



4-2010

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### Recommended Citation

Davis A.K., and Milanovich J.R. 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), p. 238-243.

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# Lead-phase and red-stripe color morphs of red-backed salamanders *Plethodon cinereus* differ in hematological stress indices: A consequence of differential predation pressure?

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**Abstract** Throughout the animal kingdom there are species that have two or more phenotypic forms or ‘morphs’, and many of these are amphibians. In North America, the red-backed salamander *Plethodon cinereus* can have either a red dorsal stripe or no dorsal stripe (lead-phase form), and evidence to date indicates the lead-phase form incurs a greater number of attacks from predators. In a recent collection of 51 *P. cinereus*, blood smears of both color morphs (35 red-stripe, 16 lead-phase) were examined to obtain numbers of circulating leukocytes (via light microscopy), which can be used to indirectly estimate levels of stress hormones in vertebrates via a ‘hematological stress index’, which is the ratio between the number of two leukocyte types (neutrophils and lymphocytes). Our results showed that lead-phase salamanders tended to have greater numbers of circulating neutrophils and lower numbers of circulating lymphocytes than red-stripe morphs, leading to higher average neutrophil-lymphocyte ratios in lead-phase individuals. Since the salamanders were held (refrigerated) for 7 days before sampling, we cannot be certain if this effect is a stress reaction to the captivity or the normal level for this morph. However comparison with two sets of related salamanders that were captured and sampled immediately indicates the red-stripe salamanders were either not stressed from the captivity at all, or their white blood cell distributions had returned to normal after 7 days of captivity. Taken together, our results indicate that lead-phase forms of *P. cinereus* have higher stress levels than the red-stripe forms, which may be a consequence of their higher exposure to, and/or attacks from, predators. They may also indicate that the lead-phase form is less-suited to captivity than the red-stripe form of this species [*Current Zoology* 56 (2): 238–243, 2010].

**Key words** Red-backed salamander, *Plethodon cinereus*, Color polymorphism, Hematological stress index, Corticosterone

There are many examples in the animal kingdom of species with color polymorphisms, where individuals have either one phenotypic character or another. These include many insects (e.g. Bots et al., 2009), birds (e.g. Maney et al., 2009), reptiles (e.g. Vercken and Clobert 2008a) and amphibians (Venesky and Anthony, 2007; Anthony et al., 2008). Within the ectothermic animals, this subject has been well-studied, especially in lizards, in which there are many species with colored skin patches that show polymorphism (e.g. Vercken and Clobert, 2008a; Huyghe et al., 2009). Recent research has shown that individuals with one color can differ from those with another color in a suite of ecological and physiological ways (Vercken and Clobert, 2008b). One physiological difference recently found is in levels of the stress hormone, corticosterone; in this case, Huyghe et al. (2009) showed that within a polymorphic

lizard species *Podarcis melisellensis* where individuals have either orange, yellow or white ventral color, each color morph differed in baseline stress hormone level. This intriguing finding adds a new dimension to our understanding of color polymorphisms in animals, and engenders further questions about stress level variations in other polymorphic species.

The red-backed salamander *Plethodon cinereus* is a New World terrestrial plethodontid salamander commonly found in eastern deciduous forests of North America. This species exhibits two color forms, a red-stripe form, characterized by a dorsal orange-red stripe running the length of the animal, and a lead-phase form (also called ‘unstriped’), where this stripe is absent (Fig. 1). While not as extensively-studied as polymorphic lizards, there is recent evidence that both forms differ in certain ecological characteristics (Anthony et

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Received Aug. 20, 2009; accepted Nov. 16, 2009

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al., 2008), such as food acquisition, with red-stripe forms generally obtaining food with higher nutritional value than lead-phase forms. There is evidence that the two morphs also have different temperature thresholds for above-ground activity, perhaps as a result of different metabolic rates (Moreno, 1989). In this case, lead-phase salamanders have a lower metabolism and therefore are capable of remaining active longer during warm temperatures because of the low energetic cost. Importantly, these forms also appear to experience differential predation pressure, as evidenced by more frequent tail autotomy in lead-phase forms (Moreno, 1989; Venesky and Anthony, 2007). Venesky and Anthony (2007) further showed in an experimental setting that lead-phase forms were more likely to flee from predators and were generally more mobile than red-stripe forms. The combined evidence suggests then, that lead-phase forms have a greater susceptibility to predation and have a different escape strategy than red-stripe forms. Given this evidence, it stands to reason that lead-phase forms may also have different baseline levels of stress hormones than red-stripe forms, reflecting their greater predation pressure.



