



## Physiological indices of stress in wild and captive garter snakes: Correlations, repeatability, and ecological variation<sup>☆</sup>



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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.

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### 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  $-80^{\circ}\text{C}$  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  $^{\circ}\text{C}$  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 $\times$  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).

To test whether CORT and H:L ratios were repeatable over time within individuals, we calculated the intraclass correlation coefficient ( $r$ ) (Lessells and Boag, 1987) for each pair of sampling periods for individuals that were sampled repeatedly—i.e., June/August, August/October, and June/October.

While the majority of snakes were bled within 10 min, a high capture rate resulted in some snakes being bled at later time points. To investigate the effects of sampling time on CORT and H:L ratios, samples taken upon capture in the field in June were divided into three categorical time intervals: bled within 10 min or less (CORT:  $N = 73$ ; H:L:  $N = 77$ ), bled between 11 and 27 min from the time of capture (CORT:  $N = 22$ ; H:L:  $N = 29$ ), or bled upon arrival to our processing camp at greater than 43 min from the time of capture (CORT:  $N = 11$ ; H:L:  $N = 13$ ). Note that no individuals were bled between 27 and 43 min. The maximum bleed time was 113 min after capture. Animals that were not bled within approx. 10 min of capture were kept individually in snake bags until the time of sampling. Analyses of variance (ANOVA) were conducted with time interval as the independent factor variable, and CORT and H:L as response variables.

#### 2.4.2. Ecotype and season in the field

We have previously reported a pattern of higher CORT levels in slow-living meadow than in fast-living lakeshore snakes in the field in some years, whereas other years no ecotypic differences have been observed (Palacios et al., 2012). To determine whether H:L ratios show a similar pattern to CORT, we tested for ecotype differences in both CORT and H:L ratios in the field. Other variables tested in the models included population nested within ecotype, sex, gravidity (gravid/non-gravid), SVL, body condition, time of day, and two-way interactions. Population nested within ecotype represents the effect of the seven different lakeshore and meadow populations. Population is treated as a fixed rather than a random effect to reflect microhabitat variation among populations within ecotypes. Body condition was defined as the residuals of the regression of  $\log_{10}$  body mass on  $\log_{10}$  SVL (Weatherhead & Brown 1996). Since body condition could not be accurately estimated in this way for gravid females due to the confounding effect of embryo mass, we excluded gravid females from a preliminary analysis that included body condition as a covariate. However, since there was no relationship between CORT or H:L ratios and body condition in non-gravid individuals, we re-included gravid females for the full analysis without body condition in the model. Analysis of covariance (ANCOVA) models were constructed in a stepwise manner, with the final model selected according to the lowest Akaike's Information Criterion (AIC) value (Burnham and Anderson, 2002).

We tested for seasonal changes in CORT by conducting an ANCOVA comparing June and September samples of wild-caught adult females of both ecotypes. Both gravid and non-gravid females were used from June, as well as all adult females from September—a sample which was likely a mixture of females that were not reproductive in that year, or had given birth in August.

#### 2.4.3. Repeated measures

A repeated measures analysis of CORT and H:L in adult gravid females was conducted via the restricted maximum likelihood (REML) method performed by PROC Mixed in SAS, which allows inclusion of subjects for whom observations are missing at random for one or

more time points. The analysis included repeated measures of females sampled upon capture in the field in June (CORT:  $N = 21$ ; H:L:  $N = 15$ ), and pre-parturition in August (CORT:  $N = 42$ ; H:L:  $N = 43$ ), and post-parturition in October (CORT:  $N = 43$ ; H:L:  $N = 43$ ) as the dependent variables. We tested for effects of time and ecotype, and the interaction between them.

