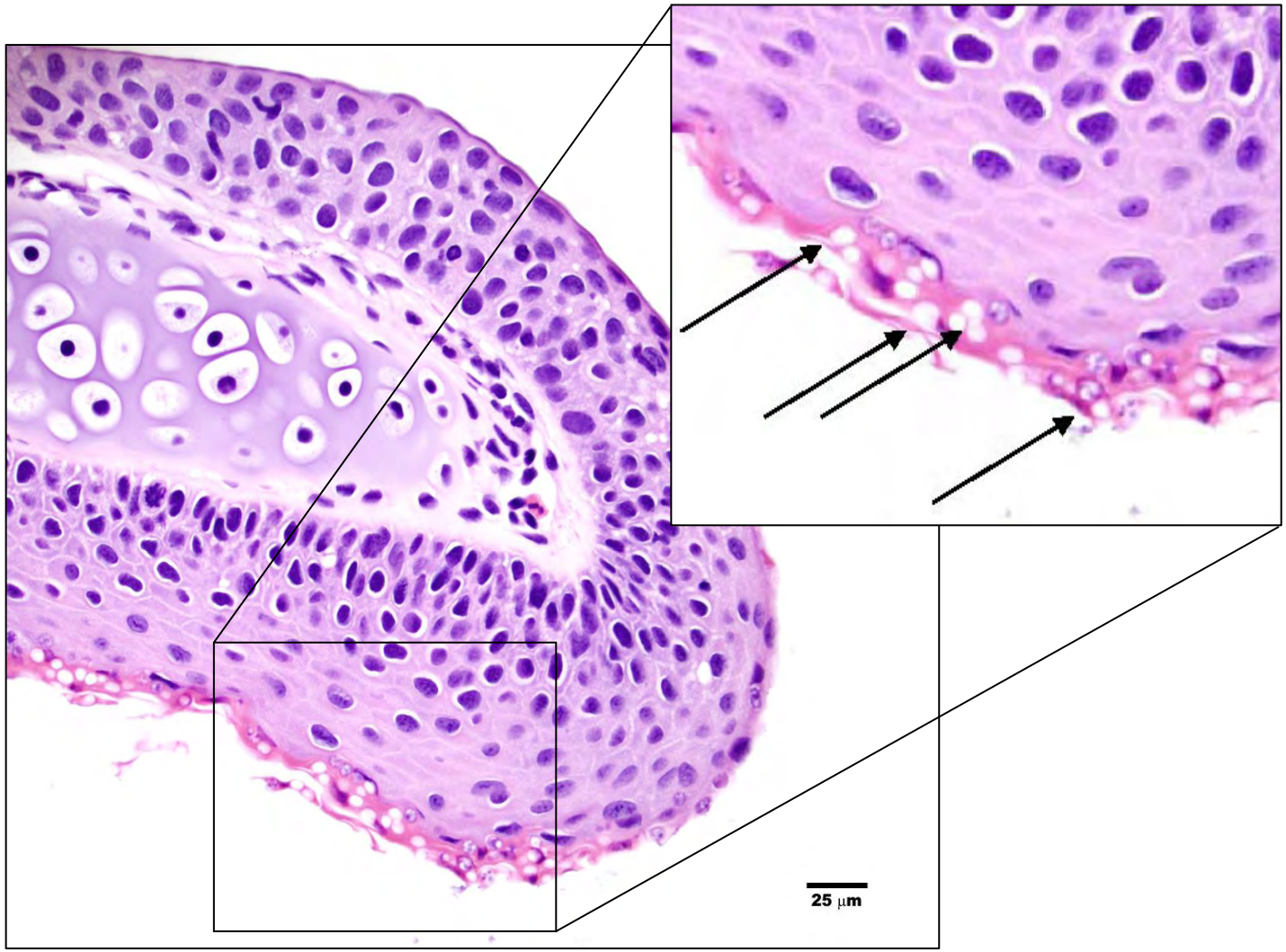

Figure 6.3

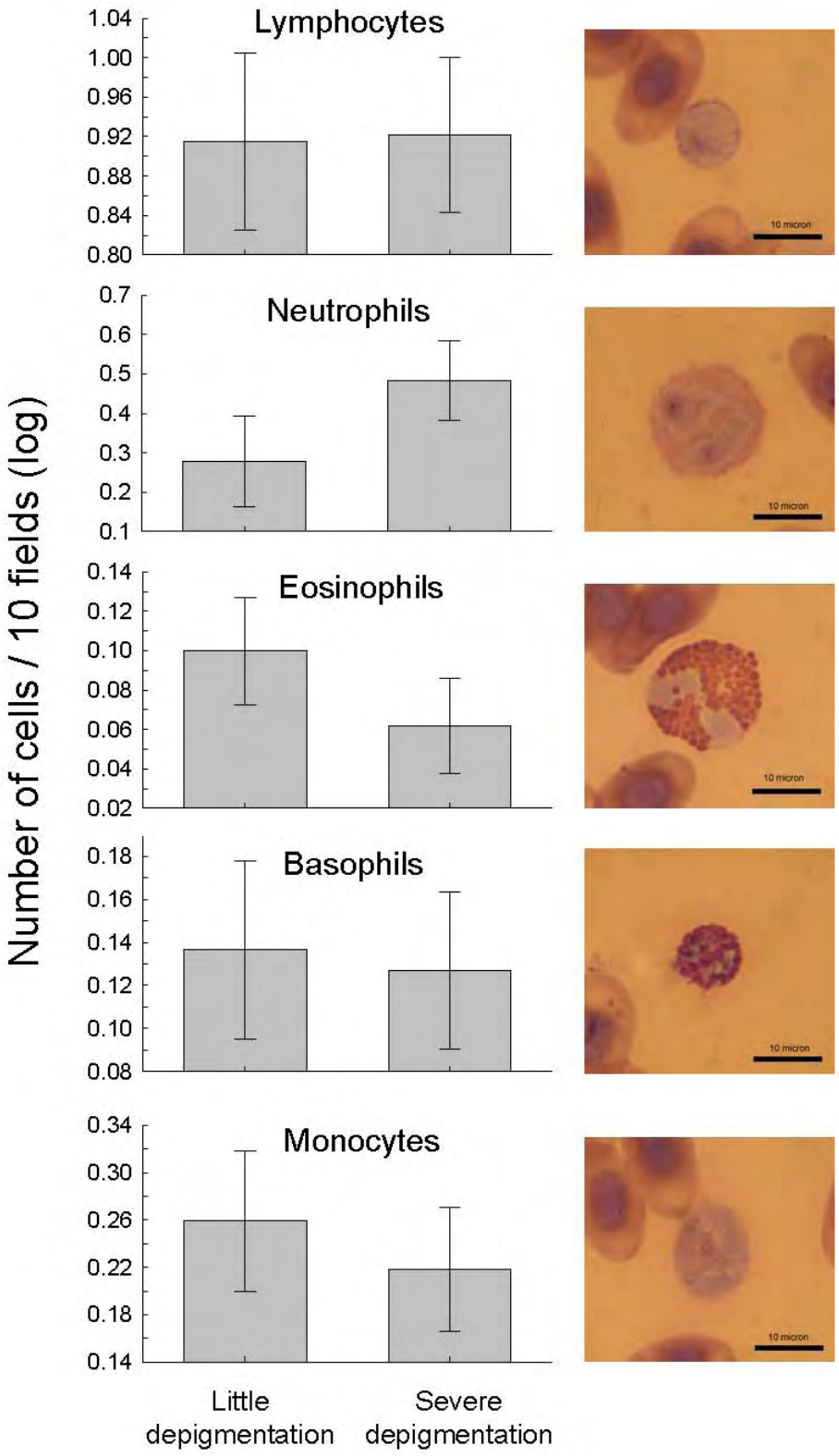
Lymphocytes
Neutrophils
Eosinophils
Basophils
Monocytes
Number of cells / 10 fields (log)
1.04
1.00
0.96
0.92
0.88
0.84
0.80
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.18
0.16
0.14
0.12
0.10
0.08
0.34
0.30
0.26
0.22
0.18
0.14
Little depigmentation
Severe depigmentation
10 micron

Figure 6.4

CHAPTER 7

ARE AMPHIBIANS REARED FOR CONSERVATION INITIATIVES CAPABLE OF MOUNTING A STRESS RESPONSE? AN EXPERIMENTAL ASSESSMENT USING LEUKOCYTE DATA[1]

[1]Davis, A.K. and J.C. Maerz. To be submitted to *Conservation Biology*.

Abstract

Global declines in amphibian populations have led to an increase in conservation initiatives focused on rearing amphibians in captivity to release into the wild. While this is often a logical course of action, there has been little work done to ascertain if captive-reared amphibians are functionally equivalent to their wild counterparts. In particular, compared to those in the wild, amphibians reared in captivity may develop in relatively stress-free environments, since they are usually fed *ad libidum*, raised in the absence of predators and pathogens and in controlled environments. If so, then are captive-reared individuals capable of reacting to normal stressors? We addressed this question by rearing two non-threatened amphibian species (*Rana sphenocephala* and *Ambystoma opacum*) from eggs and 10-day old larvae through to late larval stages in artificial pond environments, then subjecting the animals to a standard stress procedure. We performed the same procedure on wild-caught larvae of similar developmental stages and from the same source pond as the reared larvae. For all individuals we counted the number of two white blood cells, neutrophils and lymphocytes, from blood smears to derive individual neutrophil:lymphocyte (N:L) ratios, which prior research demonstrates can serve as an index of the level of corticosterone. Captive-reared *R. sphenocephala* and *A. opacum* each showed a threefold increase in N:L ratios from baseline to stress-induced levels, which was the same magnitude as wild individuals. Baseline and stress-induced N:L ratios of *R. sphenocephala* were statistically similar to wild individuals, although those of reared *A. opacum* were slightly higher than wild individuals. We conclude that captive-reared amphibian larvae are indeed capable of mounting an appropriate stress response, but further work may be needed to fine-tune rearing procedures of *Ambystoma* salamanders to minimize potential stressors.