**Fig. 1** Photograph of both color forms (lead-phase [left] and red-stripe [right]) of the red-backed salamander *Plethodon cinereus* (Photo taken by Victor S. Lamoureux)

During a recent study of erythrocyte morphology of red-backed salamanders (Davis et al., 2009), blood smears were made of sets of red-stripe and lead-phase individuals. While it is not widely-utilized in herpetological studies, blood smears can provide information as to the stress level of amphibians. Like all vertebrates, amphibians have five types of leukocytes in their blood, and the numbers of two of them (neutrophils and lymphocytes) can be used to estimate levels of stress hormones (reviewed in Davis et al., 2008a). In fact, because of the small amount of blood required, this hema-

tological approach to measuring stress is highly appropriate for studying small animals such as salamanders (Davis and Maerz 2008 a, b, 2009). Thus, since our prior collection of blood samples from *P. cinereus* contained both color forms of the species, we had the opportunity now to explore possible differences in stress between the two (which we estimated from blood smears), which to our knowledge, has never been explored in this species. Given that lead-phase forms are known to have higher predation pressure, we hypothesized that stress levels would be higher in this form.

## 1 Materials and Methods

### 1.1 Capture and handling of salamanders

On 4 October 2007, we collected 51 *P. cinereus* from within two deciduous forest sites in central Pennsylvania, USA. A total of 27 salamanders (19 red-stripe, 8 lead-phase) were collected from a mountainside in Hawk Mountain Sanctuary (HMS) and 24 salamanders (16 red-stripe, 8 lead-phase) were collected in a valley in Hopewell Furnace National Historic Park (HFP). None of the salamanders collected were in breeding condition, which would be evidenced by enlargement of the mental gland in males, and absence of enlarged ova in females. All salamanders were hand-collected from under rocks and cover objects, then placed singly into petri dishes (along with a moist tissue) and put on ice for transport to the lab, where they were held in the refrigerator (at  $\sim 10^{\circ}\text{C}$ ) for 7 days. On the 7<sup>th</sup> day we processed the salamanders following established procedures (Davis et al. 2009). Briefly, on the day of processing, each salamander was removed from its dish, blotted dry, weighed using an electronic balance and its snout-vent-length was measured to the nearest millimeter. We assigned each salamander to sex using external characteristics or from later dissection, then recorded if the salamander was a red-striped ( $n=35$ ) or lead-phase ( $n=16$ ) morph. Next, each was killed via overdose of MS-222 as specified under our university animal care protocol (# AUP2006-10041) which allowed for killing and collection of tissues from this species. Immediately after killing, the animals were decapitated and blood that welled from the heart region was blotted onto a microscope slide and a second slide was used to draw the blood into a standard blood film for light microscopy. All blood films were air-dried then later stained with giemsa. For each salamander the time from removal from its holding dish until euthanasia was approximately 5 minutes, although we point out that for ectotherms, the hematological stress index takes 24

hours to manifest after any stressful event (reviewed in Davis et al., 2008a), so that the processing of salamanders here should not have affected the results in this study.

### 1.2 Reading blood films

Each blood film was examined with a standard light microscope under 1000 $\times$  (oil) and leukocytes counted following Davis and Maerz (2008a, b, 2009). For this, slides were examined in a zig-zag pattern so that all parts of the films were sampled, and for each field of view, all leukocytes were counted. Counting continued until 100 white blood cells was reached or 150 fields were examined. Leukocytes were identified as lymphocytes, neutrophils, eosinophils, basophils and monocytes following Thrall et al. (2004) and Wright (2001), although the focus here was on lymphocytes and neutrophils (Fig. 2), which are the most common leukocytes in amphibians (Davis, 2009). For each salamander we calculated the percentage of each cell type out of the total number observed for that individual (which is the 'leukocyte profile' of the individual), as well as its neutrophil-lymphocyte ratio, based on the percentages of these cells (Davis et al., 2008b; Davis and Maerz, 2008a, b; Davis et al., 2008a). The neutrophil-lymphocyte ratio serves to estimate baseline levels of stress hormones because of the tight relationship between the two parameters (reviewed in Davis et al., 2008a). Briefly, when corticosterone is elevated due to stress, it causes a number of physiological alterations in vertebrate bodies, one of which is to redistribute certain leukocytes within the body. It causes neutrophils (or heterophils in birds and reptiles) to enter the bloodstream from tissues in greater numbers, while causing lymphocytes to egress from circulation into certain organs and tissues. The effect is an increase in numbers and proportions of circulating neutrophils and a decrease in circulating lymphocytes, so that the ratio of the two cells is linearly related to the magnitude of the hormonal stress increase (Gross and Siegel, 1983). This approach is highly appropriate for estimating stress in small animals because of the small amount of blood required.

