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Physiological indices of stress in wild and captive garter snakes: Correlations, repeatability, and ecological variation ☆

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ABSTRACT

Glucocorticoids and leukocyte ratios have become the most widespread variables employed to test hypotheses regarding physiological stress in wild and captive vertebrates. Little is known, however, regarding how these two indices of stress covary in response to stressors, their repeatability within individuals, and differences in response time upon capture. Furthermore, few studies compare stress indices between captive and wild populations, to assess potential alteration of stress physiology in captivity. To address these issues, we examined corticosterone (CORT) and heterophil to lymphocyte (H:L) ratios in two ecotypes of the garter snake *Thamnophis elegans*. We found that CORT and H:L ratios were not correlated within individuals, and both variables showed little or no repeatability over a period of months. CORT levels, but not H:L ratios, were higher for individuals sampled after 10 min from the time of capture. However, both variables showed similar patterns of ecotypic variation, and both increased over time in gravid females maintained in captivity for four months. We suggest that CORT and H:L ratios are both useful, but disparate indices of stress in this species, and may show complex relationships to each other and to ecological and anthropogenic variables.

1. Introduction

The most widely-measured indicators of physiological stress in free-living vertebrates are glucocorticoids, which generally increase within minutes of capture in wild-caught animals and may remain elevated with sustained handling and/or captivity (Romero and Reed, 2005; but see Cree et al., 2000; Kahn et al., 2007). Glucocorticoids also play pivotal roles in other diverse aspects of physiology, ranging from a role in mobilizing foraging behavior, to seasonal variation associated with mating and/or reproduction, making an understanding of natural variation in glucocorticoids essential to understanding stress physiology both in the wild and in captivity (Johnstone et al., 2012).

While glucocorticoids are often assayed to evaluate the physiological response to stress by the hypothalamic–pituitary–adrenal (HPA) axis, another increasingly common method of assessing physiological stress involves quantification of circulating leukocyte ratios—neutrophil to lymphocyte ratios in mammals, amphibians, and some fishes, and heterophil to lymphocyte (H:L) ratios in birds, reptiles, and other fish species (reviewed in Davis et al., 2008). Studies in poultry have demonstrated a causal relationship between the main circulating glucocorticoid, corticosterone (CORT), and H:L ratios (Gross and Siegel, 1983), with higher CORT resulting in increased movement of lymphocytes out of circulation into more peripheral tissues, thereby—in association with a rise in circulating heterophils—generating an increase in circulating H:L ratios (Dhabhar et al., 1994, 1996). This relocation of leukocytes is thought to be a mechanism for redistributing cells to locations where their actions are required during a stress response.

Changes in circulating leukocyte ratios have been linked to a range of ecological and anthropogenic stressors in wild species (reviewed in Davis et al., 2008). Assessment of leukocyte ratios has also been considered a promising method for the assessment of physiological stress due to advantages such as a slower response time than that of glucocorticoids, the need for only a very small amount of blood to make a blood smear, and little monetary expense (Davis, 2005). However, recent studies have suggested that the relationship between CORT and leukocyte ratios is not always straightforward, and may vary across taxa (Vleck et al., 2000; Case et al., 2005; Müller et al., 2011; Seddon and Klukowski, 2012). In general, little is yet known regarding (1) natural variation in the relationship between CORT and leukocyte ratios,

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particularly in non-avian species, (2) repeatability of both CORT and leukocyte ratios within individuals, (3) how quickly leukocyte ratios change in response to capture stress, (4) how leukocyte ratios covary with ecological and demographic factors in the wild and (5) how long-term captivity affects both CORT and leukocyte ratios.

To contribute to a greater understanding of these issues, we examined both CORT and H:L ratios in populations of the western terrestrial garter snake *Thamnophis elegans*, which reside in the vicinity of Eagle Lake in Lassen County, California. These populations of garter snake can be classified into two ecotypes that differ with regard to life-history strategies, resource availability, predation pressure, parasite load, and numerous physiological measures (e.g., Bronikowski and Arnold, 1999; Bronikowski, 2000; Sparkman and Palacios, 2009; Sparkman et al., 2009; Robert and Bronikowski, 2010; Sparkman et al., 2013). Populations of a fast-living ecotype live along the shore of Eagle Lake, and exhibit fast growth, high annual reproduction, and low annual survivorship; in contrast, populations of a slow-living ecotype reside in nearby montane meadows and exhibit slow growth, low annual reproduction, and high annual survival (Bronikowski and Arnold, 1999; Miller et al., 2011). Two previous studies have examined CORT in these two divergent ecotypes in both captive and wild conditions (Robert et al., 2009; Palacios et al., 2012). The first reported that slow-living gravid females had significantly higher CORT than fast-living gravid snakes in the field, and the second confirmed this pattern across individuals of both sexes, but by extending the study to multiple years found that this phenomenon was not always observed. Robert et al., 2009 also reported significantly higher CORT values in females in captivity than in the field, irrespective of ecotype.