Introduction

One of the most important issues in animal conservation today is the global decline in amphibian populations (e.g. Daszak *et al.*, 1999; Kriger & Hero, 2009; Lips *et al.*, 2005). While the cause of these declines may be varied and complex (e.g. D'Amen & Bombi, 2009; Kerby *et al.*, 2009; Rohr *et al.*, 2008), a common management response to these declines is to establish captive colonies or captive-rearing and reintroduction initiatives to save or help bolster declining populations (Gagliardo *et al.*, 2010; Griffiths & Pavajeau, 2008). In such programs, eggs from captive or wild amphibians are typically hatched in captivity and aquatic larvae are reared in containers through to metamorphosis, to ultimately be released to the wild or replaced back into the captive colony. While these actions are neccessary in the face of imminent population failures, few have asked if the larvae reared in such circumstances are functionally similar to their wild counterparts (but see Calisi & Bentley, 2009). Consider that when larvae are reared from an early age in captivity, they are usually raised in artificial ponds, mesocosms, or 'cattle tanks'. Generally these are structurally simple environments with no other species competitors, predators or pathogens present, and high quality food is readily available (usually *ad libidum*). While this is intended to maximize survival, and to ultimately provide the greatest number of metamorphosed animals, it must be remembered that these are all issues that wild larvae must contend with on a daily basis throughout their development. In other words, compared to their wild counterparts, captive-raised larvae are usually reared in relatively stress-free environments. If so, then would they be capable of dealing with normal stressors when they are released back into the wild? Put another way, do they develop normal physiological stress responses?

Like all vertebrates, amphibians have a physiological reaction to stressful events that develops at an early stage of life, and that begins with an increased production of glucocorticoid hormones (corticosterone in amphibians) in the bloodstream (Moore & Jessop, 2003). This hormone then orchestrates a cascade of events in the body that prepare the animal for coping with the stressor (reviewed in Wingfield & Romero, 2001). One of these events is a temporary redistribution of circulating leukocytes (white blood cells), which is thought to shunt certain cell types to where they would most be needed during the stress period (Dhabhar *et al.*, 1996; Dhabhar *et al.*, 1994). In this case, high levels of glucocorticoids cause an increase in the proportion of neutrophils and a decrease in the proportion of lymphocytes in circulation, such that the ratio of the two cells (neutrophil:lymphocyte, or N:L ratio) is positively associated with the magnitude of the hormonal increase (reviewed in Davis *et al.*, 2008b). For assessing the impact of stressful events in vertebrates, the N:L ratio (or H:L ratio in reptiles and birds, as the heterophil replaces the neutrophil in these animals), which is derived via cell counts from blood smears, can be used as an alternative to direct measurement of plasma corticosterone (e.g. Polo-Cavia *et al.*, 2010), and it is especially useful for studying small amphibians, since less blood is required and the time frame for obtaining baseline samples is on the order of hours for ectotherms (Aguirre *et al.*, 1995; Davis *et al.*, 2008b). Previously, we have shown that N:L ratios in amphibians increase when adults are brought into short-term captivity (Davis & Maerz, 2008a), when females are in reproductive status (Davis & Maerz, 2008b), when larvae are reared in high densities (Davis & Maerz, 2009), and when exogenous corticosterone is directly injected (Davis & Maerz, 2010). Collectively, these studies demonstrate the effectiveness of neutrophil:lymphocyte ratios in gauging the level of stress incurred by amphibians in a variety of contexts.

Here, we report the results of an experiment that utilizes this hematological approach to measuring stress and that was designed to ask if captive-reared amphibians are able to mount a stress response of equal magnitude as their wild-reared counterparts. Using two non-threatened amphibian species from the southeastern United States, southern leopard frogs (*Rana sphenocephala*) and marbled salamanders (*Ambystoma opacum*), we reared sets of larvae through to a late developmental stage (in a manner typical of conservation initiatives) and compared the magnitude of their baseline and stress-induced (i.e. after submitting the animals to a standardized stressor) N:L ratios to those of wild individuals. The results of this experiment should provide amphibian conservation practitioners with important information regarding the ability of captive-reared amphibians to cope with real-world stressors.

Methods

Rearing amphibians.- On January 16, 2009, we collected (via dipnetting) 150+ *A. opacum* larvae from an ephemeral pond near the University of Georgia campus in Athens, GA (Fig. 7.1A). This pond had been dry for most of the prior year, and had filled after heavy rains just two weeks prior to collecting these larvae (Davis, *pers. obs.*). Since females of this species lay eggs on the bottom of dry ponds and eggs hatch when the pond fills (Conant & Collins, 1998; Noble & Brady, 1933), we are confident that all collected larvae were approximately 10-14 days old. Collected larvae were returned to the lab and placed in two 40L aquaria for temporary storage. On January 24, the larvae were transferred to four 1000L UV-resistant polyethylene aquaculture tanks (Fig. 7.1B) that had each been filled with 900L of dechlorinated tap water and enough leaf litter to cover the bottom. Each bin received 25 larvae total, which based on prior

work with *Ambystoma* salamanders, is a moderately-low stocking density and should result in minimal crowding stress (Davis & Maerz, 2009). Moreover, prior study showed that higher densities than this lead to extremely low survival in *Ambystoma* larvae (i.e. <10%, Davis & Maerz, 2009). Bins were situated outdoors near our field lab, and were left uncovered.

On February 15, we collected 10+ clutches of *R. sphenocephala* eggs from the same pond the salamander larvae were collected from. Collected eggs were brought to the lab, where they were placed into aquaria filled with dechlorinated tap water (one clutch per aquarium) until hatching. Hatching of all eggs was completed by February 22. On March 2, we selected 5 of the most advanced clutches and haphazardly selected larvae, which were transferred to four additional 1000L bins. As before, bins had been filled to 900L with dechlorinated tap water and a layer of leaf litter covered their bottoms. Each bin received 24 larvae from each of the 5 clutches (120 total larvae per bin).This higher density of leopard frog larvae was based on prior studies in our lab that showed very high survival of this species (>80%) even at greater densities than this (Maerz, unpubl data).

All rearing bins were monitored daily and food was added weekly. Food for larval frogs consisted of Reptomin floating food sticks, in amounts proportional to the size of the larvae, and always enough to ensure their supply did not run out over the week. In other words, their food was provided *ad libidum*. Salamander larvae were fed zooplankton collected from nearby ponds via a plankton net, and by the natural colonization of insects such as mosquitoes. As with frog larvae, the amount of zooplankton provided was proportional to the size of the larvae, and we aimed to ensure the larvae were never without food. In the meantime, larval frogs and salamanders in the original pond were monitored weekly (via random dipnetting) to gauge their

developmental progress. Here we were only interested in determining when larvae were large enough to sample (below).

Sampling/Stress procedure.- On April 9, 50 larval *A. opacum* salamanders were collected from the original pond. At this time, larvae were approximately 5-7cm in length and had fully-formed gills (Fig. 7.2A). Larvae were immediately transported to the lab (10 minutes away). There they were divided into two groups of 25. The first group was processed immediately, which entailed euthanization via a 2% solution of neutral-buffered MS-222, weighing with an electronic balance, and obtaining a blood sample following Davis and Maerz (2008a; 2008b; 2009). Standard blood films were made for each salamander. We considered the data from these initial blood films to represent 'baseline' leukocyte data (see below), because the effects of capture, handling and transport are negligible on N:L ratios since the time lag between stress-induced glucocorticoid increase and subsequent leukocyte redistribution in amphibians and other ectotherms is typically 24 hours (Aguirre *et al.*, 1995; Bennett & Alspaugh, 1964; Bennett *et al.*, 1972; Bennett & Harbottle, 1968; Bennett & Newell, 1965), which leaves ample time for obtaining initial, 'baseline' blood samples.

Salamander larvae from the second group were placed individually into 1L plastic containers that had been filled with dechlorinated tap water (Fig. 7.2B). Then, each salamander was gently agitated by hand for 1 minute, similar to the 30-second 'chase' procedure used in Langkilde and Shine (2006) on captive skinks. Thereafter the salamanders were left undisturbed for 24 hours, which is enough time to allow glucocorticoid-induced changes to leukocytes (reviewed in Davis *et al.*, 2008b). After this time they were removed and processed following the procedure above. This group is hereafter referred to as the 'stressed' treatment. We point out here that the salamander larvae in this group no doubt perceived the capture alone as stressful

(Davis & Maerz, 2010) and as such it may not have been necessary to even perform the agitation procedure. However, since the object here was to elucidate the leukocyte 'stress' response of reared and wild larvae, we felt it necessary to ensure all larvae underwent an additional, standardized, stressor besides capture. Finally, the entire process was repeated for a second batch of 45 *A. opacum* larvae later that week, so that in the end, 95 wild salamander larvae were examined (45 larvae in the baseline group, 50 in the stressed group).

At the time the wild salamander larvae were examined, larvae in the rearing bins were not as developed, so we waited an additional 20 days for them to reach a similar size before we sampled the captive-reared larvae. On that day, all surviving salamander larvae from the rearing bins were removed and transported to the lab. There were 11, 20, 14, and 11 larvae from the four bins (56 total), and the larvae from each bin were divided into the two treatment groups, so that each bin was represented in both treatments (27 in the baseline group, 29 in the stressed group). The first group was processed immediately while the second underwent the stress procedure outlined above and processed 24 hours later.

