White Blood Cell Profiles in Long-Term Captive and Recently Captured Eastern Tiger Salamanders (*Ambystoma tigrinum*)

Heather L. Waye[1], Peter C. Dolan[1], and Alexis Hernandez[1]

Physiological responses to stress are important indicators of the effects of environmental disruption on individuals and, therefore, a way to determine the health of populations. These responses can be measured in a variety of ways, including the survey of differential counts of white blood cells. Baseline cell numbers and neutrophil to lymphocyte ratios have been determined for a number of species in the genus *Ambystoma*, but not for *A. tigrinum*, and baseline values are necessary for assessments of stress in natural and captive populations. We counted white blood cells in blood smears from long-term captive and recently captured Eastern Tiger Salamanders (*A. tigrinum*) and compared the proportions of each cell type between these two samples. We also compared our results to the published values for other post-metamorphic or paedomorphic ambystomatids. Mean neutrophil to lymphocyte ratios, a measure of stress, were higher in our captive salamanders (0.81) than in the wild sample (0.41), as were the mean number of basophils (36.0 for captive and 10.3 for wild). The cell counts for our wild salamanders were comparable to those for other unstressed ambystomatids. Our results suggest that our long-term captive salamanders are under a small degree of stress and are not a good source of baseline values for this species.

AMPHIBIAN populations are declining worldwide, with many species facing extinction (Wake and Vredenburg, 2008). In response, there has been increased interest in the impacts of environmental disruption on amphibian populations and the physiological mechanisms involved. These mechanisms may result in changes in performance, immune function, growth, and reproductive timing and success (Navas et al., 2016). One important physiological mechanism is the vertebrate stress response, which has been described as a generalized neuroendocrine response to challenging situations. It involves rapid sympathetic activation and a more delayed and sustained release of glucocorticoids (Hill et al., 2016). In amphibians, the release of glucocorticoids (primarily corticosterone) is mediated through the hypothalamic-pituitary-interrenal axis (Rollins-Smith, 2001). This "stress hormone" supports heightened activity through increased release of glucose and fatty acids into circulation for fuel. Chronic or repeated stress can result in depletion of energy stores, inhibition of reproduction, and suppression of the immune system (Hill et al., 2016). For example, increased concentrations of corticosterone have been shown to inhibit lymphocyte production and function in anurans (Rollins-Smith, 2001).

We are interested in habitat use of tiger salamanders in wetland habitats of West-Central Minnesota. Eastern Tiger Salamanders (*Ambystoma tigrinum*) can be found in a variety of habitats across eastern North America (Moriarty and Hall, 2014). Although Western Tiger Salamanders (*A. mavortium*) have been reported in western Minnesota (Moriarty and Hall, 2014), we will refer to our study animals as the more common Eastern Tiger Salamanders (*A. tigrinum*) until we are able to resolve species identification of individual tiger salamanders in this area. The tallgrass prairie environment of western Minnesota is a highly modified landscape, with much of the historic prairie pothole habitat converted to agriculture over the last century (Johnson et al., 2008). Salamanders occupying these wetlands are subject to many potential environmental stressors, including agricultural chemicals (e.g., Rohr and McCoy, 2010), road salt (e.g., Milotic et al., 2017), and increased susceptibility to disease (e.g., Kirschman et al., 2018). Sublethal responses to these stressors can impact population survival through changes in physiological function that negatively affect individual growth, reproduction, and immune function (Navas et al., 2016).

One approach toward understanding the effects of these stressors is to survey individuals for physiological signs of chronic stress. A widely used technique involves measurement of the concentration of glucocorticoids in circulation as a direct assessment of the stress response (Romero, 2004). Another approach is to measure the relative proportion of leukocytes in circulation. When vertebrates experience stress, the proportion of circulating neutrophils (or heterophils in birds and reptiles) increases while the proportion of circulating lymphocytes decreases, leading to an elevated neutrophil to lymphocyte (N:L) ratio (reviewed in Davis et al., 2008).