### 1.3 Data analysis

Since the goal of this study was to explore possible differences in stress levels between color forms of red-backed salamanders, the variables of interest to us were the proportions of lymphocytes and neutrophils, as well as their ratio (Davis et al., 2008a). Proportions of neutrophils and lymphocytes were arcsin-squareroot transformed prior to analyses to approximate normal

distributions. Neutrophil-lymphocyte ratios were log-transformed (+1). We also confirmed that the assumption of homogeneity of variance was met in these three variables (between color form groups) with Levene's tests. We then compared the proportions of lymphocytes and neutrophils, along with their ratio, between lead-phase and red-stripe forms using Student's t-tests. We note that the sex ratios of both color form groups were approximately equal and initial comparisons indicated no difference in body sizes between color forms ( $t=1.17$ ,  $df=49$ ,  $P=0.247$ ). Analyses were conducted using Statistica software 6.1 (Statistica 2003).

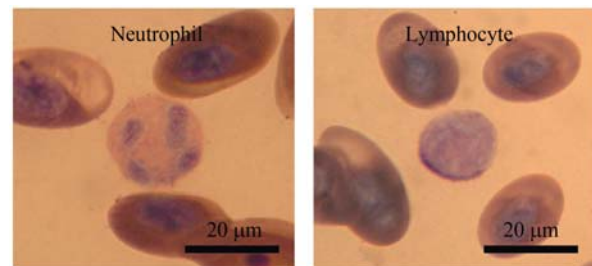


Fig. 2 Photomicrographs of two leukocytes from blood smears of *P. cinereus*: neutrophil and lymphocyte

## 2 Results

Lymphocytes were the most common leukocyte observed overall in *P. cinereus*, followed by neutrophils (Table 1). When leukocyte profiles of each color form were considered separately however, differences in the percentages of each of these cells became evident. On average, lead-phase salamanders had significantly greater percentages of circulating neutrophils (Student's t-test,  $t=-2.02$ ,  $df=49$ ,  $P=0.049$ ) and significantly lower percentages of lymphocytes ( $t=2.28$ ,  $df=49$ ,  $P=0.027$ ). Both of these patterns are consistent with elevated corticosterone in the lead-phase form (Davis et al. 2008a). Moreover, these differences in cell numbers resulted in lead-phase individuals having an average neutrophil-lymphocyte ratio (0.69) that was double that of red-stripe salamanders (0.31). This difference was significant ( $t=2.87$ ,  $df=49$ ,  $P=0.006$ ). Since these data were obtained after the salamanders were held for 7 days, we also present leukocyte data from separate collections of two related species (*Eurycea wilderae* and *Eurycea cirrigera*), in which the individuals were captured and sampled immediately (Table 1). In both species, average neutrophil-lymphocyte ratios were close to 0.30.

**Table 1** Summary of leukocyte profiles (percentages of circulating white blood cells) of both color forms of *Plethodon cinereus*

| Color Form                    | <i>n</i> | Lymphocytes  | Neutrophils  | Eosinophils | Basophils | Monocytes | N/L Ratio     |
|-------------------------------|----------|--------------|--------------|-------------|-----------|-----------|---------------|
| Lead-phase <i>P. cinereus</i> | 16       | 58.4 (20.0)  | 27.5 (18.9)  | 5.3 (6.7)   | 9.3 (4.2) | 0.2 (0.5) | 0.68 (0.68)   |
| Red-stripe <i>P. cinereus</i> | 35       | 68.3 (14.8)* | 18.0 (10.9)* | 3.5 (4.2)   | 9.6 (6.6) | 0.7 (1.1) | 0.31 (0.24)** |
| <i>Eurycea wilderae</i>       | 11       | 68.7 (20.2)  | 22.3 (15.0)  | 1.5 (2.4)   | 5.0 (8.8) | 2.4 (3.8) | 0.32 (0.25)   |
| <i>Eurycea cirrigera</i>      | 5        | 70.9 (12.8)  | 19.3 (10.5)  | 1.0 (1.2)   | 2.9 (2.7) | 5.9 (4.2) | 0.30 (0.20)   |

Shown are the means ( $\pm$ 1SD) of each group. All individuals of *P. cinereus* were captured in central Pennsylvania, USA, and held in a refrigerator for 7 days before obtaining blood samples. Also shown are profiles from two separate collections of related plethodontid species, which were sampled immediately after capture (A. Davis, *unpubl. data*).

\* Significant difference between color forms (Student's t-test,  $P < 0.05$ ), \*\* Significant difference between color forms (Student's t-test,  $P < 0.01$ ).