On 19 April and 7 May, a total of 56 larval leopard frogs were collected from the same pond and brought to the lab where similar procedures were performed. At this time, most larvae were between Gosner (1960) stage 30-39. No larvae were beyond stage 39, so all were pre-metamorphosis. Over the two sampling periods, 30 of these larvae were examined immediately after arrival (i.e. for baseline samples), and 17 were subjected to the stress procedure (placement into individual containers, agitation for 1 minute, sampling 24 hours later). From 20-25 April, frog larvae from the rearing bins were collected for the same procedures. Since survival of leopard frog larvae was much higher than *Ambystoma* larvae (survival was between 77-83% in our four frog bins), we haphazardly selected 40 frog larvae from each bin for study. These larvae

ranged in Gosner stage from 29 to 35. Upon arrival to the lab, each set of 40 was divided equally into the two treatment groups and sampled accordingly. Certain larvae were processed but we were not able to obtain sufficient blood for a film. In the end, a total of 63 frog larvae were processed and sampled in the baseline group, while 74 were sampled in the stressed group.

Leukocyte counting.- Blood films from frog and salamander larvae were examined with a standard light microscope under 1000X (oil), following procedures used previously (Davis, 2009a; Davis *et al.*, 2004; Davis *et al.*, 2008a). Only fields of view with relatively uniform cell distributions were examined, and all leukocytes within each field were identified as neutrophils, lymphocytes, eosinophils, basophils and monocytes following Hadji-Azimi et al. (1987) and Thrall (2004). At least 100 leukocytes were counted for each individual, and from these data we calculated the percentage of all cell types, although we focus here on neutrophils and lymphocytes. Neutrophil:lymphocyte ratios for all individuals were calculated based on the percentages of both cell types.

Data analyses.- Neutrophil:lymphocyte ratios were log-transformed (+1) to approximate a normal distribution. There was a small degree of variation in N:L ratios of frog larvae among bins, though this was not significant at the 0.05 level (one-way ANOVA, $F_{3,133}$=2.3, p=0.080), and there was no effect of bin on salamander N:L ratios ($F_{3,52}$=0.49, p=0.692). We therefore pooled the data from all bins for salamanders and similarly for frogs in further analyses. Then, to address the objective of this study (do captive-reared larvae differ in stress response from wild), we examined transformed N:L ratios of salamanders and frogs using ANCOVA, where the larval environment (reared or wild) and sample treatment (baseline or stressed) were categorical explanatory variables, while body mass was a covariate. We also included all two-way interaction terms in the initial models, and selected the final model that best described the data

from all candidate models using AIC values. All analyses were conducted using Statistical 6.1 software (Statistica, 2003).

Results

The overall leukocyte profiles of *A. opacum* and *R. sphenocephala* differed considerably, especially with respect to the relative abundance of circulating neutrophils and to a lesser extent, circulating lymphocytes. Baseline data from wild-caught larval marbled salamanders showed an average neutrophil percentage of 13.6% (±9.6% SD) and an average of 61.0% (±13.8% SD) lymphocytes, which is within the range reported in most other amphibians (Davis & Durso, 2009). Meanwhile, the average percentage of neutrophils and lymphocytes in wild-caught, unmanipulated, *R. sphenocephala* larvae was 5.2% (± 4.5% SD) and 77.0% (±11.3% SD), respectively. This especially-low abundance of neutrophils in leopard frog larvae, led to much lower overall N:L ratios of larval leopard frogs than marbled salamanders.

The ANCOVA model that best described the variation in N:L ratios of larval marbled salamanders (i.e. with the lowest AIC score) was one that contained the larval environment (wild or reared), sample treatment (baseline or stressed) and their interaction, which was not significant (Table 7.1A). Body size was not included in the final model. The final model showed that regardless of rearing environment, salamanders that underwent the stress treatment had significantly higher N:L ratios than the baseline group (Fig. 7.3A). Further, salamanders reared in captivity had slightly, but significantly, higher N:L ratios than those from the wild (Table 7.1A, Fig. 7.3A).

The model that best described the N:L ratio data for larval leopard frogs contained the sample treatment (baseline or stressed) and body mass, but not rearing environment (Table 7.1B). Thus, there was no improvement in our ability to predict N:L ratios of leopard frog larvae when the larval environment (wild or reared) was included in the final model. As we expected, frog larvae subjected to the stress procedure had significantly higher N:L ratios than did those in the baseline group (Fig. 7.3B). The effect of body mass was negative, such that larger individuals tended to have lower N:L ratios, although this effects appeared to be driven mostly by the data from the stress group (Fig. 7.4). In other words, larger larvae appeared to have a smaller leukocyte response to the stress treatment than did smaller larvae.

Discussion

The results from this experiment show that the leukocyte response of captive-reared leopard frog larvae to a stressor is equivalent to that of their wild counterparts. In other words, captive-reared leopard frogs appeared capable of mounting a normal physiological stress response, which for all vertebrates typically involves increases in the proportion of circulating neutrophils and decreases in proportions of circulating lymphocytes (reviewed in Davis *et al.*, 2008b). We are confident in this result since both the wild-caught and captive-reared larvae were of similar developmental stages, and both groups ultimately originated from the same pond. We only examined one species of frog, so generalizing to all species is premature; however, if the results can be generalized, this would speak to the suitability of conservation-related captive-rearing initiatives for producing frogs that have normal physiological reactions to stressful events.

While tangential to the goals of this study, we also discovered a slight, but significant negative relationship with body size and N:L ratio in leopard frog larvae (Fig. 7.4). Since we had only intended to statistically *control* for possible effects of variation in body size, we did not have any *a priori* predictions regarding the direction of the effect, if any. There is in fact, little research into the relationship between body size and stress physiology in any animal, let alone amphibians. Nevertheless, it appears that larger larvae had lower N:L ratios. Given that all larvae hatched at roughly the same time (within a few days), this pattern may indicate a correlation between rapid growth and lower circulating stress hormones as indicated by N:L ratios. Similar relationships have been reported for nestling birds, where low ratios were associated with fast growth (Moreno *et al.*, 2002).

Results concerning N:L ratios of larval marbled salamanders differed somewhat from those of frog larvae, and in an unexpected direction. Our analysis of these data suggest that although the captive-reared larvae appeared capable of mounting a stress response of a similar *magnitude* as their wild-reared counterparts (i.e. their average N:L ratios increased threefold from the baseline to the stress group, as did the wild group), their overall N:L ratios were slightly, but significantly *higher*, not lower, than those from the wild. This was especially pronounced in the baseline group (Fig. 7.3A). In fact, comparison of only baseline N:L ratios of captive-reared versus wild salamander larvae showed a significant difference (two-sample t-test, $t=3.21$, $df=70$, $p=0.002$), but a similar comparison of stress-induced N:L ratios showed no significant difference ($t=0.83$, $df=77$, $p=0.408$). Thus, rather than being 'less stressed' in the presumably benign rearing environment, captive-reared salamander larvae had *higher* resting stress levels than normal.

Interestingly, this is not the first case of higher-than-normal resting stress levels found in mesocosm-reared *Ambystoma* salamanders. In a recent study examining the effects of larval density on *A. maculatum*, we found that even in the lowest density used in the experiment (12 larvae initially in 1000L bins), salamanders emerged with an average resting N:L ratio of 0.57, which is nearly twice as high as typical ratios of wild-caught *Ambystoma* salamanders, which tend to be close to 0.30 (Davis, 2009b). Furthermore, while there are no published comparisons of actual corticosterone level differences between reared and wild salamanders, a recent doctoral project did address this using *A. jeffersonianum* (Chambers, 2009). In this case, late-stage, mesocosm-reared larvae were found to have moderately elevated resting corticosterone levels compared to wild larvae. Meanwhile, stress-induced levels in both groups were equivalent (and much higher), consistent with the present study.

Apparently, there is something about the captive-rearing environment that larval *Ambystoma* salamanders perceive as 'mildly stressful'. One possibility is that due to their carnivorous feeding behavior, they are simply aggressive in nature toward each other in captivity. Support for this idea comes from an earlier study examining effects of rearing density on rates of tail and appendage injuries of *A. talpoideum* (Semlitsch & Reichling, 1989). Even in the lowest density used in that study, at least 20% of larvae had injuries caused by other larvae, and the authors noted considerable rates of intraspecific predation and cannabalism. Indeed, even in our experiment, we initially placed 25 larvae into each bin (100 total), but after nearly 4 months, only 56 were left. It is possible that some of these larvae were cannibalized by other larvae. It may be that when in captivity and when there are few other stimuli present, these salamanders act aggressively toward each other, but in the wild, where there is a greater variety of stimuli and prey items, they do not. Whatever the reason, this is an issue that must be

considered in conservation-related initiatives involving rearing of similar species, especially those that are carnivorous and might display some degree of intraspecific aggression.

Despite the mildly-increased basal stress level of the captive-reared salamanders, it is important to remember that their *response* to stress (i.e. the difference between the basal and stress-induced N:L ratios) was of equal magnitude as their wild counterparts, which was the original question in this study. Thus, both species examined here appeared to show 'normal' physiological reactions to stress when reared in captivity, which supports the use of captive-rearing for conservation initiatives. However, we do suggest that future research efforts should be aimed at identifying factors that help to minimize stress levels during larval rearing, especially for species that may naturally be aggressive. Given the increasing reliance on captive-rearing and captive-breeding programs to protect declining or endangered amphibian species, this issue should be given a high priority.

Acknowledgements

We thank the many graduate students and field assistants in the Maerz lab for their assistance in the field. We also thank Joe Milanovich for his help with agitating the salamanders. AKD was supported by a grant from the Morris Animal Foundation (#D08ZO-409). All procedures in this study were approved by the University of Georgia Animal Care and Use Committee (AUC #A2010 2-015).

Literature cited

Aguirre, A. A., G. H. Balazs, T. R. Spraker, and T. S. Gross. 1995. Adrenal and hematological responses to stress in juvenile green turtles (*Chelonia mydas*) with and without fibropapillomas. Physiological Zoology 68: 831-854.

Bennett, M. F., and J. K. Alspaugh. 1964. Some changes in the blood of frogs following administration of hydrocortisone. The Virginia Journal of Science 15: 76-79.