These two techniques are not interchangeable as they measure different aspects of the stress response (Davis and Maney, 2018). An increase in glucocorticoids results in many physiological responses including the change in circulating leukocytes (Davis et al., 2008). Levels of glucocorticoids increase rapidly upon exposure to a stressor and decline over time, even under continued exposure to the stressor (Romero, 2004). The leukocyte response may take hours to days and does not decrease over time (Davis et al., 2008). The endocrine response is therefore particularly useful in the study of acute, short-term stressors or peak stress, but could be problematic when measuring baseline levels in certain sampling situations, as the hormone sample must be obtained within minutes of capture. Conversely, changes in proportions of leukocytes could be useful in studies of chronic, long-term stress, or to generate baseline information for species that may spend some time in a trap before a sample can be obtained (Davis and Maney, 2018). Regardless of the technique used, baseline measurements for the species of interest are necessary.

The purpose of our study was two-fold: 1) To compare leukocyte numbers and N:L ratios in long-term captive tiger salamanders to recently captured ones. Salamanders maintained in captivity for years are fed and handled regularly and are presumably habituated to the captive environment. The stress status of target wild populations is largely unknown

[1] University of Minnesota Morris, Morris, Minnesota 56267; Email: (HLW) wayex001@morris.umn.edu. Send reprint requests to HLW.
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beforehand, so leukocyte numbers from captive animals could be a better measure of baseline "low stress."

2) To compare captive and recently captured tiger salamander leukocyte numbers with published values for other ambystomatid species. If our results are dramatically different from the range of values for other ambystomatids, our samples may not present a representative baseline for Eastern Tiger Salamanders.

MATERIALS AND METHODS

The tiger salamanders we used in this study were all collected from the Pepperton Waterfowl Production Area or the surrounding area near Alberta, Minnesota. The 12 captive salamanders were obtained in October 2011 and maintained at the University of Minnesota Morris. Salamanders were individually housed in ten-gallon aquaria with a substrate of damp moss on cage carpeting and a water bowl filled with dechlorinated tap water that was changed and cleaned every two weeks or as needed. Lighting (10L:14D) was provided by a 13-watt helical fluorescent bulb placed over one end of each aquarium and by indirect sunlight ambient to the room. Ambient lighting, temperature, and humidity in the room tend to fluctuate seasonally, but all cages were exposed to the same room temperature (21 to 24°C) and relative humidity (44 to 77%). These salamanders have been maintained on a diet alternating between crickets, earthworms, and fish, and infrequently waxworms, offered twice weekly and occasionally supplemented with Herptivite™ multivitamin (Rep-Cal Research Labs, Los Gatos, CA). They were checked daily and appeared to be in good health prior to the sampling period.

We captured seven free-living (wild) post-metamorphic salamanders using minnow traps that were deployed for another study between 10 June and 23 June 2018. We checked the traps every day between 1100 hrs and 1400 hrs. Several times the traps were checked again around 1900 hrs and were found to be empty of amphibians at that time, suggesting that the salamanders entered the traps at night or in the morning. Captured wild salamanders were transported to the laboratory at the University of Minnesota Morris for blood collection. The maximum possible time between the previous trap check and when a salamander from that trap was sampled was 27 hours. All salamanders were weighed and measured for snout–vent (SVL) and total length.

Blood collection and slide preparation.—Salamanders were anesthetized by partial immersion in 0.1% MS-222 (tricaine methanesulfonate). Once they had lost the righting reflex, we collected 0.05 mL of blood from the caudal vein using a syringe with a 27-gauge needle. We used small drops of blood to make two or three standard blood smears for each salamander. The salamanders were then placed in dechlorinated water and, once recovered, either transferred to their home tank or transported to the study site and released at the site of capture. All salamanders took no more than 30 minutes to fully recover from anesthesia. Blood smears were allowed to air dry, then were stained using Camco Quik Stain II (Wright-Giemsa, Cambridge Diagnostic Products, Inc.).