### 3. Results

#### 3.1. Correlations, repeatability & sampling time

There was no correlation between CORT and H:L ratios in either field animals as a whole, or in the subset of gravid females brought into captivity and sampled in August and October (Table 1, Fig. 1). Interestingly, however, the four field individuals that had CORT levels that exceeded the highest concentrations of our standard curve (i.e., 450 ng/mL) and were consequently removed from our analyses, were also the ones showing the highest H:L ratios (data not shown). There was weak repeatability of CORT levels within individuals in captivity, with August significantly related to October ( $r = 0.27$ ;  $F_{39,40} = 1.72$ ;  $P = 0.045$ ), but all other repeatabilities were highly non-significant (CORT: June/August— $r = -0.14$ ;  $F_{19,20} = 0.679$ ;  $P = 0.798$ ; June/October— $r = -0.27$ ;  $F_{19,20} = 0.257$ ;  $P = 0.998$ ; H:L June/August— $r = -0.27$ ;  $F_{14,15} = 0.261$ ;  $P = 0.992$ ; August/October— $r = -0.48$ ;  $F_{39,40} = 0.350$ ;  $P = 0.999$ ; June/October— $r = -0.29$ ;  $F_{14,15} = 0.230$ ;  $P = 0.995$ ). There was a significant effect of sampling interval for CORT, with levels significantly higher in blood samples taken at more than 10 min post-capture (Fig. 2;  $F_{2,103} = 6.08$ ,  $P = 0.003$ ). In contrast, there was no evidence of change in H:L ratios between 10 min, 11–27 min and >43 min sampling intervals (Fig. 2;  $F_{2,116} = 0.244$ ,  $P = 0.784$ ).

#### 3.2. Ecotype and season in the field

The final model for June field CORT included ecotype, population nested within ecotype, gravidity, and SVL, whereas the final model for H:L ratios included ecotype and population nested within ecotype. Meadow snakes had higher H:L ratios than lakeshore snakes (Table 2, Fig. 3). While there was also a trend for higher CORT levels in meadow snakes, it was not significant (Table 2, Fig. 3). Gravid females had lower CORT levels than non-gravid individuals (male or female), and CORT increased with SVL (Table 2).

With respect to seasonal effects, CORT differed significantly between meadow, but not lakeshore females between the two field sampling dates, with meadow females exhibiting higher levels in September than in June (see Fig. 4a; ecotype:  $F_{1,36} = 13.86$ ,  $P = 0.001$ ; month:  $F_{1,36} = 6.39$ ,  $P = 0.016$ ; month \* ecotype:  $F_{1,36} = 5.97$ ,  $P = 0.020$ ). To aid in the interpretation of this finding, we also conducted an

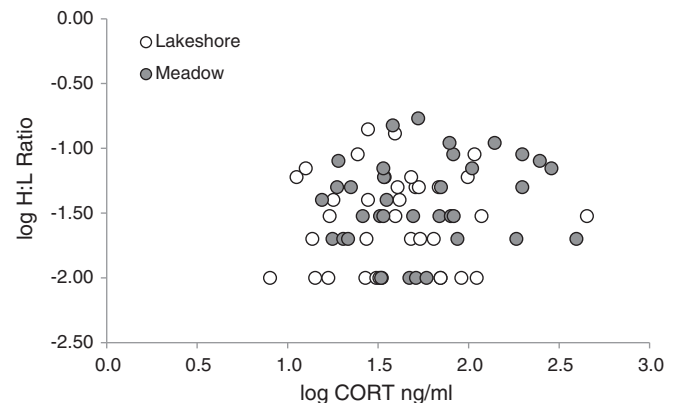
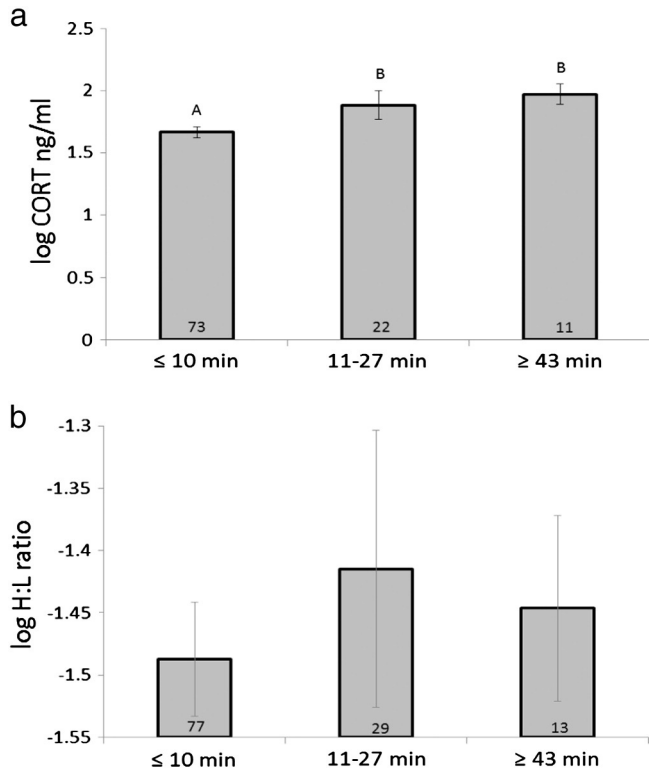


