

# A meta-analysis of plasma corticosterone and heterophil : lymphocyte ratios – is there conservation of physiological stress responses over time?

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## Summary

1. There is growing need for reliable biomarkers of the physiological stress response in basic ecological investigations and conservation-specific applications. One effect of stress hormone secretion is the alteration of circulating leucocytes, specifically an increase in the heterophil : lymphocyte ratio (HLR). There have been numerous studies and reviews of the relationship between the stress response and HLR, yet the data have not been systematically analysed across studies.

2. Here, we performed meta-analyses of studies in which paired values of plasma corticosterone (CORT) and HLR were available to investigate the relationship between these parameters. Specifically, we analysed CORT and HLR as baseline measures between treatment (or ‘stressed’ populations) and control (or ‘reference’ populations). Additionally, we investigated the relationship within CORT and HLR to identify temporal patterns of the response as a result of the duration of the stressor.

3. Across studies, we identified that CORT and HLR are significantly elevated in populations subjected to environmentally stressful conditions. While not significantly different, CORT tended to be more elevated than HLR in the stressed populations.

4. We found that there is a significant, negative relationship between CORT and the temporal duration of environmental stress. Additionally, we found a significant break point in this response at 85 days of stress duration, above which there is not a clear relationship between CORT and duration. This indicates that the CORT response to environmental stress attenuates.

5. We found a small but positive significant relationship between HLR and temporal duration of environmental stress. This suggests that the elevation of HLR in response to environmental stress does not decrease over time.

6. Our data identified that data gaps of wild populations of species exist and that more studies pairing physiological stress responses will further our understanding of how populations respond to environmental challenges. We found that CORT and HLR responded differently to stress, depending on captivity status, whether the study population was the domestic chicken, and the sex of the study population.

**Key-words:** differential leukocytes, environmental stressor, global change, glucocorticoid, immune system, stress responses

## Introduction

Advances in physiological techniques have blurred traditional divisions between physiology and ecology such that

ecologists increasingly use the tools of physiologists to answer fundamental ecological questions (e.g. Sapolsky, Romero & Munck 2000; Romero 2004; Davis, Maney & Maerz 2008). For example, ecologists have been interested in determining physiological markers of ‘stress’ in an environment to examine effects of global change (McEwen & Wingfield 2003), which include anthropogenic disruptions

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that may reduce the fitness of populations (Bonier *et al.* 2009). Assays of the physiological stress response are not only important in conservation-driven questions, but also useful for basic ecological investigation as physiological coping mechanisms to environmental challenges are a natural process within all populations (Romero & Wikelski 2001; Wikelski & Cooke 2006). In the presence of increased sublethal anthropogenic stressors on natural systems, including habitat modification and degradation, anthropogenic contaminants and human-mediated spread of pathogens (Wingfield *et al.* 1997), biomarkers of long-term stressors (i.e. chronic stress) are of added importance to understanding negative effects of global change.

The use of the term 'stress' remains difficult to define as it may either indicate environmental constraints that decrease the fitness of individuals or populations (herein referred to as a 'stressor'), or it may refer to altered allostasis (McEwen & Wingfield 2003) resulting from an individual responding to the stressor (herein referred to as the 'stress response'). Moreover, understanding the results of stress responses is controversial as the stress response itself may either enhance fitness by protecting individuals from negative consequences of the stressor (Romero, Dickens & Cyr 2009) or it may reduce fitness through altering energetic allocation (especially in response to long-term stressors) from physiological parameters such as growth, maintenance and/or reproduction (Thierry, Ropert-Coudert & Raclot 2013).

In vertebrates, the stress response is regulated by the hypothalamic–pituitary–adrenal (HPA) axis; activation of the HPA axis causes glucocorticoid (GC) secretion which further elicits a cascade of physiological effects. Activation of this response is generally considered adaptive in the short term, as it allows an individual to maintain homeostatic control while confronting a discrete threat. However, chronic stress (and thus potential chronic elevation of GC concentration) can be detrimental to individuals (Wingfield *et al.* 1998; Sapolsky, Romero & Munck 2000; Greenberg, Carr & Summers 2002; Busch *et al.* 2008). The Cort-Fitness Hypothesis (Bonier *et al.* 2009) suggests that baseline circulating GC concentration is negatively correlated with fitness; the rationale for this hypothesis is that GC concentration is a measure of environmental challenge that individuals and populations experience. While circulating GC may correlate with the degree of encountered stressors, inconsistencies within individuals are commonly observed, thus causing skepticism of the degree of interpretation warranted by this measure (e.g. Bonier *et al.* 2009; Ouyang, Hau & Bonier 2011).