Leukocyte counting.—We examined one slide for each salamander using a light microscope under 400X, switching to 1000X oil immersion to verify cell identity when necessary. We scanned the slide in a zig-zag pattern and counted and identified the first 100 leukocytes encountered. For each cell, one person made the initial identification, then moved aside and allowed another to view the cell and either verify or dispute the identity. When consensus was reached on the identity of the cell, it was recorded and the scan continued. We identified leukocytes based on the descriptions in Hadji-Azimi et al. (1987), Heatley and Johnson (2009), and Campbell (2015).

Data analysis.—Statistical analyses were performed using R (R Core Team, 2018) and visualized using ggplot2 (Wickham, 2016). Proportions of the different cell types for captive and wild salamanders were compared using Pearson's chi-squared test. Several sources (e.g., Davis and Maerz, 2008a) use ANCOVAs to control for other factors such as sex, mass, etc. In our data there was a strong association between the status of the specimen (wild vs. captive) and mass and SVL. As a result of this association, it is inappropriate to use these covariates as part of an ANCOVA (Miller and Chapman, 2001). Because of the non-normality of the N:L ratios and the small sample sizes, we performed a non-parameteric Kruskal-Wallis rank sum test to compare the ratios for the wild and captive salamanders.

RESULTS

The 12 captive salamanders ranged in size from 10.5 to 12.5 cm SVL (mean 11.9 cm) and 71.1 to 120.8 g (mean 95.1 g). The seven wild salamanders ranged in size from 9.1 to 11.5 cm SVL (mean 10.6 cm) and 27.0 to 68.6 g (mean 46.4 g). The sex of the captive salamanders was known, but as it was difficult to confidently determine the sex of the wild salamanders, we did not include sex in the analysis.

We saw statistically significant differences between the distribution of white blood cell types in captive vs. wild samples ($\chi^2 = 200.28$, df = 4, $P < 0.001$; Table 1). Examination of standardized residuals showed that in wild salamanders, eosinophils were strongly over-represented (9.412636), lymphocytes were over-represented (5.35035), neutrophils under-represented (–3.382957), and basophils were strongly under-represented (–12.24709). In captive individuals this was reversed, with basophils strongly over-represented, neutrophils over-represented, lymphocytes under-represented, and eosinophils strongly under-represented (Fig. 1).

The N:L ratio for captive salamanders was statistically greater than that of wild salamanders (H = 4.6528, df = 1, P = 0.031; Table 2). To be consistent with the literature, we report the observed means of the two groups, but outliers in both the wild and the captive groups inflate the means, particularly for the wild group. The medians (wild = 0.29, captive = 0.75) are likely a better representation of the group N:L ratios. (Fig. 2).

DISCUSSION

We hypothesized that tiger salamanders that had been housed in a stable captive environment for almost seven years would demonstrate low levels of stress and therefore provide a baseline "unstressed" sample. Our results indicate that wild tiger salamanders, in the study population at least, have lower levels of stress than our captive animals. Although there was a range of N:L ratios within each treatment group, the wild salamanders generally had lower ratios than the captive ones, even after spending up to a day in a trap before sampling. This appears to be due to higher lymphocyte numbers in wild salamanders rather than differences in neutrophils. In addition, wild salamanders

Table 1. Summary of white blood cells for 19 Eastern Tiger Salamanders, by captivity status. The numbers are presented as a percent of the total leukocytes counted.

	Mean	Minimum	Maximum	SD
Captive				
Lymphocytes	17.1	8	27	7.6
Neutrophils	13.3	3	29	7.9
Eosinophils	30.5	12	70	18.0
Basophils	36.0	1	68	23.3
Monocytes	3.2	0	12	3.3
N:L	0.81	0.26	1.69	0.4
Wild				
Lymphocytes	27.4	10	44	12.0
Neutrophils	8.1	4	13	3.8
Eosinophils	52.3	33	71	14.3
Basophils	10.3	2	18	5.6
Monocytes	1.9	0	5	1.9
N:L	0.41	0.10	1.20	0.4

had higher eosinophil numbers than captive salamanders. *Ambystoma talpoideum* injected with corticosterone showed a reduction in circulating lymphocytes and eosinophils (Davis and Maerz, 2010), which supports the conclusion that the differences in leukocyte numbers between our captive and wild salamanders were due to an increased stress response by the captive individuals.