Fig. 1. There was no correlation between log-transformed field CORT and H:L ratios in garter snakes (*T. elegans*), of both ecotypes in June ( $N = 36$  lakeshore, 35 meadow).

Table 1  
Correlations between CORT and H:L within individual garter snakes (*T. elegans*).

	N	t	P
June (field; all individuals)	71	1.20	0.233
June (field; gravid only)	14	1.09	0.297
August (captive; preparturition)	41	-0.77	0.444
October (captive; postparturition)	42	1.18	0.245



**Fig. 2.** Mean log-transformed CORT and H:L ratios for garter snakes (*T. elegans*) sampled during different time intervals post-capture. Each time interval contains unique individuals. Standard errors of the means are shown. Letters denote significant differences between time points, and sample sizes for each time point are indicated at the base of columns.

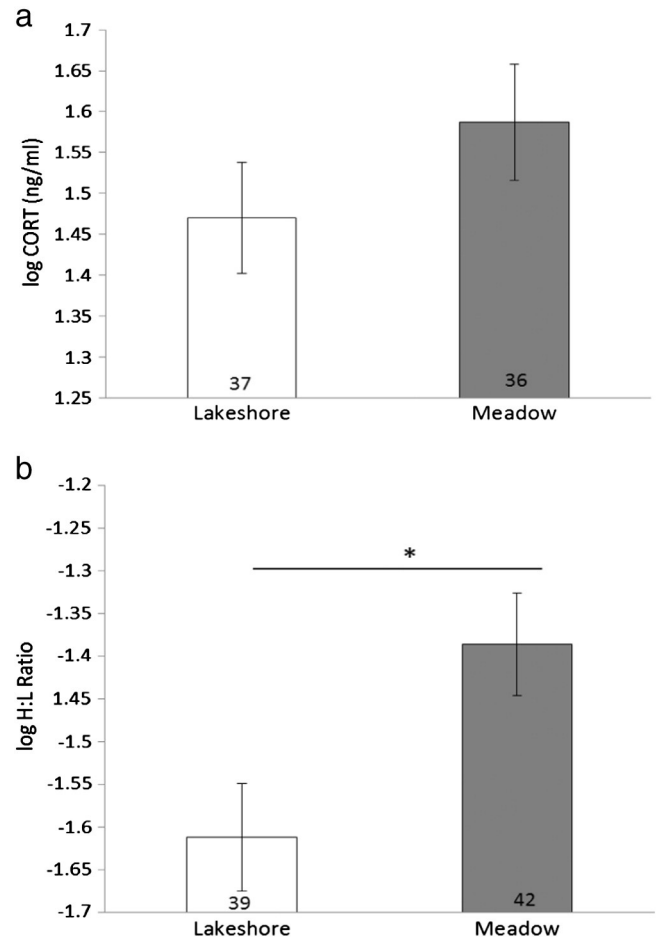
ANCOVA to test for a difference in body condition between months for non-gravid females of both ecotypes. Higher levels of CORT in meadow snakes were accompanied by a reduction in body condition seen in meadow, but not lakeshore snakes (ecotype:  $F_{1,34} = 2.16$ ,  $P = 0.151$ ; month:  $F_{1,34} = 0.21$ ,  $P = 0.651$ ; month \* ecotype:  $F_{1,34} = 4.96$ ,  $P = 0.0327$ ).