Bennett, M. F., and N. C. Newell. 1965. A further study of the effects of hydrocortisone on the blood of frogs. The Virginia Journal of Science 16: 128-130.

Bennett, M. F., and J. A. Harbottle. 1968. The effects of hydrocortisone on the blood of tadpoles and frogs, *Rana catesbeiana*. Biological Bulletin 135: 92-95.

Bennett, M. F., C. A. Gaudio, A. O. Johnson, and J. H. Spisso. 1972. Changes in the blood of newts, *Notophthalmus viridescens*, following administration of hydrocortisone. Journal of Comparative Physiology A 80: 233-237.

Calisi, R. M., and G. E. Bentley. 2009. Lab and field experiments: Are they the same animal? Hormones and Behavior 56: 1-10.

Chambers, D. M. 2009. Abiotic factors underlying stress hormone level variation among larval amphibians. Doctoral Dissertation. Virginia Polytechnic Institute and State University.

Conant, R., and J. T. Collins. 1998. A Field guide to reptiles and amphibians of Eastern and Central North America (Peterson Field Guide Series). Houghton Mifflin Company, New York.

D'Amen, M., and P. Bombi. 2009. Global warming and biodiversity: Evidence of climate-linked amphibian declines in Italy. Biological Conservation 142: 3060-3067.

Daszak, P., L. Berger, A. Cunningham, H. AD, G. DE, and S. R. 1999. Emerging infectious diseases and amphibian population declines. Emerging Infectious Diseases 5: 735-748.

Davis, A. K. 2009a. The Wildlife Leukocytes Website: The ecologist's source for information about leukocytes of wildlife species. www.wildlifehematology.uga.edu.

Davis, A. K. 2009b. Metamorphosis-related changes in leukocyte profiles of larval bullfrogs (*Rana catesbeiana*). Comparative Clinical Pathology 18: 181-186.

Davis, A. K., and J. C. Maerz. 2008a. Comparison of hematological stress indicators in recently captured and captive paedomorphic mole salamanders, *Ambystoma talpoideum*. Copeia 2008: 613-617.

Davis, A. K., and J. C. Maerz. 2008b. Sex-related differences in hematological stress indices of breeding, paedomorphic mole salamanders. Journal of Herpetology 42: 197-201.

Davis, A. K., and J. C. Maerz. 2009. Effects of larval density on hematological stress indices in salamanders. Journal of Experimental Zoology Part A-Ecological Genetics and Physiology 311A: 697-704.

Davis, A. K., and A. M. Durso. 2009. Leukocyte differentials of northern cricket frogs (*Acris c. crepitans*) with a compilation of published values from other amphibians. Herpetologica 65: 260-267.

Davis, A. K., and J. C. Maerz. 2010. Effects of exogenous corticosterone on circulating leukocytes of a salamander (*Ambystoma talpoideum*) with unusually abundant eosinophils. International Journal of Zoology 2010. DOI:10.1155/2010/735937.

Davis, A. K., K. C. Cook, and S. Altizer. 2004. Leukocyte profiles of House Finches with and without mycoplasmal conjunctivitis, a recently emerged bacterial disease. Ecohealth 1: 362-373.

Davis, A. K., D. L. Maney, and J. C. Maerz. 2008a. The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. Functional Ecology 22: 760-772.

Davis, A. K., N. E. Diggs, P. P. Marra, and R. J. Cooper. 2008b. Hematological stress indices show no effect of radio-transmitters on wintering Hermit Thrushes. Journal of Field Ornithology 79: 293-297.

Dhabhar, F. S., A. H. Miller, B. S. McEwen, and R. L. Spencer. 1996. Stress-induced changes in blood leukocyte distribution - Role of adrenal steroid hormones. Journal of Immunology 157: 1638-1644.

Dhabhar, F. S., A. H. Miller, M. Stein, B. S. McEwen, and R. L. Spencer. 1994. Diurnal and acute stress-induced changes in distribution of peripheral blood leukocyte subpopulations. Brain Behavior and Immunity 8: 66-79.

Gagliardo, R., E. Griffith, R. Hill, H. Ross, J. R. Mendelson, E. Timpe, and B. Wilson. 2010. Observations on the captive reproduction of the horned marsupial frog *Gastrotheca cornuta* (Boulenger 1898). Herpetological Review 41: 52-58.

Gosner, K. L. 1960. A simplified table for staging anuran embryos and larvae. Herpetologica 16: 183-190.

Griffiths, R. A., and L. Pavajeau. 2008. Captive breeding, reintroduction, and the conservation of amphibians. Conservation Biology 22: 852-861.

Hadji-Azimi, I., V. Coosemans, and C. Canicatti. 1987. Atlas of adult *Xenopus laevis laevis* hematology. Developmental and Comparative Immunology 11: 807-874.

Kerby, J. L., K. L. Richards-Hrdlicka, A. Storfer, and D. K. Skelly. 2009. An examination of amphibian sensitivity to environmental contaminants: are amphibians poor canaries? Ecology Letters 13: 60-67.

Kriger, K. M., and J. M. Hero. 2009. Chytridiomycosis, amphibian extinctions, and lessons for the prevention of future panzootics. Ecohealth 6: 6-10.

Langkilde, T., and R. Shine. 2006. How much stress do researchers inflict on their study animals? A case study using a scincid lizard, *Eulamprus heatwolei*. Journal of Experimental Biology 209: 1035-1043.

Lips, K. R., P. A. Burrowes, J. R. Mendelson, and G. Parra-Olea. 2005. Amphibian population declines in Latin America: A synthesis. Biotropica 37: 222-226.

Moore, I. T., and T. S. Jessop. 2003. Stress, reproduction, and adrenocortical modulation in amphibians and reptiles. Hormones and Behavior 43: 39-47.

Moreno, J., S. Merino, J. Martinez, J. J. Sanz, and E. Arriero. 2002. Heterophil/lymphocyte ratios and heat-shock protein levels are related to growth in nestling birds. Ecoscience 9: 434-439.

Noble, G. K., and M. K. Brady. 1933. Observations on the life history of the marbled salamander, *Ambystoma opacum* Gravenhorst. Zoologica 11: 89-132.

Polo-Cavia, N., T. Engstrom, P. López, and J. Martín. 2010. Body condition does not predict immunocompetence of western pond turtles in altered versus natural habitats. Animal Conservation Online Early - DOI: 10.1111/j.1469-1795.2009.00329.x.

Rohr, J. R., T. R. Raffel, J. M. Romansic, H. McCallum, and P. J. Hudson. 2008. Evaluating the links between climate, disease spread, and amphibian declines. Proceedings of the National Academy of Sciences of the United States of America 105: 17436-17441.

Semlitsch, R. D., and S. B. Reichling. 1989. Density-dependent injury in larval salamanders. Oecologia 81: 100-103.

Statistica. 2003. Statistica version 6.1, Statsoft Inc.

Thrall, M. A. 2004. Hematology of amphibians. *In* M. A. Thrall, D. C. Baker and E. D. Lassen [eds.], Veterinary Hematology and Clinical Chemistry: Text and Clinical Case Presentations. Lippincott Williams & Wilkins, Philadelphia, PA.

Wingfield, J. C., and L. M. Romero. 2001. Adrenocortical responses to stress and their modulation in free-living vertebrates, pp. 211-234. *In* B. S. McEwen and H. M. Goodman [eds.], Handbook of physiology, section 7: The endocrine system, Vol. IV: Coping with the environment: neural and endocrine mechanisms. Oxford University Press, New York.

Table 7.1. Final ANCOVA models explaining variation in neutrophil:lymphocyte ratios (response variable) of larval marbled salamanders and southern leopard frogs examined in this study. Models shown reflect those with the lowest AIC values of all candidate models (see methods).

A. Marbled Salamanders

Explanatory Variable	df	MS	F	p
Baseline/Stressed	1	1.32	71.55	0.0000
Reared/Wild	1	0.08	4.50	0.0356
1*2	1	0.01	0.46	0.5008
Error	147	0.02		
Total	150			

B. Leopard Frogs

Explanatory Variable	df	MS	F	p
Baseline/Stressed	1	0.06	33.03	0.0000
Wt	1	0.01	4.69	0.0316
Error	181	0.00		
Total	183			

CHAPTER 7 FIGURES

7.1 Ephemeral pond in northeast Georgia where larval *Ambystoma opacum* and *Rana sphenocephala* were collected (A) and 1000L UV-resistant polyethylene aquaculture tanks used to raise larvae (B).

7.2 Photograph of a marbled salamander larva collected from the study pond in mid-April 2009 (left) and stress procedure setup (right). Salamanders were gently agitated by hand for 1 minute after placement into individual containers, then sampled 24 hours later to determine neutrophil:lymphocyte ratios.

7.3 Average neutrophil:lymphocyte ratios of reared and wild larval marbled salamanders (A) and southern leopard frogs (B), examined either immediately after capture ('Baseline') or 24hours after stress procedure ('Stressed'; see methods). Error bars represent 95% confidence intervals.

7.4 Relationship between body mass and neutrophil:lymphocyte ratios in larval southern leopard frogs in this study, in both the baseline and stressed groups.