Our captive salamanders also showed a higher number of circulating basophils compared to the wild salamanders. It is not clear what role basophils perform in amphibians, but they appear to be involved in inflammatory reactions as in other vertebrates (Claver and Quaglia, 2009). Captive amphibians may be particularly susceptible to disease, including those caused by bacteria, viruses, and fungi

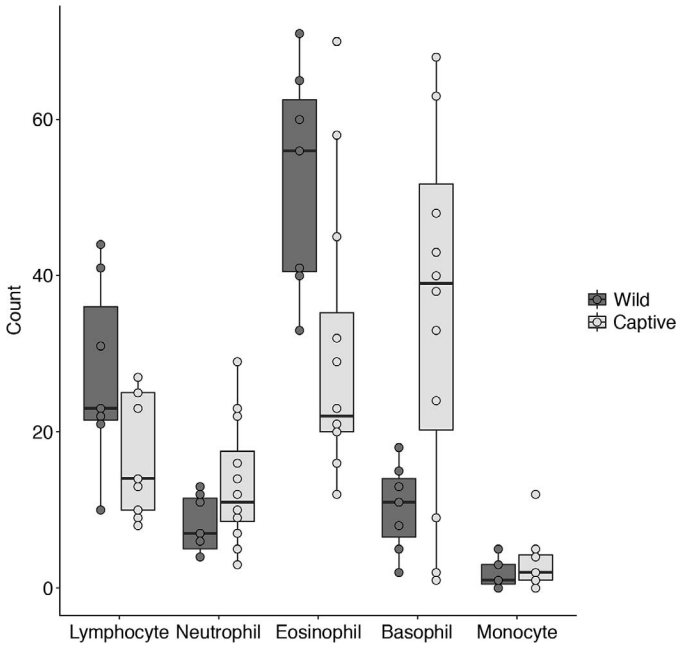

Fig. 1. Number of circulating leukocyte cell types obtained from wild and captive tiger salamanders, out of a total count of 100 leukocytes for each individual. Box and whisker plots with data points overlaid; boxplots show first quartile, median, and third quartile. Whiskers denote highest and lowest data points that satisfy the 1.5 interquartile range rule.

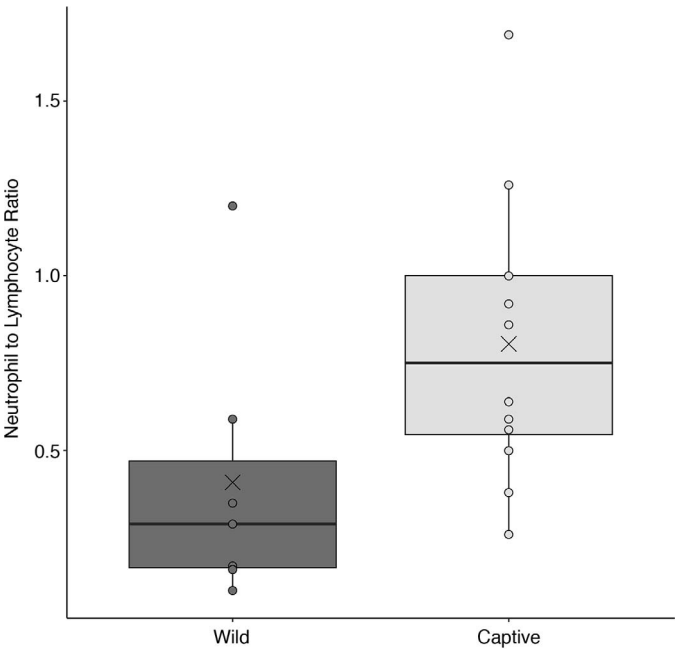

Fig. 2. Comparison of neutrophil to lymphocyte ratios for wild and captive tiger salamanders. Box and whisker plots with data points overlaid; boxplots show first quartile, median, and third quartile. Whiskers denote highest and lowest data points that satisfy the 1.5 interquartile range rule. The X indicates the mean N:L ratio for each group.