### 3.3. Repeated measures

CORT levels in both ecotypes increased over time spent in captivity, with August levels significantly higher than field levels in June, and October levels significantly higher than both August and June (Table 3; Fig. 4a; least square means comparisons all  $P < 0.001$ ). H:L ratios also increased over time (Table 3; Fig. 4b). A comparison of least square means show that H:L in the August sample was significantly greater than field levels ( $P = 0.012$ ), and was followed by an even greater increase between August and October sampling times ( $P < 0.0001$ ). Furthermore, lakeshore snakes showed a marginally significant ( $P = 0.085$ ) trend for a greater increase in H:L ratios overall than meadow females (Table 3; Fig. 4b). There was no overall ecotype difference in CORT in the repeated measures analysis (Table 3).

**Table 2**  
ANOVA of CORT and H:L ratios in garter snakes (*T. elegans*) in June in the field.

	Effect	df	F	P
CORT	Ecotype	1,64	1.84	0.179
	Population (ecotype)	5,60	0.26	0.050
	Gravidity	1,64	8.90	0.004
	SVL	1,64	7.14	0.010
H:L	Ecotype	1,74	6.77	0.011
	Population (ecotype)	5,74	2.58	0.096



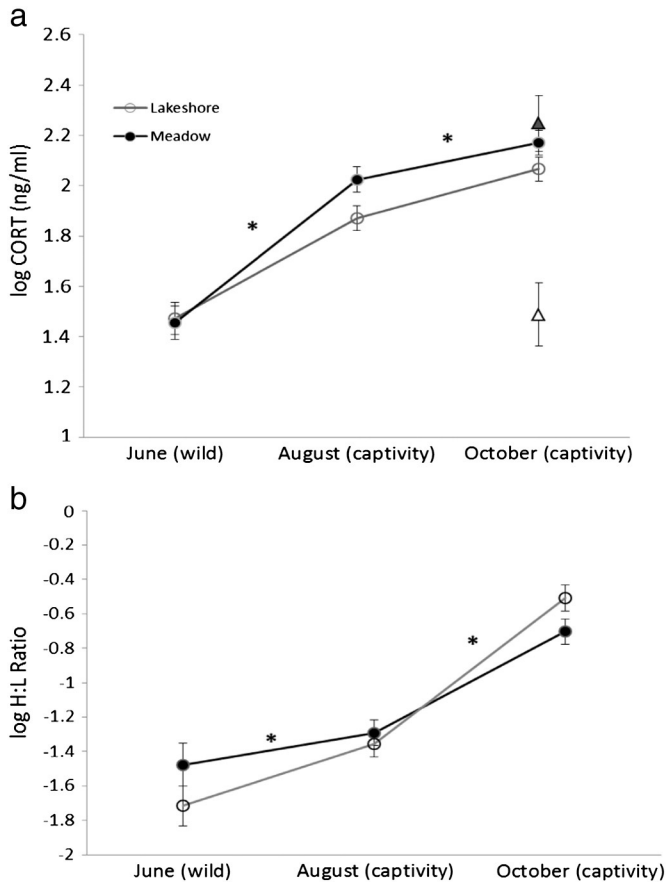
**Fig. 3.** Least square means and standard errors of the means of log-transformed CORT (a) and H:L ratios (b) in the field in both ecotypes of garter snakes (*T. elegans*). Sample sizes for each time point are indicated at the base of columns. Statistics are given in Table 2.

## 4. Discussion

We found that CORT and H:L ratios were not correlated within individual garter snakes, either in the wild or in captivity (Fig. 1, Table 1). This finding is consistent with recent studies in birds and other reptiles that suggest that a correlation between CORT and H:L ratios may be absent or weak, and these indices represent at least partially independent responses to stress or other factors (Vleck et al., 2000; Case et al., 2005; Müller et al., 2011; Seddon and Klukowski, 2012). Interestingly, however, we also found that CORT and H:L ratios do exhibit some of the same general trends with regard to both ecotype and temporal changes in captivity. Meadow snakes had higher H:L ratios overall than lakeshore snakes in the field (Fig. 3), a finding which—though meadow snakes did not show significantly higher CORT in this study—is consonant with higher levels of CORT in meadow snakes reported in other years (Palacios et al., 2012). Furthermore, both ecotypes exhibited an increase in CORT and H:L ratios over time spent in captivity (Fig. 4). The latter in particular suggests that H:L ratios may indeed respond to environmental stressors, as demonstrated in other taxa (Davis et al., 2008), in spite of not showing a correlation with CORT within individuals.