The classic effects of GC secretion are alterations in energy usage and storage to enable an individual to initiate an emergency life history strategy (Wingfield & Kitaysky 2002). In addition to these metabolic effects, GC secretion has immuno-modulatory effects, such as altering the circulating leucocyte profile (Sapolsky, Romero & Munck 2000). Following the perception of a stressor, circulating GC reaches a maximum peak within a few minutes to

hours (i.e. the 'acute stress' response). This surge in GC causes a change in circulating leukocytes within a few hours, wherein phagocytes (such as neutrophils) increase in abundance and lymphocytes decrease in abundance either through altered distribution (Dhabhar *et al.* 1996) or GC-mediated lymphoid cell apoptosis (Cidlowski *et al.* 1996). This change in circulating leukocyte profile is likely adaptive for initiating an emergency life history strategy, as the increase in phagocytes helps combat immediate infection (such as from the result of tissue damage from a predatory attack), while the decrease in circulating lymphocytes prevents an individual from unnecessarily wasting resources of the adaptive immune system. While this change in circulating leukocytes occurs relatively quickly, it is largely unknown how long these changes remain in an individual following GC elevation. Thus, rather than assaying GC concentration directly, an alternative assay of the stress response focuses on this specific immuno-modulatory effect of GC by measuring the heterophil (or neutrophil) : lymphocyte ratio (HLR; Davis, Maney & Maerz 2008). A review by Davis, Maney & Maerz (2008) demonstrated the benefits of measuring this predictable change in circulating leukocytes as a response of exposure to environmental stressors. Because there remains uncertainty in interpreting what baseline GC concentration indicates regarding chronic stress responses, Davis, Maney & Maerz (2008) suggested that HLR may have added benefits to indicate generalized responses of individuals to environmental stressors. This supposition is supported by a series of studies wherein individuals predictably alter their circulating leukocytes profile in response to exogenous GC administration (e.g. Dhabhar *et al.* 1994). However, a clear positive relationship between elevated GC and HLR has remained difficult to detect, especially in wild populations, as Müller, Jenni-Eiermann & Jenni (2011) failed to show a significant positive relationship between these two parameters.

In this study, we used meta-analysis to test which biomarker (i.e. increase in GC concentration or HLR) is more reliable for determining sustained responses to environmental stressors. We performed a meta-analysis across available published studies to test (i) whether there is a difference between the magnitudes of altered baseline GC concentration and HLR between stressful and non-stressful conditions and (ii) whether there is a temporal component to these stress responses that may suggest differences in their nature as a result of the duration of stressors.

Meta-analysis represents a powerful tool to assess large-scale patterns in data that may otherwise be obscured by high variability within studies (Osenberg *et al.* 1999). By pooling data across studies, conclusions can be inferred based on common features within studies, such as similar response parameters measured in different experimental subjects. Meta-analysis was the ideal approach for our study, as it allowed us to evaluate empirical data independently of the specific details of each stress study, and yet

we could still subdivide our data to test for potential variance due to experimental differences. We tested whether we could determine generalizations in the stress response of GC and HLR to better understand the nature of these assays of physiological stress.

## Materials and methods

### LITERATURE SEARCH AND DATA COLLECTION

We performed a systematic data base search in the Web of Science spanning all available, published studies up to 9 March 2012. Based upon a preliminary search, it was clear that there is an abundance of publications in which the study organisms use corticosterone (CORT) as the main GC, rather than cortisol. Thus, to prevent confounding our conclusions between differences in GC of the subject taxa, we limited our search to those taxa that utilize CORT and produce heterophils (i.e. amphibians, reptiles, crocodilians and birds). Thus, the search term employed in our study was 'corticosterone heterophil : lymphocyte'.