### 3 Discussion

Results of this simple exploration provide a new insight into the nature of polymorphism in this terrestrial amphibian, and are generally consistent with those of prior studies involving direct measurement of stress hormones. As we expected, the lead-phase form of *P. cinereus*, which is more mobile and may experience greater predation risk (Moreno, 1989; Venesky and Anthony, 2007), had significantly higher estimated stress levels (N/L ratios) than the red-stripe form. While we interpret this difference between color forms to be a function of their differential predation pressure, we admit that this may not be the only explanation for this pattern. This could simply be a manifestation of an underlying physiological difference between the two forms. Indeed, the two forms are already known to differ in one other physiological trait, their metabolic rates (Moreno, 1989), where lead-phase individuals have lower rates than red-stripe forms. In fact, this difference in metabolism could very well have influenced the results we found, especially since the animals were all sampled 7 days after capture. On the day the animals were captured, their circulating white blood cells were no doubt affected by the rise in plasma corticosterone that occurred in response to the capture (Cash et al., 1997; Lindström et al., 2005; Langkilde and Shine, 2006), with neutrophils migrating into the bloodstream from tissue and circulating lymphocytes migrating from blood to tissue (reviewed in Davis et al., 2008a). However, the time-course of this effect can be influenced by the metabolic rate of the animal. This is thought to be the reason why it takes at least 24 hours for the N/L ratio to become elevated after experimental administration of corticosterone in ectothermic amphibians (Bennett and Harbottle, 1968; Bennett et al., 1972), but it only takes 1–2 hours in endothermic mammals (Morrow-Tesch et al., 1993). By extension, we could envision the effect of the capture stress taking longer to manifest (in terms of redistributing their white blood

cells) in the lead-phase forms because of their lower metabolism than red-stripe forms (Moreno 1989). And importantly, it could also take longer for the circulating white blood cells of lead-phase salamanders to return to normal numbers.

After 7 days of captivity, the red-stripe salamanders had an average N/L ratio (0.31, Table 1) that was typical of unstressed individuals (Davis, 2009). Further, their mean ratio was consistent with the ratios of the two other Plethodontid species examined (Table 1), in which all individuals were sampled *immediately* after capture. It is not unreasonable to assume, therefore, that the red-stripe forms in this study were not 'stressed' after the 7 day holding period. This result in itself is rather surprising, since captivity of a similar duration results in elevated N/L ratios in other salamanders *Ambystoma talpoideum* (Davis and Maerz, 2008b). As mentioned above, it is not clear if leukocyte numbers in these individuals quickly returned to normal after alteration by the capture stress, or if they simply never became stressed from the capture and captivity. We suspect the former, and point out that if indeed red-stripe salamanders display little evidence of stress after short-term captivity, they could be considered well-suited for herpetological research where animals must be held captive.

Results generated here show a degree of consistency with those from polymorphic lizard species, despite different approaches being used to measure stress. First, detection of differences in N/L ratios between color forms of *P. cinereus* is consistent with recent evidence of corticosterone differences among color forms of the lizard *P. melisellensis* (Huyghe et al., 2009). Secondly, if we consider these differences to be differential stress reactions to capture and holding, this too would be consistent with work on another polymorphic lizard *Uta stansburiana* (Comendant et al., 2003), where certain color forms show stronger stress reactions (i.e. corticosterone increases) in response to a stressor than do others.

Finally, we point out that the differences in stress levels between color forms of the red-backed salamander could have consequences to the lives of both, but especially for the lead-phase salamanders. While studies on amphibians are few, we know from work with other taxa that the consequences of having chronically high baseline stress levels (measured either directly or with blood cells) include low growth rates (Moreno et al., 2002), increased disease susceptibility (Al-Murrani et al. 2006), and low survival during unfavorable environmental conditions (Romero and Wikelski 2001). If lead-phase *P. cinereus* salamanders do indeed have higher baseline stress levels than red-phase individuals, then these consequences might be realized. Interestingly, recent work showed that lead-phase forms are actually increasing in number relative to red-stripe forms (Gibbs and Karraker, 2006), which is in contrast to what one might expect based on results from the current study. We hope that additional research into the stress physiology of this species will help to reconcile these issues.

**Acknowledgements** We thank J. Maerz, J. DeVore and K. Cecala for help collecting salamanders. Funding for AKD during this project was provided by the D.B. Warnell School of Forestry and Natural Resources and a grant from the Morris Animal Foundation. Support for the field work of this project came from an NSF grant (NSF-DEB DEB0542974) to John C. Maerz.

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