It does appear that H:L ratios have a demonstrably lower response time to capture stress than CORT in *T. elegans*, as H:L ratios did not differ significantly between animals bled within 10 min or less and those bled between 11–27 and 43–113 min post-capture (Fig. 2). Note, however, that variation among individuals was high, and repeated measures of the same individuals over time should be conducted in the future, to ascertain whether there is indeed no significant rise in H:L ratios over this time. If this proves to be the case, it may be easier for researchers





**Fig. 4.** Least square means of repeated measures of adult female garter snake (*T. elegans*) log-transformed CORT (a) and H:L ratios (b) in the wild and at two time points in captivity. For comparative purposes, triangles indicate mean September CORT for wild adult meadow (closed) and lakeshore (open) females. Asterisks (\*) indicate significant differences between consecutive time points for both ecotypes. Note that the full sample of wild-caught females (data not shown) have similar mean CORT values for June as the subsample brought into captivity and portrayed here. Statistics are given in Table 3.

to collect baseline data on this index of stress than others with a more rapid response time. However, the lack of a correlation between H:L ratios and CORT within individuals confirms that for this species, the former cannot be used as a proxy for the latter.

The lack of repeatability in H:L ratios from repeated measures of gravid females sampled in the field in June and then again in captivity in August and October is also of interest. It is similar to the findings in female tree swallows, *Tachycineta bicolor*, where H:L ratios were not significantly repeatable over multiple breeding seasons (Ochs and Dawson, 2008). We did find marginal repeatability of CORT between the two captive sampling time points for these gravid females, which suggests some consistency in ranking among individuals pre- and post-parturition while in captivity. Studies of repeatability of CORT in wild species have primarily been conducted on birds and amphibians, with varying results: some show repeatability only in baseline values whereas others only in stress-induced values; yet others show variation in repeatability according to sex, developmental stage, or breeding

**Table 3**

Repeated measures analysis of gravid garter snakes (*T. elegans*) for samples taken in the field, pre-parturition, and post-parturition.

	Effect	df	F	P
CORT	Ecotype	1,42	3.31	0.076
	Time	2,60	89.32	<0.0001
H:L	Ecotype	1,41	0.87	0.331
	Time	2,54	31.19	<0.0001
	Ecotype * time	2,54	2.58	0.085

season (Wada et al., 2008; Rensel and Schoech, 2011; Ouyang et al., 2011; Narayan et al., 2012; Narayan and Hero, 2013; Narayan et al., 2013). Thus, the low or absent repeatability in these stress indices in garter snakes is not unprecedented in other groups, and care must be taken when using such measures as proxies for overall fitness. Nevertheless, further research must be done in our system to determine whether the lack of repeatability in CORT and H:L ratios between June and August in this species is due to individual variation in response to captivity, or whether these indices are equally unrepeatable in the field over long time intervals (i.e., a matter of weeks or months). In addition, assessment of the potential effects of sex and other factors in repeatability of these stress indices is warranted.

The effects of captivity on indices of physiological stress vary widely across species, with some species showing signs of a return to pre-capture status within days of confinement (e.g., greenfinches, Sepp et al., 2010; leopard frogs and tiger salamanders, Davis and Maerz, 2011; Fijian ground frogs, Narayan et al., 2011), but others showing more sustained changes (e.g., skinks, Jones and Bell, 2004; mole salamanders, Davis and Maerz, 2008; Davis and Maerz, 2011; spider monkeys, Rangel-Negrín et al., 2009; blackbirds, Adams et al., 2011; ground frogs, Narayan and Hero, 2011; lynx, Fanson et al., 2012). In our study, adult gravid females returned to the lab showed increases in both CORT and H:L ratios over time spent in captivity (Fig. 4). The most straightforward interpretation of this pattern is that captivity generates sustained changes in stress physiology, lasting from weeks to months. Another species in the same natricine subfamily as *Thamnophis*, the northern water snake *Nerodia sipedon*, also shows elevated levels of CORT in captivity relative to wild values when assessed 5–8 days post-capture (Sykes and Klukowski, 2009), however longer term data of captive stress for this species were not available. It is surprising that CORT and H:L levels rose not only between June in the field and August in captivity, but also between August and October in captivity (Fig. 4b). However, a gradual increase in CORT over three weeks spent in captivity has been reported in tree lizards, *Urosaurus ornatus* (Moore et al., 1991), and it may be that some captive reptiles take weeks or months to reach maximum CORT levels. It is also interesting to note the different time dynamics shown by the two indices of stress, with CORT showing the largest increase between June and August and then slowing down, while the rise in H:L ratios was initially slow and became more pronounced between August and October. These findings are also in accordance with the notion that H:L ratios change slower than CORT levels in circulation, which supports the hypothesis that it is the elevation of CORT that drives the subsequent re-distribution of leukocytes (Davis et al., 2008).