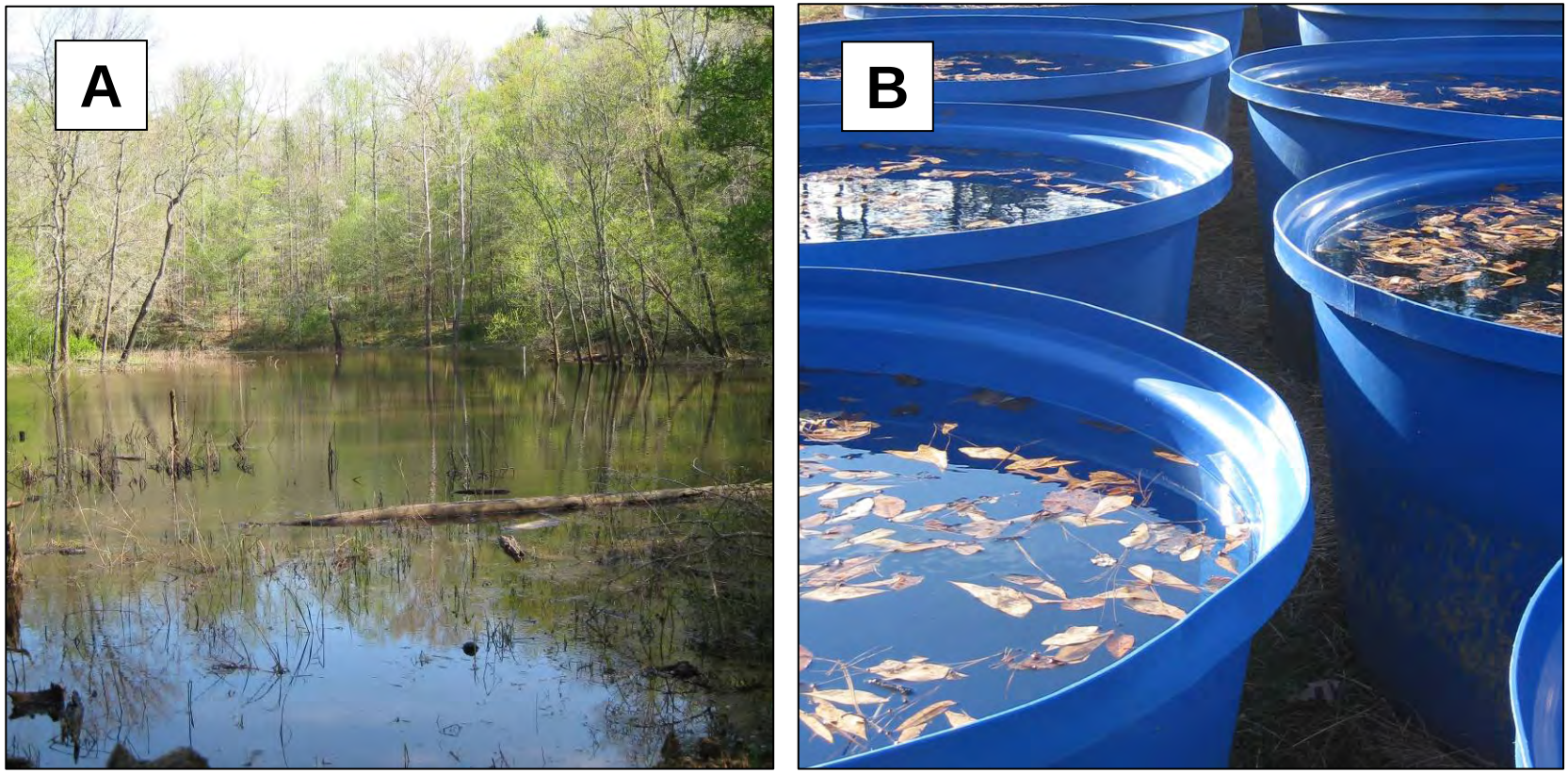

Figure 7.1

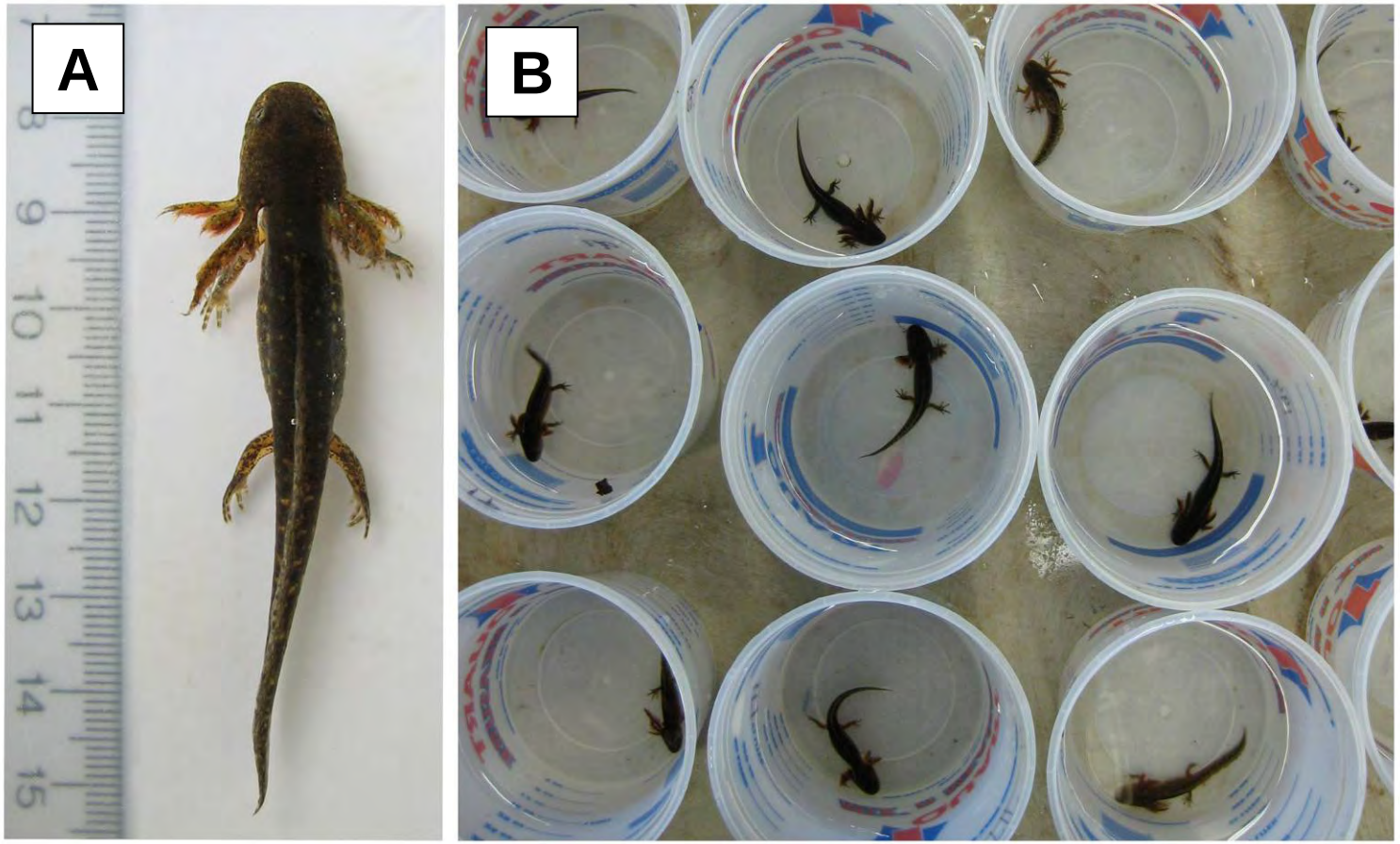

Figure 7.2

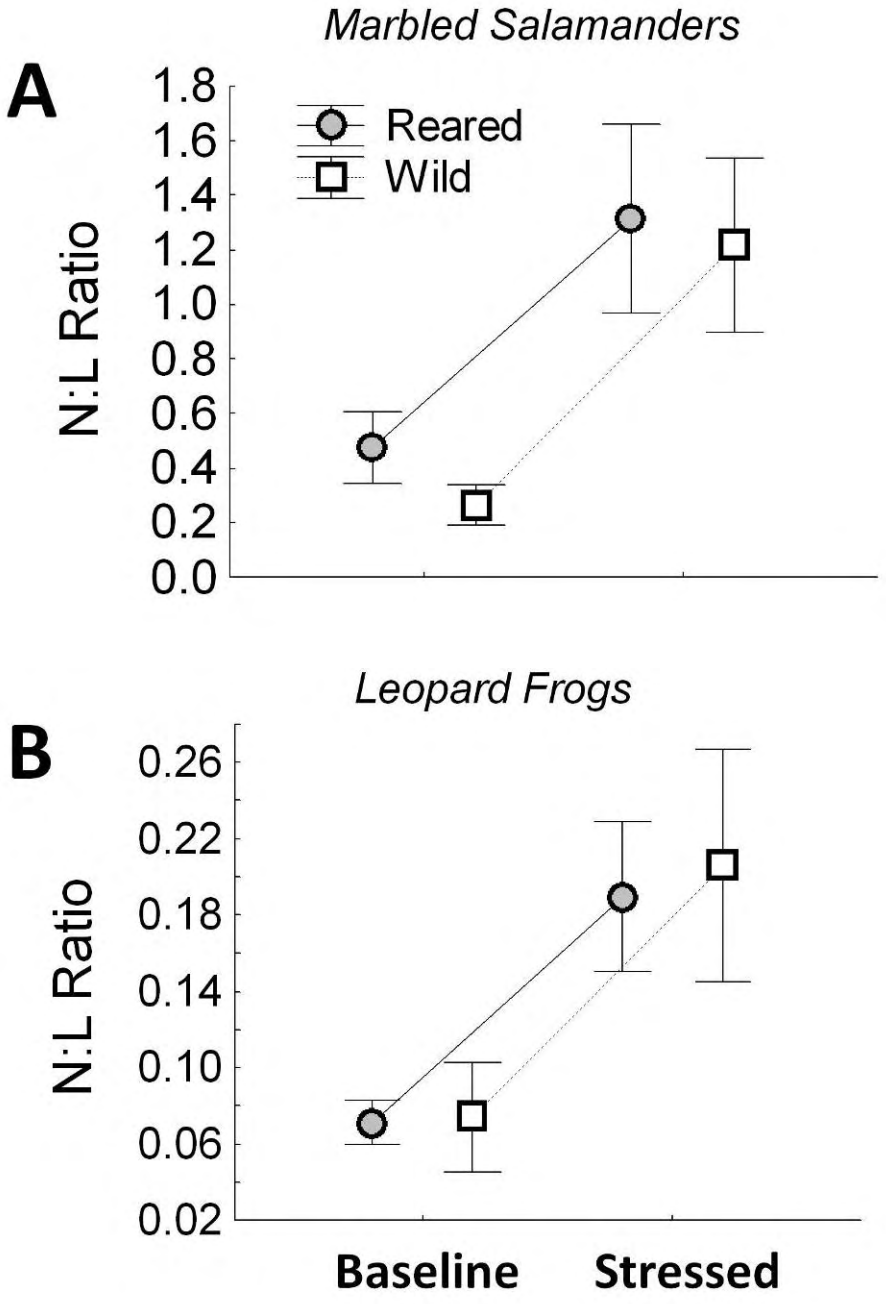
Marbled Salamanders
A
N:L Ratio
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Reared
Wild
Leopard Frogs
B
N:L Ratio
0.26
0.22
0.18
0.14
0.10
0.06
0.02
Baseline
Stressed