(Densmore and Green, 2007). The higher basophil numbers, plus the increased phagocytic leukocytes (neutrophils and monocytes), could indicate that our captive salamanders are responding to increased exposure to pathogens in their environment (Claver and Quaglia, 2009). The salamanders appear to be in good health, so these higher leukocyte counts may reflect a successful immune response to environmental challenges.

Another possible stressor is that of inadequate nutrition. Captive animals typically are not offered as varied a diet as they might encounter in the wild, and captive amphibians are therefore prone to nutritional diseases (Densmore and Green, 2007). Our salamanders have been fed only three different food items. We have been adding vitamins to their food, but will do so more consistently in case their repetitive diet is a source of stress. In addition, our captive salamanders had a higher mass to length relationship than the wild salamanders, which could indicate that the captive animals are somewhat overweight. There is a strong connection between obesity and inflammation in humans, which results in significantly higher levels of circulating monocytes, lymphocytes, and neutrophils in overweight individuals compared to controls (e.g., Marzullo et al., 2014). However, our captive animals had lower circulating lymphocyte counts than our wild samples (Table 1), suggesting that the differences are primarily due to stress (Davis et al., 2008) and not direct indicators of inflammation.

Comparing our results to those reported for paedomorphic or post-metamorphic ambystomatid salamanders (Table 2), we find that while our captive salamanders have a higher mean N:L ratio than both freshly caught *A. talpoideum* (0.17) and those held in captivity for ten days (0.39; Davis and Maerz, 2008b), it is well below *A. talpoideum* injected with corticosterone (4.42; Davis and Maerz, 2010) or *A. maculatum* raised in high densities (2.24; Davis, 2012). This could

Table 2. Neutrophil to lymphocyte ratios for ambystomatid salamanders. The mean and median ratios for captive and wild tiger salamanders examined in this study compared to the mean N:L ratios reported for other ambystomatid species.

Status	Mean (this study)	Median (this study)	Davis and Maerz, 2008b	Davis and Maerz, 2010	Davis, 2012
Captive or "stressed"	0.81	0.75	0.39	4.42	2.24
Wild or "low stress"	0.41	0.29	0.17	0.56	0.44
Source of stress	captivity		captivity	corticosterone injection	crowding

indicate that while our captive salamanders were experiencing a greater degree of stress than our wild salamanders, it was still only slightly elevated. The N:L ratio for our wild salamanders was higher than that for the freshly caught *A. talpoideum* and similar to those in captivity (Davis and Maerz, 2008b) and to *A. maculatum* raised at low densities (0.44; Davis, 2012). However, our wild salamander N:L ratio was lower than that for reference *A. talpoideum* (0.56; Davis and Maerz, 2010).

Although the change in N:L ratios is thought to take hours or days in ectotherms (Davis and Maney, 2018), the extended period of time between our capture of wild salamanders and sampling their blood was of concern. After extensive searching of the literature, we found few studies on amphibians that measured N:L ratios immediately after capture and then at various points up to 24 hours later. Narayan and Hero (2011) found elevated N:L ratios in Fijian ground frogs after six hours in transport. In contrast, DuRant et al. (2015) immediately sampled wild-caught Hellbenders, and then injected them with ACTH or saline. They sampled again at 6 and 28 hours after injection, and found the N:L ratio to be no different from the control at 6 hours, but elevated at 28 hours. Similarly, Bennett et al. (1972) did not see a significant change in neutrophil and lymphocyte numbers 48 hours after injecting Eastern Newts with hydrocortisone acetate. These studies suggest that we might expect to see a hematological stress response in tiger salamanders around or possibly before 24 hours, yet our wild salamanders had low N:L ratios. Clearly more work needs to be done to describe changes in circulating leukocytes during the first 24 hours of exposure to a stressor.