Our goal in this study was to extend our understanding of the comparative physiology of stress in free-living vertebrates through assessing the dynamics of CORT and H:L ratios in garter snakes. To this end, we examine (1) whether CORT and H:L are correlated within individuals in the field and/or in captivity, (2) whether CORT and H:L ratios are repeatable within individuals sampled both in the wild and in captivity, and (3) whether time elapsed between capture and bleeding affects H:L ratios as it does CORT. We also test for (4) natural variation in H:L ratios in the wild, to determine whether they vary between ecotypes as previously reported for CORT (Palacios et al., 2012). Finally, we test for (5) changes in CORT and H:L ratios over time in gravid adult females captured in the field and maintained in captivity for four months.

In general, if CORT and H:L ratios can be considered redundant indices of stress in garter snakes, then we predict that CORT and H:L will be correlated within individuals, and show similar patterns with respect to ecotype and overall. If H:L ratios increase in circulation in response to the increased glucocorticoid levels elicited by a stressor, as demonstrated in other taxa, then we predict that H:L ratios will show slower changes in response to stress than CORT. Finally, if captivity acts as a physiological stressor but individuals are able to adapt quickly to novel conditions, then we predict that captive individuals will initially show higher CORT and H:L levels than free-living individuals, but levels will return to field baseline in the long-term.

2. Materials and methods

2.1. Ethical procedures

All animal handling procedures were carried out in accordance with the standard animal care protocols and approved by the Iowa State University Animal Care and Use Committee (IACUC #: 3-2-5125-J). The State of California Department of Fish and Wildlife granted scientific research permits.

2.2. Field methods

Free-ranging garter snakes (*T. elegans*) were captured by hand from three lakeshore and four meadow populations in the vicinity of Eagle Lake in Lassen County, California in their summer reproductive season (June 11–22) and fall non-reproductive season (September 14–20) in 2010. All snakes were bled from the caudal vein, and time between capture and sampling was recorded. Blood samples were centrifuged in a field centrifuge, and plasma was snap-frozen in liquid nitrogen and stored at −80 °C until analysis. In June, blood smears were made from freshly drawn blood, fixed in methanol, and stained with Wright–Giemsa stain at the College of Veterinary Medicine at Iowa State University. Blood smears were not made for September samples. All snakes were sexed via hemipene eversion, weighed, measured for snout-vent-length (SVL), and palpated for the presence of embryos. After processing, snakes were released at site of capture except for forty-four gravid females representing two lakeshore and four meadow populations that were shipped overnight to Iowa State University in June.

2.3. Laboratory methods

Gravid females were housed individually in 40 L glass aquaria with corncob substrate and water dishes with hollow rims for shelter. Aquaria were positioned with one end on a heating element, which produced a permanent thermal gradient within each aquarium between 25 and 34 °C at opposite ends to allow thermoregulation. Snakes were maintained on 12:12 light–dark daily cycles with the thermal gradient available continuously. Females were fed thawed frozen mice once per week to satiation. All females were bled twice while in captivity: pre-parturition on August 9, and post-parturition on October 27. Blood smears were made at each of these time points. Each female gave birth on a single day between 12 August and 19 September.

Relative abundances of lymphocytes, monocytes, heterophils, and basophils were estimated from both field and captive-derived blood smears by classifying the first 100 leukocytes encountered under 1000× magnification using a compound microscope as described in Palacios et al. (2009). The proportion of heterophils and lymphocytes was used to calculate the H:L ratio for each individual.

Levels of plasma CORT were determined from field and captive samples using double-antibody radioimmunoassay kits (Catalog # 07–102103, MP Biomedical, Orangeburg, NY, USA) that had already been validated for use in our study system (Robert et al., 2009). We followed the protocol of Robert et al. (2009), except that plasma samples were diluted 1:80 (instead of 1:40) because this dilution proved optimal for the range of samples in the present study. Four samples exceeded the highest value of our standard curve (i.e. 450 ng/mL) and thus were excluded from our analyses. We used the kit-provided low control to calculate intra- and inter-assay coefficients of variation of CORT concentrations. Mean intra-assay variation was 9% whereas mean inter-assay variation was 8%. All samples were run in duplicate.

2.4. Statistical analyses

Plasma CORT and H:L ratios were $\log_{10}$-transformed to achieve normality, and transformed values were used throughout. With the exception of the analysis of the effects of time elapsed between bleeding and capture, all analyses of field samples were restricted to individuals bled within 10 min of capture, during which timeframe CORT values do not positively correlate with sampling time and are therefore considered as baseline values (Palacios et al., 2012). All analyses were conducted with SAS software (SAS 9.3, SAS Institute Inc., Cary, NC, USA).

2.4.1. Correlations, repeatability & sampling time

To test whether CORT and H:L ratios were related within individuals we conducted simple correlation analyses between the two variables for snakes sampled upon capture in the field in June, as well as for captive adult females sampled in August (pre-parturition) and October (post-parturition).