Figure 7.3

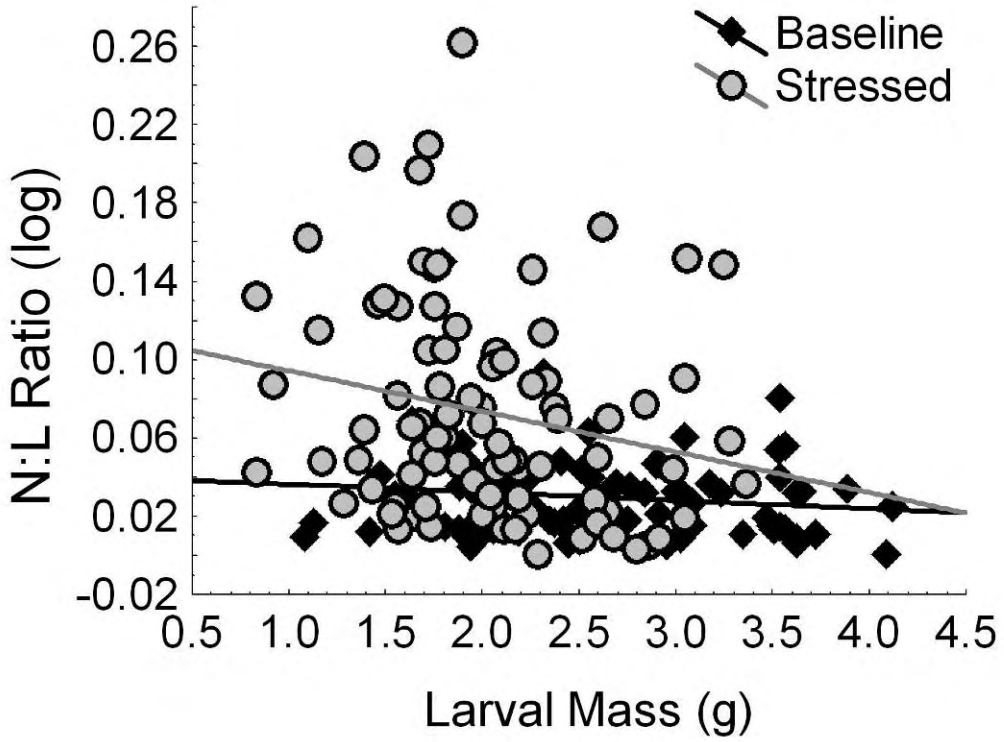
Baseline
Stressed
0.26
0.22
0.18
0.14
0.10
0.06
0.02
-0.02
N:L Ratio (log)
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Larval Mass (g)

Figure 7.4

CHAPTER 8

CONCLUDING REMARKS

One of the overarching goals of this entire body of work has been to solidify and establish the concept and methodology of 'hematological stress markers' in the scientific community. This goal was set early by the author and his advisor, primarily in response to many reviewer comments of initial manuscripts and grants, as well as several critical discussions with colleagues. It is the author's hope that this thesis, as well as several related projects that were completed during his doctoral program, accomplished that goal, and in fact, parts of this work (such as the experimental administration of stress hormone – Chapter 3) were directly in response to these critiques. However, it should be pointed out that certain chapters in this thesis, along with the related manuscripts not appearing here (Davis and Maerz 2008, Davis 2009, Davis and Durso 2009, Davis and Milanovich 2010), still encountered resistance during the review stage (some were rejected from initial journals), and the author still occasionally receives some critical comments about this line of research despite the publication of these chapters and other manuscripts, although thankfully, these comments are now becoming fewer. Because of the persistent nature of these criticisms, however, and the fact that they all appear to revolve around similar themes, it is worthwhile to discuss these themes here.

There appear to be two main groups of individuals who remain resistant to using N:L or H:L ratios to index levels of stress in animals. The first appear to be ecologists who either still favor the use of direct hormone assays, despite their extreme time-sensitivity, or who do not work on stress-related questions, and know only of the conventional, direct measurement,

approach. For example, the larval density paper (Chapter 5) was initially rejected by Canadian Journal of Zoology with the following reviewer comments:

"This paper by Davis and Maerz describes experiments that tested the hypothesis that crowding in salamanders leads to alterations in the immune system as measured by changes in blood neutrophil:lymphocyte ratios. The authors assume that such changes are caused by elevations in stress hormones, although this was not directly tested."

"The authors do not provide support that elevated plasma stress hormone concentrations played a role in this high mortality or the altered N:L ratios. It is also not explained why in the medium density treatment, if so-called 'stress levels' were higher than the low density treatment, that this is not reflected in the N:L ratio. Whether elevated corticosterone was cause or consequence (if there was indeed elevations in corticosterone) was not tested." This issue of 'validation' was one of the most frequent criticisms of this entire body of work (not just Chapter 5), appearing in many manuscript and grant proposal reviews. Usually, the reviewer was unaware (or skeptical) that stress could be measured by any other means than by direct sampling of hormones in blood, and they wanted to know if this 'new' technique has been 'validated' using controlled experiments. I was constantly frustrated by this comment, since I was aware of numerous studies (although some were older) that had indeed done this, and I also knew that this technique is frequently used by modern ornithologists who do not present 'validation' data with every manuscript written that uses H:L ratios. This ultimately led to the review paper (Chapter 2) where these many studies were highlighted, although these comments still persisted somewhat after that paper was published. To further address this problem, I also created a website (see the Appendix of this thesis) where these numerous validation studies (38 so far) are listed online, and I frequently tell reviewers to view it (usually in response to comments about 'validation').

And, the experimental administration of corticosterone (Chapter 3) was also in response to these criticisms. Furthermore, this paper went through several overhauls in an attempt to make it novel, since this idea had been experimentally tested so often before(!). That is why it was finally accepted after its focus was shifted to the eosinophil and the effects of stress hormones on that cell, rather than the N:L ratio *per se*. In fact, one reviewer of that paper actually wrote *"I find it hard to argue that we need yet another study demonstrating that stressed vertebrates have a shift in the N:L ratio."* Thus, I am pleased to see that at least there has been *some* momentum gained on this issue of validation.

The other group that remains critical of this research tend to be veterinarians, no doubt because the utilization of white blood cell counts here is similar to that used in veterinary health-screenings of animals. The comments from this group generally fall into three categories, the first of which is criticizing the lack of 'total white blood cell counts' in the projects where relative cells counts were obtained, like those in this thesis (although estimated absolute counts *were* obtained in all chapters). This is exemplified by the following response to a related paper that the author initially submitted to a veterinary journal, Journal of Zoo and Wildlife Medicine (which was later successfully submitted to Herpetologica - Davis and Durso 2009): *"Two of the three reviewers found the lack of total white blood cell counts made this article unusable in clinical medicine. Additionally, the term absolute counts is misused throughout the article. Absolute counts are measured in cells per microliter and require that the relative count (%) be multiplied by the WBC counts to produce an absolute differential cell count. This is what is typically medically used."* For some reason, the veterinarians I speak to and communicate with seem adamant that all hematological investigations MUST use this total white blood cell technique and no other. This may stem from the idea that by doing so one can compare results

from one study/species to another on a cells-per-volume basis. Or it is because only the volume of cells per liter is useful in clinical settings, which was pointed out by reviewer above, who made multiple references to *clinical medicine*. Perhaps this last statement is the most poignant and highlights the major difference between taking white blood cell data from blood smears, as was done in this thesis, and conducting veterinary-type health screenings of animals. In the former, the data on cell counts is estimated (which is all that is necessary for this work), and in the latter, it is absolute.

The second category of criticisms by veterinarians relates to the issue of statistical inference. These involve questions about sampling animals, such as the comment from the same paper above (Davis and Durso 2009): *"How do the investigators avoid bias in sampling? Perhaps all frogs caught were ill?"* This seems like an issue that could be raised for all studies everywhere that involve capturing wild animals, not just those conducted in this thesis. While the criticism is valid (it may indeed be possible that all animals captured were sick, although unlikely), I would argue that this is why statistics must be used to evaluate patterns in data, and that inferences be drawn on comparisons of means between treatment groups. Another comment from veterinarians is *"How can you use H:L ratios to tell if a single bird is stressed?"* The answer to this is you cannot, nor has it ever been advocated in the studies by the author. Again, all of the studies in this thesis and in this entire line of research rely on comparisons of the mean N:L ratios across groups of animals.