After performing the systematic literature search, we collected the following data of moderator variables (or covariates) from each publication: study species, sample size, error, sex, stress treatment type, duration of stressful treatment and captivity status (i.e. whether or not the study population was maintained in a captive setting or laboratory). The response variables collected from each study were mean baseline CORT and HLR in both control (or reference; i.e. 'unstressed') and treatment (i.e. 'stressed') populations. Because we were interested in contrasting simultaneous CORT and HLR responses to a stressor, we only collected data from those studies that presented paired values of CORT and HLR in both control (or reference) and treatment (or stressed) groups. If stress treatments (or populations) were not clearly identified in the study, we used descriptions included in the studies to classify the populations *a priori*. For example, if a toxin was used as the treatment, the population given the toxin was treated as 'stressed' and the negative control population was treated as the control. Based on the protocol of the included studies, we classified stressors into the following categories: food restriction, water restriction, altered density, altered climate, differing habitats (in free-ranging populations), exogenous non-steroid chemical, handling stress, bacterial challenge, non-bacterial parasite or pathogen, altered food composition and between-population differences in body condition (in free-ranging populations). Studies that were included in our meta-analysis are listed in Appendix S1 (Supporting information).

### CALCULATIONS AND ANALYSES

We used all weighted meta-analyses, except for identifying the temporal break point in the segmented regression. Weighted analyses were conducted in Comprehensive meta-analysis version 3 (CMA; Borenstein *et al.* 2014). The unweighted analysis was performed in the basic R package with 'segmented' package loaded (version 3.1; R Core Team 2014).

The effect size metric we used was the log response ratio (Osenberg *et al.* 1999), which was calculated as the ratio of ln-transformed treatment population means divided by ln-transformed control population means (hereafter, stress response effect sizes will be referred to as 'CORT' and 'HLR'). We used this effect size metric because it normalized our data in preliminary analyses, and the ratio represents a measure of the relative degree of change between the treatment and control groups. We considered responses significant if the 95% confi-

dence interval (CI) of the stress responses did not cross zero. Furthermore, we considered it a significantly positive response (i.e. lower 95% CI >0) when the physiological parameter (e.g. CORT or HLR) was increased in response to the stressor. Interpretation of this type of change indicates that the physiological parameters were significantly increased in the stressed population above the unstressed population. Confidence intervals for the overall physiological responses were calculated in CMA. Calculations in CMA utilized a mixed-effects model, in which a random-effects model is used to group studies within covariates and a fixed-effects model is used to generate overall effects among covariates. This model did not assume equal study-to-study variance among covariates. All comparisons among moderator variables (i.e. study covariates) were performed in CMA using mixed-effects models.

We compared the degree of change in the two measures of stress responses; thus, when an included study presented data from factorial block designs (i.e. exposed the same treatment groups to several concurrent stressors, yet presented those data separately), we only included the data with the greatest treatment effect. We selected the populations with the greatest mean effect sizes from factorial designs, as our interest was to identify the relationship between temporal duration of physiological responses to stress, and not simply if there is a physiological response to stress. Due to pseudoreplication, we could not include all data from factorial designs. While we did not include pseudoreplicates from factorial designs, we did include multiple effect sizes from single studies if the study included multiple independent population means for CORT and HLR. While most studies included specific times for the stress treatment (i.e. stress duration), if a study did not clearly identify a time for each treatment (e.g. some studies gave multiple times for a single stress response mean or presented a range of days over which the responses were measured), we treated the longest time published with the response data as that effect size's stressor duration. Additionally, we did not include effect sizes in the regression analyses if there was not an identifiable temporal duration of treatment (such as in free-ranging populations not exposed to experimental treatments).

We used regression analyses to identify temporal patterns in the responses. A simple linear regression tested the relationship between a response (either CORT or HLR) and the duration of the stress treatment. This linear model was made in CMA using weighted effect size data and a random-effects model that accounts for both within and among study variance. Linear regression parameters were contrasted using a standard major axis (SMA) analysis. Initial regression analyses examined the overall range of responses to stressful treatments of varying duration. Additionally, we were interested in further identifying discrete differences in the stress responses at different scales of stress duration (such as acute versus chronic). When we identified a significant negative effect of stress duration on stress response using the simple linear model, we subsequently performed a segmented regression analysis to identify the temporal location of the regression break point (i.e. at what duration of environmental stress the physiological response changed). A Davies' test of the segmented regression was used to test the significance of the change in slope. To identify the segmented regression relationship, we first used all of our data in the 'segmented' package in R. This analysis generated a significant break point, which we then used to test for the presence of this relationship using only the weighted data in CMA. To test this relationship, we ran a regression between response and stressor duration, with an interaction component of duration  $\times$  treatment greater or less than the break point identified in the segmented regression analysis. Thus, this secondary analysis allowed us to validate both the existence of the segmented regression relationship and the location of the temporal break point using the weighted data.