Nevertheless, there are other potential interpretations of the observed elevation in CORT and H:L ratios between June and October in these gravid snakes. In fact, the maximum values of CORT measured in this study were from meadow animals bled in the field in September. Thus, it is also conceivable that the change in CORT and H:L ratios over time is associated with (1) a change from gravid to non-gravid reproductive status, (2) seasonality, and/or (3) vitellogenesis.

A change in reproductive status did indeed occur over the sampling period. However, gravid red-sided garter snakes, *Thamnophis sirtalis parietalis*, in the lab have been shown to have decreasing rather than increasing CORT over the gestation period (Whittier et al., 1987). Furthermore, a previous study from our laboratory (Robert et al., 2009) shows CORT is much higher than field baseline in gravid females a mere six days after arrival in captivity in July. Nevertheless, a study with either males or non-reproductive females will be necessary to resolve whether the observed changes in CORT and H:L ratios over time are due to captive stress rather than to changes in reproductive physiology.

The potential for seasonality in CORT and H:L (2) that persists in captivity is also a factor to consider. Seasonality in CORT has been widely reported (Romero, 2002), and there is evidence that H:L ratios can also change seasonally (e.g., Owen and Moore, 2006; Ochs and Dawson, 2008; Machado-Filho et al., 2010; Pap et al., 2010). Our sample of

meadow snakes suggests that field levels in September of 2010 were indeed higher than those in June (see Fig. 4). However, this was not the case for lakeshore snakes, for whom values in the field were comparable during both time points. So at least for lakeshore snakes, pre-programmed seasonal variation in CORT levels cannot explain the increase we observe in captivity.

The third hypothesis, that the change in CORT and H:L ratios over time is due to involvement in vitellogenesis (3), is more easily dismissed, as all *Thamnophis* and *Nerodia* (both members of the subfamily Natricinae) species examined to date exhibit secondary vitellogenesis in the spring (type I vitellogenesis) (Whittier and Crews, 1986; Ford and Karges, 1987; reviewed in Aldridge, 1979), suggesting that *T. elegans* is unlikely to be an exception. Thus in general, though effects of gravidity and seasonality may certainly be in play, it seems likely that captivity itself is contributing to changes in stress physiology, achieving CORT levels that approximate stress response levels documented in June in the wild (Palacios et al., 2012). It remains to be determined whether these animals are indeed under chronic stress, or whether the HPA axis has been reset to a new optimum in novel laboratory conditions (see Fanson et al., 2012).

Even were our findings not a result of captivity per se, but a result of gravidity or seasonality, it would be important to understand how such dramatic changes in stress physiology over time are affecting other data collected in captivity. Given these findings, along with our report regarding minimal repeatability, especially in H:L ratios, and only moderate evidence of any correspondence between H:L ratios and CORT (and none within individuals), we emphasize that more work must be done not only to learn more about the ecology and evolution of the stress response, but also to make decisions regarding the placement of animals in captivity, to ascertain that physiological stress is not posing a threat to their general welfare, and to the validity of scientific findings (especially if the purpose of studying captive animals is to infer what they would experience or how they would respond in the wild). We have presented a level of complexity here that may be present in many wild species brought into captivity, and therefore urge that great care be taken when interpreting data on indices of stress, their relationships to each other, and the positive, neutral, and/or negative effects of captivity.

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