The last criticism by veterinarians of this line of research is the idea that other things besides stress hormones can cause changes to neutrophil or lymphocyte levels in circulation, such as infections or diseases. This is indeed a valid point, and it *is* well-known that infections and inflammation cause increases in the phagocytic cells – heterophils and monocytes (Latimer

et al. 1988, Jain 1993, Davis et al. 2004, Thrall et al. 2006), and an infected animal would no doubt have an increased H:L ratio. However, it is also true that infections can cause increases in stress hormones, either as a result of the infection itself (i.e. to stimulate the immune system) or the symptoms of the infection (Lindström et al. 2005). This was seen in Chapter 6, where results showed that infections with chytridiomycosis alters counts of white blood cells in anuran tadpoles a manner similar (although not entirely) to that of stress. This can be taken to mean that the tadpoles were stressed because the chytrid infection damaged their mouthparts and they could not eat properly (the argument we preferred), or that the infection caused alterations to the host immune system and redistributed the cells to fight the infection. Clearly, the two processes (infection and stress) are tightly linked – everyone knows that when humans get stressed they tend to get sick – so it is indeed difficult to separate out the effects of infection from the effects of stress in the studied conducted here, and it *is* possible that some animals sampled for the current work were fighting unseen infections which altered their cell counts. However, once again, this issue is really a statistical one. By sampling groups of animals, and basing all conclusions on comparisons of *mean* N:L ratios between treatment groups (which was done in all projects here), the effect of a few animals having infections would only cause increased variation within the treatment groups.

A final area of recurring contention (or perhaps frustration?) with this collective work is the difficulty faced in convincing colleagues that it has conservation applications. This fact is emphasized by the recent rejection (an editor's decision to decline to review) of Chapter 7 from *Animal Conservation* (and later *Conservation Biology*). The editor's comments stated *"The Editors are particularly looking for rigorous quantitative studies of an empirical or theoretical nature, which may relate to populations, species or communities and their conservation. I have*

enjoyed reading your paper, but I do not feel that it meets with the criteria required by the Editors." This casual and perhaps indifferent (or maybe ignorant?) attitude towards this paper, which clearly DOES have conservation importance to amphibians, is one that is taken by many and underscores the work that still remains to convince these individuals otherwise. This may be an issue that is faced by many of my colleagues with aspects of their own research, although it does at times seem like an uphill battle for this line of research. It is, however, encouraging to see several recent studies and review articles now in print that highlight the importance of stress physiology in animal conservation in general (Reeder and Kramer 2005, Rodl et al. 2007, Franceschini et al. 2008, Busch and Hayward 2009). With these publications becoming more frequent, and with more and more ecologists considering the importance of stress physiology in their work, the emerging field of 'conservation physiology' (Wikelski and Cooke 2006) may soon become commonplace and a rewarding field of study.

Literature Cited

Busch, D. S., and L. S. Hayward. 2009. Stress in a conservation context: A discussion of glucocorticoid actions and how levels change with conservation-relevant variables. Biological Conservation 142: 2844-2853.

Davis, A. K. 2009. Metamorphosis-related changes in leukocyte profiles of larval bullfrogs (*Rana catesbeiana*). Comparative Clinical Pathology 18: 181-186.

Davis, A. K., and J. C. Maerz. 2008. Sex-related differences in hematological stress indices of breeding, paedomorphic mole salamanders. Journal of Herpetology 42: 197-201.

Davis, A. K., and A. M. Durso. 2009. Leukocyte differentials of northern cricket frogs (*Acris c. crepitans*) with a compilation of published values from other amphibians. Herpetologica 65: 260-267.

Davis, A. K., and J. R. Milanovich. 2010. Lead-phase and red-stripe color morphs of red-backed salamanders (*Plethodon cinereus*) differ in hematological stress indices: a consequence of differential predation pressure? Current Zoology 56: 238-243.

Davis, A. K., K. C. Cook, and S. Altizer. 2004. Leukocyte profiles of House Finches with and without mycoplasmal conjunctivitis, a recently emerged bacterial disease. Ecohealth 1: 362-373.

Franceschini, M. D., D. I. Rubenstein, B. Low, and L. M. Romero. 2008. Fecal glucocorticoid metabolite analysis as an indicator of stress during translocation and acclimation in an endangered large mammal, the Grevy's zebra. Animal Conservation 11: 263-269.

Jain, N. C. 1993. Essentials of Veterinary Hematology. Blackwell Publishing, Philadelphia.

Latimer, K. S., K. N. Tang, M. A. Goodwin, W. L. Steffens, and J. Brown. 1988. Leukocyte changes associated with acute inflammation in chickens. Avian Diseases 32: 760-772.

Lindström, K., D. Hawley, A. K. Davis, and M. Wikelski. 2005. Stress responses and disease in three wintering house finch (*Carpodacus mexicanus*) populations along a latitudinal gradient. General and Comparative Endocrinology 143: 231-239.

Reeder, D. M., and K. M. Kramer. 2005. Stress in free-ranging mammals: Integrating physiology, ecology, and natural history. Journal of Mammalogy 86: 225-235.

Rodl, T., S. Berger, L. M. Romero, and M. Wikelski. 2007. Tameness and stress physiology in a predator-naive island species confronted with novel predation threat. Proceedings of the Royal Society B-Biological Sciences 274: 577-582.

Thrall, M. A., D. C. Baker, E. D. Lassen, T. W. Campbell, D. DeNicola, A. Rebar, M. J. Fettman, and G. Weiser. 2006. Veterinary hematology and clinical chemistry. Blackwell Publishing, Ames, Iowa USA.

Wikelski, M., and S. J. Cooke. 2006. Conservation physiology. Trends in Ecology & Evolution 21: 38-46.

APPENDIX

Listing of published and unpublished leukocyte profiles of amphibian species. Data shown here are also presented in an online resource created by the author, the 'Wildlife Leukocytes Website', www.wildlifehematology.uga.edu. This website contains information for researchers who are interested in using, or are just beginning to use, hematological techniques in their work. Users can learn how to make blood smears, identify white blood cells and view blood cell data from birds, amphibians and reptiles. In addition, there is a table listing the publications on the effects of stress on leukocytes of vertebrates.

Screenshot:

www.wildlifehematology.uga.edu

The table below lists the leukocyte profiles (percentage of cells) for amphibians from published and unpublished sources. The mean percentage of each cell type (lymphocytes, neutrophils, eosinophils, basophils and monocytes) is given, along with the neutrophil/lymphocyte ratio, which is useful to gauge the stress level of the animals (see review paper in further reading page). Reference range of N/L ratios is shown below table. If the study was an experiment, only the values from control animals is listed. References are listed below the table.

Species	Age	N	Status*	Lymph	Neut	Eosin	Baso	Mono	N/L	Source
Hyla versicolor	Adult	11	W	83.3	9.4	2.6	3.2	1.4	0.13	Davis, unpubl. data
Osteopilus septentrionalis	Adult	7	C	63.6	24.6	6.2	1.5	4.2	0.39	Robert Huran's Study
Acris c. crepitans	Adult	79	W	68.3	22.4	1.6	5.0	2.7	0.33	Davis and Durso 2009
Rana catesbeiana	Adult	14	C	62.9	22.0	8.9	2.5	0.6	0.35	Cathers et al. 1997
Rana catesbeiana	Adult	302	C	26.8	60.9	5.8	3.5	2.9	2.27	Coppo et al. 2005
Rana catesbeiana	Larvae	40	W	73.0	23.8	3.6	2.3	0.3	0.33	Davis 2009
Rana catesbeiana	Adult	7	W	67.4	8.8	11.6	10.5	1.8	0.13	Davis 2009
Rana pipiens	Adult	50	C	53.4	26.5	7.3	4.4	11.0	0.50	Rouf 1969
Rana pipiens	Adult	18	C	55.4	11.3	10.1	19.2	4.1	0.20	Maniero and Carey 1997
Rana pipiens	Adult	14	C	25.4	61.8	7.0	1.8	5.2	2.43	Bennett and Alspaugh 1964
Rana pipiens	Adult	93	U	64.5	20.7	8.0	6.7	0.0	0.32	Kaplan 1952

Rana sphenocephala	Adult	69	C	63.6	13.8	4.2	12.4	5.9	0.22	Davis, unpubl. data
Rana sphenocephala	Adult	61	C	60.7	23.9	2.0	9.9	3.5	0.39	Davis, unpubl. data
Rana esculenta	Adult	*	U	75.2	17.1	5.7	1.9	0.0	0.23	Jordan 1938
Rana esculenta	Adult	136	W	57.6	15.2	14.4	12.4	0.5	0.26	Romanova and Romanova 2003
Rana clamitans	Adult	35	W	66.0	16.0	17.0	1.0	1.0	0.24	Shutler et al 2009
Bufo arenarum	Adult	12	C	60.9	27.3	3.7	3.8	1.7	0.45	Chiesa et al. 2006
Bufo arenarum	Adult	24	W	64.0	20.9	13.7	0.0	1.3	0.33	Cabagna et al. 2005
Bufo americanus	Adult	27	W	20.0	68.0	3.3	7.4	1.5	3.40	Forbes et al. 2006
Bufo americanus	Adult	50	W	75.9	7.0	5.9	10.5	0.6	0.09	Davis and DeVore, unpubl. data
Bufo alvarius	Adult	*	W	37.0	48.0	9.0	1.0	5.0	1.30	Cannon and Cannon 1979
Bufo fowleri	Adult	6	W	72.3	8.5	8.5	9.8	0.8	0.12	Davis unpubl. data
Bufo vulgaris	Adult	*	U	73.0	18.3	1.3	6.7	0.0	0.25	Jordan 1938
Bombina bombina	Adult	31	W	51.6	25.0	3.9	7.6	12.6	0.48	Wojtaszek and Adamowicz 2003
Glyphogloossus molossus	Adult	18	W	41.6	26.3	1.1	8.3	22.7	0.63	Ponsen et al. 2008
Xenopus laevis	Adult	10	C	30.1	26.5	1.2	40.5	1.6	0.88	Hadji-Azimi et al. 1987
Cryptobranchus alleganiensis	Adult	9	W	66.5	20.7	4.0	0.0	9.3	0.31	Jerrett and Mays 1973
Cryptobranchus alleganiensis	Adult	18	W	60.7	25.9	4.9	0.0	8.4	0.43	Jerrett and Mays 1973