## Results

Our literature search yielded 135 total studies; however, only 43 studies contained data appropriate for our analyses which included 152 effect sizes for both CORT and HLR (Table 1). Within the entire data set, 35 studies included sample sizes and equivalent error terms to produce the 124 effect sizes used in weighted analyses. Data included in our analysis included 14 species between two taxonomic classes (reptiles and birds). Because of the high frequency of publications related to poultry production, most of our data were from the domestic chicken (e.g. 64% of effect sizes from the domestic chicken).

### OVERALL RESPONSE

The overall responses of both CORT and HLR were significantly positive. And, although we did not detect a significant difference between CORT and HLR, CORT was marginally greater than HLR (Fig. 1).

Additionally, we tested whether the responses of either CORT or HLR were influenced by variation in experimental covariates (i.e. taxonomic class, captivity status, whether the model organism was the domestic chicken, sex of the population and stressor type). We found that there were significant differences among all of these parameters for CORT, except for taxonomic class (e.g. there was no difference between birds and reptiles, Table 2). CORT was more significantly elevated (among grouping variables) in stressed populations that were captive (over wild), in populations that were not the domestic chicken, and in populations in which only females were studied. We found that

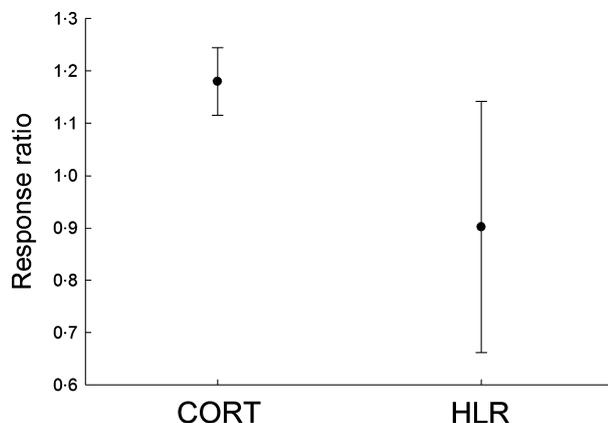
there were no differences among any of the covariates for HLR (Table 2) except for stressor type. For both CORT and HLR, stressor type had a significant effect on the physiological response (CORT and HLR, respectively:  $Q_{18} = 221.5$ ,  $P \leq 0.0001$ ;  $Q_{18} = 168.3$ ,  $P < 0.0001$ ). For CORT, the analyses indicated significant differences among experimental treatment parameters, yet there was no clear trend by which any specific treatment type yielded a response different than all others. The most notable differences among stressor treatments included data from one study that combined food restriction and altered thermal regime, which produced a CORT response that was significantly lower than the others, and two studies wherein an exogenous drug treatment was used, which produced a CORT response that was significantly elevated above the others (all experimental covariate differences listed in Appendix S2). For HLR, the mean effect size for five experimental treatments that included food restriction and six experimental treatments that included combined effects of handling and a bacterial challenge was elevated above all other stressor types. As with differences among treatment parameters for CORT, we cannot clearly indicate whether this difference is due to variance in sample sizes among treatment parameters, or whether it in fact is of biological significance.

### STRESS RESPONSE OVER TIME

Corticosterone effect sizes decreased over time while a slight positive relationship was observed between HLR and time (Fig. 2, Table 3). The SMA analysis of the two linear relationships indicated that there were significant

**Table 1.** Number of studies included (Studies) and effect size estimates (ES) in the meta-analyses identified by taxonomic class and species. Not all studies from which we collected data had sufficient error terms to perform weighted analyses, thus columns for studies and ES used in the weighted analyses are lower than columns for all data of studies and ES.