Looking at all five leukocyte types, our results are not wildly different from those published for other ambystomatid salamanders (Table 3). Davis and Durso (2009) summarize ambystomatids separately from other amphibians due to the elevated number of eosinophils reported for this genus. While eosinophils in other urodeles make up on average 4.7% of all leukocytes, in ambystomatids eosinophils are on average 32.1% of the leukocyte count. The tiger salamanders surveyed in this study also showed high numbers of eosinophils, within the range reported for other ambystomatids. Our lymphocyte numbers are on the low side, while basophils and monocytes are elevated, especially for the captive salamanders. This, plus the higher N:L ratio compared to our wild salamanders, indicates that captive animals, even those held for multiple years, may not be a good model for wild populations.

We were unable to examine the effect of body size on leukocyte counts because of the relationship between body size and captivity status. In addition, we were unable to confidently determine the sex of the wild salamanders. However, other studies have found that sex of nonreproductive individuals (Davis and Maerz, 2008a) and body size (Davis and Maerz, 2008a, 2008b) do not have a significant effect on neutrophil or lymphocyte numbers. It is not clear how comparable leukocyte counts or N:L ratios are between species, or even for the same species examined by different investigators. Results on leukocyte proportions reported for the three studies of *A. talpoideum* listed in Table 3 are fairly consistent, but they were also conducted by the same authors. However, the consistently high numbers of eosinophils, and relatively similar N:L ratios for unstressed treatments, does suggest some validity to this approach. Additional studies on these species in a variety of environments will help to further establish useful baseline values.

In conclusion, our captive animals did not prove to be a good model for "unstressed" tiger salamanders. Others have also expressed caution about using laboratory results based on captive animals to describe processes of natural systems without a good understanding of these systems (e.g., Gregory, 2001). Further research on stress in natural populations of tiger salamanders in prairie pothole habitats will provide us with stronger baseline data for this species and a better understanding of their response to local stressors. Capture should not result in a significant change in the number of circulating leukocytes for at least a few hours (Davis et al., 2008), which makes this a useful technique to measure chronic levels of stress in natural

Table 3. Average white blood cell percentages for ambystomatid salamanders. The tiger salamanders examined in this study are compiled with those reported by others for putatively unstressed post-metamorphic or adult ambystomatid salamanders (adapted from Davis and Durso, 2009).

Species	Status	*n*	Lymphocytes	Neutrophils	Eosinophils	Basophils	Monocytes	Source
A. tigrinum	captive	12	17.1	13.3	30.5	36.0	3.2	this study
A. tigrinum	wild	7	27.4	8.1	52.3	10.3	1.9	this study
A. californiense	captive	34	48.7	25.2	19.7	4.6	1.3	Brady et al., 2016
A. maculatum	newly metamorphed	11	31.7	18.1	25.5	24.2	0.6	Davis and Maerz, 2009
A. mexicanum	captive	7	20.1	21.7	52.0	4.9	1.0	Ussing and Rosenkilde, 1995
A. mexicanum	captive	15	59.0	13.5	22.5	4.0	1.0	Deparis and Beetschen, 1967
A. rivulare	wild	163	77.4	7.9	11.2	2.3	1.2	Barriga-Vallejo et al., 2015
A. talpoideum	paedomorphic	34	41.5	12.7	45.7	0.0	0.2	Davis and Maerz, 2008a
A. talpoideum	paedomorphic	16	39.0	5.7	51.2	3.9	0.1	Davis and Maerz, 2008b
A. talpoideum	wild	11	40.5	21.3	32.0	4.9	1.3	Davis and Maerz, 2010

populations of amphibians. However, better characterization of the time course of the hematological response, especially over the first 24 hours or so after exposure to a stressor, would help to inform sampling methodology and improve our understanding of the amphibian immune system.

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