Cryptobranchus alleganiensis	Adult	22	W	37.5	43.5	14.0	4.2	0.1	1.16	Solis et al. 2007
Cryptobranchus alleganiensis	Adult	11	W	35.5	42.5	18.5	3.2	0.2	1.20	Solis et al. 2007
Cryptobranchus alleganiensis	Adult	31	W	49.5	33.0	11.5	5.1	0.8	0.67	Solis et al. 2007
Cryptobranchus alleganiensis	Adult	14	W	48.0	36.5	3.3	11.4	0.9	0.76	Solis et al. 2007
Notophthalmus viridescens	Adult	120	C	63.5	24.3	6.2	3.2	2.8	0.38	Bennett and Daigle 1983
Notophthalmus viridescens	Adult	13	C	55.6	37.6	1.8	0.8	4.2	0.68	Bennett and Johnson 1973
Notophthalmus viridescens	Adult	70	W	68.2	23.5	0.9	2.2	5.2	0.34	Davis and Erickson, unpubl. data
Taricha granulose	Adult	13	C	88.0	7.0	2.0	1.0	1.0	0.08	Friedmann 1970
Triton cristatus	Adult	*	U	42.9	52.2	3.7	1.2	0.0	1.22	Jordan 1938
Cynops pyrrhogaster	Adult	23	C	3.0	28.0	4.0	57.0	6.0	9.33	Pfeiffer et al. 1990
Plethodon cinereus	Adult	63	W	65.0	21.7	3.6	8.8	0.8	0.33	Davis and Milanovich 2010
Plethodon cinereus	Adult	25	W	59.6	35.9	1.5	2.1	1.0	0.60	Davis unpubl. data
Eurycea cirrigera	Adult	5	W	70.9	19.3	1.0	2.9	5.9	0.27	Davis and Milanovich 2010
Eurycea wilderae	Adult	11	W	68.7	22.3	1.5	5.0	2.4	0.32	Davis and Milanovich 2010
Ambystoma maculatum	Adult	1	C	51.1	19.6	19.6	9.8	0.0	0.38	Davis, unpubl. data
Ambystoma maculatum	Adult	10	C	31.7	18.1	25.5	24.2	0.6	0.57	Davis & Maerz In Press

Ambystoma maculatum	Larvae	4	C	51.7	14.1	26.9	6.8	0.6	0.27	Davis, unpubl. data
Ambystoma tigrinum	Adult	1	C	46.5	14.0	23.3	16.3	0.0	0.30	Davis, unpubl. data
Ambystoma mexicanum	Adult	7	C	20.1	21.7	52.0	4.9	1.0	1.08	Ussing and Rosenkilde 1995
Ambystoma mexicanum	Adult	15	C	59.0	13.5	22.5	4.0	1.0	0.23	Deparis and Beetschen 1967
Ambystoma talpoideum	Adult	34	W	41.5	12.7	45.7	0.0	0.2	0.31	Davis & Maerz 2008a
Ambystoma talpoideum	Adult	16	W	39.0	5.7	51.2	3.9	0.1	0.15	Davis & Maerz 2008b
Ambystoma talpoideum	Larvae	45	W	52.1	16.7	22.0	8.5	0.7	0.32	Davis, unpubl. data
Ambystoma opacum	Larvae	45	W	61.0	13.6	8.5	15.8	1.0	0.22	Davis, unpubl. data

*Status: W= Wild, C=Captive, U=Unknown

References for table above

Bennett, M.F., and J.K. Alspaugh. 1964. Some changes in the blood of frogs following administration of hydrocortisone. The Virginia Journal of Science 15: 76-79.

Bennett, M.F., and A.O. Johnson. 1973. Osmotic stress, ACTH and the white blood cell picture in newts, *Notophthalmus viridescens*. Journal of Comparative Physiology A 82: 333-338.

Bennett, M.F., and K.R. Daigle. 1983. Temperature, stress and the distribution of leukocytes in red-spotted newts, *Notophthalmus viridescens*. Journal of Comparative Physiology A 153: 81-83.

Cabagna, M.C., R.C. Lajmanovich, G. Stringhini, J.C. Sanchez-Hernandez, and P.M. Peltzer. 2005. Hematological parameters of health status in the common toad *Bufo arenarum* in agroecosystems of Santa Fe Province, Argintina. Applied Herpetology 2: 373-380.

Cannon, M.S., and A.M. Cannon. 1979. The blood leukocytes of *Bufo alvarius*: a light, phase-contrast, and histochemical study. Canadian Journal of Zoology 57: 314-322.

Cathers, T., G.A. Lewbart, M. Correa, and J.B. Stevens. 1997. Serum chemistry and hematology values for anesthetized American bullfrogs (*Rana catesbeiana*). Journal of Zoo and Wildlife Medicine 28: 171-174.

Chiesa, M.E., C.E. Rosenberg, N.E. Fink, and A. Salibian. 2006. Serum protein profile and blood cell counts in adult toads *Bufo arenarum* (Amphibia : Anura : Bufonidae): Effects of sublethal lead acetate. Archives of Environmental Contamination and Toxicology 50: 384-391.

Coppo, J.A., N.B. Mussart, S.A. Fioranelli, and P.A. Zeinsteger. 2005. Blood and urine physiological values in captive bullfrog, *Rana catesbeiana* (Anura: Ranidae). Analecta Veterinaria 25: 15-17.

Davis, A.K. 2009. Metamorphosis-related changes in leukocyte profiles of larval bullfrogs (*Rana catesbeiana*). Comparative Clinical Pathology 18(2): 181-186.

Davis, A.K., and A.M. Durso. 2009. Leukocyte differentials of northern cricket frogs (*Acris c. crepitans*) with a compilation of published values from other amphibians. Herpetologica 65(3): 260-267.

Davis, A.K., and J.C. Maerz. 2008a. Sex-related differences in hematological stress indices of breeding, paedogenic mole salamanders. Journal of Herpetology 42: 197-201.

Davis, A.K., and J.C. Maerz. 2008b. Comparison of hematological stress indicators in recently captured and captive paedogenic mole salamanders, *Ambystoma talpoideum*. Copeia 2008: 613-617.

Davis, A.K., and J.C. Maerz. 2009. Effects of larval density on hematological stress indices in salamanders. Journal of Experimental Zoology 311A: 697-704.

Davis, A.K. and J.R. Milanovich. 2010. Lead-phase and red-stripe color morphs of red-backed salamanders (*Plethodon cinereus*) differ in hematological stress indices: a consequence of differential predation pressure? Current Zoology 56(2): 238-243.

Deparis, P., and J.C. Beetschen. 1967. Comparative observations on some hematological characteristics of diploid and triploid salamanders (*Ambystoma mexicanum*). Comptes rendus hebdomadaires des séances de l'Académie des sciences. Série D: Sciences naturelles 265: 382-385.

Forbes, M.R., D.L. McRuer, and D. Shutler. 2006. White blood cell profiles of breeding American toads (*Bufo americanus*) relative to sex and body size. Comparative Clinical Pathology 15: 155-159.

Friedmann, G.B. 1970. Differential white blood cell counts for the urodele *Taricha granulosa*. Canadian Journal of Zoology 48: 271-274.

Hadji-Azimi, I., V. Coosemans, and C. Canicatti. 1987. Atlas of adult *Xenopus laevis laevis* hematology. Developmental and Comparative Immunology 11: 807-874.

Jerrett, D.P., and C.E. Mays. 1973. Comparative hematology of the hellbender, *Cryptobranchus alleganiensis* in Missouri. Copeia 1973: 331-337.

Jordan, H.E. 1938. Comparative hematology, pp. 699-839. *In* H. Downey [ed.], Handbook of hematology. Vol 2. Harper and Brothers, New York.

Kaplan, H.M. 1952. Variations in white blood cells between normal and red leg frogs. Illinois Academy of Science Transactions 45: 170-176.

Maniero, G.D., and C. Carey. 1997. Changes in selected aspects of immune function in the leopard frog, *Rana pipiens*, associated with exposure to cold. Journal of Comparative Physiology B 167: 256-263.

Pfeiffer, C.J., H. Pyle, and M. Asashima. 1990. Blood cell morphology and counts in the Japanese newt (*Cynops pyrrhogaster*). Journal of Zoo and Wildlife Medicine 21: 56-64.

Ponsen, S., N.-a. Narkkong, S. Pamok, K. Sappaso, and W. Aengwanich. 2008. Hematological values and morphological observation of blood cells in balloon frog, *Glyphogloossus molossus*. Journal of Microscopy Society of Thailand 22: 71-75.

Romanova, E.B., and O.Y. Romanova. 2003. Peculiarities of leukocytic formula of peripheral blood of green frogs under conditions of anthropogenic load. Journal of Evolutionary Biochemistry and Physiology 39: 480-484.

Rouf, M.A. 1969. Hematology of the leopard frog, *Rana pipiens*. Copeia 1969: 682-687.

Shutler, D., T.G. Smith, and S.R. Robinson. 2009. Relationships between leukocytes and hepatozon spp. in green frogs, *Rana clamitans*. Journal of Wildlife Diseases 45: 67-72.

Solis, M.E., J.M. Bandeff, and Y.W. Huang. 2007. Hematology and serum chemistry of Ozark and eastern hellbenders (*Cryptobranchus alleganiensis*). Herpetologica 63: 285-292.

Ussing, A.P., and P. Rosenkilde. 1995. Effect of induced metamorphosis on the immune system of the Axolotl, *Ambystoma mexicanum*. General and Comparative Endocrinology 97: 308-319.

Wojtaszek, J., and A. Adamowicz. 2003. Hematology of the fire-bellied toad, *Bombina bombina* L. Comparative Clinical Pathology 12: 129-134.