Total	Class	Species	Studies (all data)	ES (all data)	Studies (weighted)	ES (weighted)
			44	152	39	128
	Bird	Domestic chicken ( <i>Gallus gallus</i> )	28	106	20	82
		Domestic turkey ( <i>Meleagris gallapavo</i> )	2	7	2	7
		Domestic duck ( <i>Anas platyrhynchos</i> )	2	4	1	3
		Japanese quail ( <i>Coturnix japonica</i> )	2	11	2	11
		Adele penguin ( <i>Pygoscelis adeliae</i> )	1	2	1	2
		African grey parrot ( <i>Psittacus erithacus</i> )	1	3	1	3
		Florida scrub jay ( <i>Aphelocoma coerulescens</i> )	1	3	1	3
		Orange-winged Amazon parrot ( <i>Amazona amazonica</i> )	1	1	1	1
		Pied flycatcher ( <i>Ficedula hypoleuca</i> )	2	6	2	6
		White-crowned sparrow ( <i>Zonotrichia leucophrys</i> )	1	2	1	2
Total (birds)			41	145	32	
	Reptile	Ball python ( <i>Python regius</i> )	1	3	1	3
		Blue-tongued skink ( <i>Tiliqua scincoides</i> )	1	3	1	3
		Texas rat snake ( <i>Pantherophis obsoleta lindheimeri</i> )	1	1	1	1
Total (reptiles)			3	7	3	7



**Fig. 1.** There were significant, positive effect sizes for both log response ratios of corticosterone (CORT) and HLR (calculated as the ratio of ln-transformed treatment population means divided by ln-transformed control population means) in response to environmental stressors. While not significantly different, there was a tendency for the CORT effect size to be larger than the HLR effect size. Data points indicate the mean of each parameter and error bars represent 95% confidence intervals. HLR, heterophil : lymphocyte ratio.

differences between CORT and HLR in the slope of the regression ( $P < 0.001$ ), but that there were no differences in the elevation ( $P = 0.893$ ) or shift ( $P = 0.603$ ) of the regressions.

The segmented regression analysis indicated a break point at 85 days of stress treatment in the CORT response (Fig. 3; below this duration, a negative relationship was present

between CORT and stress duration [segmented linear model: intercept = 1.41; 0–85.4 days, slope =  $-0.013$ ; >85.4 days, slope =  $0.0005$ ;  $T_{122} = -2.628$ ,  $P = 0.0097$ ; Multiple  $R^2 = 0.124$ ]. The Davies’ test indicated that this change in slope was significant ( $P = 0.009$ ). Using the estimated break point at 85 days from the segmented regression analysis, we validated this relationship using the weighted data in CMA. This relationship was confirmed by a significant interaction between stressor duration and discrete time points below and above 85 days (break point  $\times$  duration interaction:  $Q_2 = 31.49$ ,  $P < 0.00001$ ).

Lastly, as was done by Müller, Jenni-Eiermann & Jenni (2011), we plotted CORT and HLR against each other to visually determine whether there was a direct relationship between the two parameters (Fig. 4). As found by Müller, Jenni-Eiermann & Jenni (2011), this indicated that there was not a direct relationship between elevated CORT and elevated HLR.

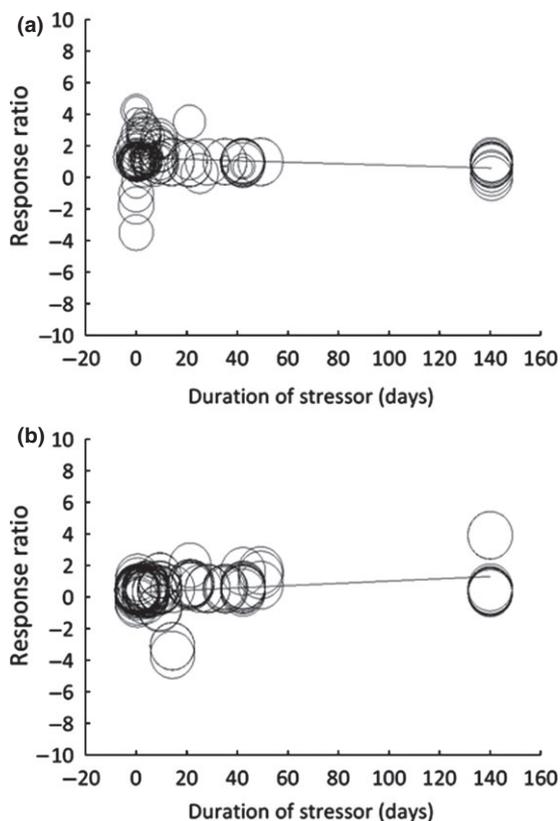
**Discussion**

Support has grown for the use of HLR as an indicator of stress in vertebrates (see Davis, Maney & Maerz 2008), yet the reliability of this measure has not been tested at large scales or compared directly to GC concentration (Müller, Jenni-Eiermann & Jenni 2011). Additionally, because of the large variation within and among individuals of circulating GC (Ouyang, Hau & Bonier 2011), questions remain regarding the utility of using baseline GC concentration to indicate chronic stressors.

**Table 2.** Results of weighted meta-analyses of effect sizes (ESs) of log response ratio CORT and log ratio HLR compared for differences among grouping variables of captivity status, taxonomic class, whether or not the study species was the domestic chicken (*Gallus domesticus*), and sex of the study population

Grouping variable	Number of ES	Estimate	Lower limit	Upper limit	Differences among groups		
					Q-value	P-value	
<b>Ln ratio CORT</b>							
Captivity	Captive	110	1.204	1.133	1.276	20.103	<0.0001
	Wild	14	1.014	0.971	1.056		
Taxonomic class	Bird	117	1.189	1.121	1.257	1.486	0.223
	Reptile	7	1.056	0.855	1.258		
Domestic chicken	Yes	82	1.101	1.023	1.180	11.478	0.001
	No	42	1.419	1.253	1.586		
Sex	Both	48	1.287	1.116	1.458	29.975	<0.0001
	Female	37	1.487	1.249	1.724		
	Male	39	0.894	0.796	0.993		
<b>Ln ratio HLR</b>							
Captivity	Captive	108	0.887	0.637	1.137	0.088	0.767
	Wild	14	1.025	0.147	1.904		
Taxonomic class	Bird	115	0.901	0.649	1.154	0.019	0.892
	Reptile	7	0.882	0.757	1.007		
Domestic chicken	Yes	79	0.795	0.460	1.130	1.433	0.231
	No	43	1.074	0.764	1.383		
Sex	Both	48	1.034	0.813	1.255	2.658	0.265
	Female	38	1.036	0.436	1.635		
	Male	36	0.541	-0.015	1.097		

CORT, corticosterone; HLR, heterophil : lymphocyte ratio.



**Fig. 2.** Linear relationships between stressor duration (days) and response ratios of (a) Corticosterone (CORT) or (b) HLR, which were calculated as the ratio of ln-transformed treatment population means divided by ln-transformed control population means. The 95% confidence interval of the regression line is in grey. Significant linear regressions were present for both stress parameters; however, the direction of this response was different between CORT and HLR. Specifically, CORT decreased over time, suggesting an attenuation of the CORT response, while there was a slight, positive relationship between HLR and stressor duration. These results are based on weighted meta-regressions, where circles represent individual study effect sizes, and the size of the circle is proportional to the relative weight of the effect size based upon sample size and error. HLR, heterophil : lymphocyte ratio.

Meta-analysis represents an approach for biologists to understand the responses of conserved features (such as the stress response) across related studies. Furthermore, Ouyang, Hau & Bonier (2011) suggested the utility of a

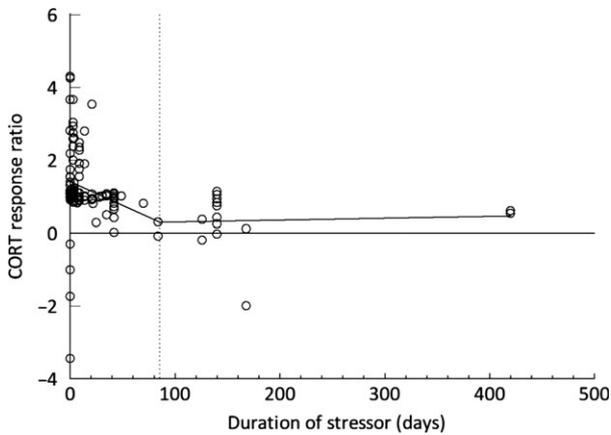
meta-analysis to identify large-scale patterns in repeatability of physiological responses to stress, yet they identified the lack of available data to perform such an analysis (see Constantini & Møller 2009; Constantini, Marasco & Møller 2011 for recent examples of meta-analysis used in stress physiology). In their study, Ouyang, Hau & Bonier (2011) refer to the sparse studies that focused on wild species that precluded the use of meta-analysis. While the review by Davis, Maney & Maerz (2008) was instrumental in providing a framework for using HLR in an ecological context, we were able to provide a quantification of this response that was not included in Davis, Maney & Maerz (2008). This study was inclusive of all available and relevant data from both wild and domesticated species. One of the analyses we performed tested for differences in the effect sizes between wild and captive populations, as well as between taxonomic class, species and sex (see Table 2); in general, these analyses revealed subtle differences among these groups, yet interpretation of these differences remains difficult. Interestingly, CORT was significantly elevated in captive populations (over wild populations), non-chicken populations (over chicken populations) and in studies that only included females (over males). For HLR, however, we were not able to detect these significant differences among study covariates. We did detect significant differences among stress treatment types for both CORT and HLR; however, an overall pattern explaining these differences is not clear. While the role that captivity status, sex and stressor type have on stress responses remains generally unclear, our analysis suggests that these parameters may affect the physiological stress axis. Due to the abundance of studies on the domestic poultry HPA axis, our study highlights the need for continued research of physiological stress in more ecologically relevant contexts.

The goal of this study was to use data across experiments to test for a generalized 'stress response' in both measures, and to compare the two measures to determine possible differences in their utility. We detected no differences in the overall effect size (i.e. magnitude of response) between HLR and CORT, yet the confidence interval for HLR was greater than the confidence interval for CORT; however, testing these overall differences in effect size between the two measures may obscure more subtle differences within

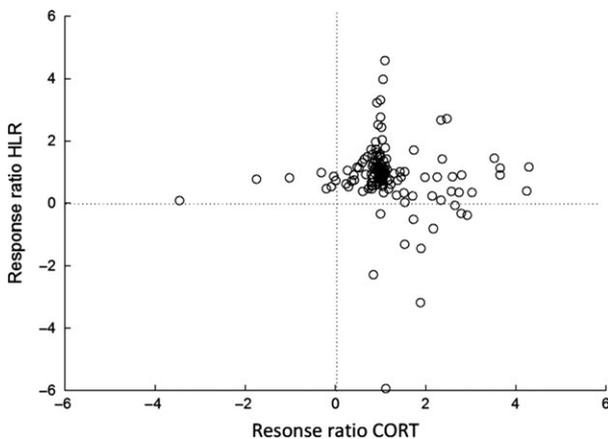
**Table 3.** Model variables for the effect of stressor duration on CORT and HLR using weighted analyses. There was a significant effect of duration on both physiological stress responses; however, these responses were in opposite directions. CORT significantly decreased in response to prolonged stressors, while HLR significantly increased. The  $R^2$  analogue was calculated as the proportion of total between-study variance explained by the model

	Estimate	Standard error	2-sided <i>P</i> -value	$R^2$ analogue
Ln ratio CORT				
Intercept	1.280	0.043	<0.00001	0.12
Duration of stressor	-0.005	0.001	<0.00001	
Ln ratio HLR				
Intercept	0.592	0.148	0.0001	0.21
Duration of stressor	0.014	0.003	<0.00001	

CORT, corticosterone; HLR, heterophil : lymphocyte ratio.



**Fig. 3.** Segmented linear relationship between stressor duration (days) and CORT response ratio. The break point was estimated to be 85 days using segmented regression analysis (intercept = 1.41; 0–85.4 days, slope =  $-0.013$ ;  $>85.4$  days, slope =  $0.0005$ ;  $T_{122} = -2.628$ ,  $P = 0.0097$ ; Multiple  $R^2 = 0.124$ ). This relationship was subsequently supported using weighted meta-analysis data (break point  $\times$  duration interaction:  $Q_2 = 31.49$ ,  $P < 0.00001$ ). CORT, corticosterone.



**Fig. 4.** Direct comparison of the response ratios of corticosterone (CORT) and HLR indicated that there was no clear relationship between the two physiological stress parameters. Specifically, either a high CORT or HLR response did not indicate that the opposite stress response was also elevated. HLR, heterophil : lymphocyte ratio.

the measures. Therefore, we performed additional analyses to isolate possible trends embedded within the data.

Because the physiological stress axis is sensitive to time across multiple scales (Romero & Reed 2005; Romero, Dickens & Cyr 2009), we were interested in whether differences exist in the response of the stress biomarkers across time of treatment duration (i.e. duration of stress). Regression analyses demonstrated differences in the stability of the two measures to indicate stress across time and that CORT attenuates over time (Cyr & Romero 2007). Specifically, when analysed on a single timeline (between 0 and 140 days), CORT has a significant, negative relationship to duration of stress treatment, while HLR is positively affected by treatment duration (Fig. 2). We interpret this

result to indicate the attenuation of the CORT response over a long time period. Acclimation to stressors as seen in a decreased GC response to stress over time has been suggested as an important consideration for ecologists measuring GC as an indicator of the degree of stress an organism is under (Romero 2004); data from our analysis thus confirm that when exposed to a chronic stressor, the GC response to that stressor decreases over time. As Romero (2004) points out, the mechanism(s) behind this acclimation of the GC response may be variable, yet this attenuation of the GC response may limit the ability of this measure to indicate stress for longer term studies. The slight but significant increase in HLR might be interpreted as increased sensitivity of physiological effectors of stress responses, independently of the attenuation of endogenous stress hormone, or as a means of coping with the secondary pathological effects of elevated GC (Sapolsky, Romero & Munck 2000).

In addition to the simple linear regression, we performed a segmented regression analysis that indicated a significant break point in the CORT response at 85 days of stress duration. Before this time point, there is a negative relationship between CORT and stress duration, while beyond it there is no temporal relationship between CORT and duration. We interpret this finding to indicate that at that break in the regression, there is an important physiological change that likely happens wherein the response transitions between the most relevant and/or adaptive stress responses. While the exact mechanism of this transition is not known, factors such as a change (specifically an increase) in the sensitivity or density of GC receptors might account for attenuation of the GC increase (Romero & Wikelski 2010) at longer time intervals without the negative consequences of elevated GC concentration. This result indicates that if one was interested in measuring long-term stress responses (i.e. to stressors  $>85$  days), circulating GC concentration may fail to positively indicate that a population is responding to a stressor (or series of stressors).

Our analysis showed that elevation of HLR in populations is positively affected by stress duration (Fig. 2). Thus, the data indicate that HLR may be more likely to indicate stressful conditions than elevated baseline GC, especially in situations where the interest is to measure responses to ecological stressors. Because of the delayed response of HLR to GC secretion (Sapolsky, Romero & Munck 2000), caution should be applied in using this interpretation in applications where the interest is to measure the response to a short-term (i.e. acute) stressor.

As was noted by Ouyang, Hau & Bonier (2011), the ecological literature still lacks a large body of studies that present data that can isolate on a finer scale the dependability of definitive biomarkers to indicate environmental stressors, especially in wild populations. The approach used in this study was to combine data across experiments (and experimental designs) to overcome this challenge.

When we directly compared CORT and HLR (Fig. 4), we found that the two measures did not indicate the same

degree of a stress response. Specifically, as was also noted by Müller, Jenni-Eiermann & Jenni (2011), elevated CORT did not indicate elevated HLR. This makes the point clear that depending on the nature of the question, and the timeline of the predicted response to a stressor, the best measures of the stress response may include several physiological parameters. Therefore, we stress the importance of measuring a broad suite of parameters if one is interested in the overall fitness or well-being of populations. While we demonstrated the stability of HLR in response to a chronic stressor, we recognize that it is unlikely that due to inherent complexities of the stress response, any single measure can completely indicate the overall environmental stress on populations. Our data indicate that HLR could represent a robust marker of physiological stress, yet further research is warranted. Specifically, identifying the mechanism of this observed trend, wherein GC concentration may be downregulated, yet the effect of GC secretion (i.e. alterations of circulating leucocytes) remains elevated. Additionally, more physiological stress parameters and understanding how these parameters relate on a chronic stress scale will be important in further understanding how populations respond to environmental stressors.

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## Data accessibility

All data in this manuscript have been published elsewhere; thus, data are not separately archived.

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## Supporting Information

Additional Supporting information may be found in the online version of this article:

**Appendix S1.** Citations of manuscripts from which data were collected for meta-analyses.

**Appendix S2.** Log response ratios of CORT and HLR identified by differences among stressor types.