A Comparison of Techniques Measuring Stress in Birds

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ABSTRACT

Free-living birds are subjected to both external and internal stresses which can affect their health, activity, and reproductive success. To study stress in free living birds, they must be captured in nets and handled by the researcher to take blood samples for commonly used measures of stress, an activity which itself can induce stress and confound results. This study compares the effects of handling time on three different measures of stress: levels of the stress hormone corticosterone (CORT), levels of Heat Shock Protein 60 (HSP 60) and the ratio of heterophils to lymphocytes (H/L ratio) in tufted titmouse *(Baeolophus bicolor)* captured at feeders between December and January (2011-2013) in Fredericksburg, VA. Blood samples collected between two and 15 minutes from 12 birds were assayed for levels of CORT and HSP and from 24 birds for H/L ratios. Relationships were examined between these stress indicators and handling time, body mass and body condition. CORT was significantly correlated with handling time ($p<0.01$), which reinforces existing evidence of CORT's sensitivity to the way subjects are handled immediately prior to blood collection. HSP or H/L ratios were not affected by handling time, suggesting that they may be preferable indicators of stress in free living birds under some circumstances.

INTRODUCTION

Free-living birds face a variety of internal and external sources of stress, which may affect physiological function and reduce fitness. Acute stress results from a specific stressful event, such as an attack by a predator or sudden storms, whereas chronic stress results from prolonged exposure to biologically challenging conditions, such as exposure to extreme temperatures (Vleck et al. 2000), periods of limited food availability (Herring et al. 2011), and anthropogenic pressures such as pollution, habitat disturbance (Arriero et al. 2008; Busch and Hayward 2009), and from prolonged psychosocial stressors (Cyr et al. 2007; Cyr and Romano 2007; Landys et al. 2011). To

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cope with such stressors, birds and other animals have a protective physiological stress response that allows them to withstand immediate threats to their homeostatic balance. When this response is elevated chronically, however, it can become biologically costly and have negative impacts on birds' fitness by weakening the immune system (Dabbert et al. 1997), which could increase susceptibility to disease, and compromising growth and reproduction (Sapolsky et al. 2000). Thus, stress levels can indicate the general physiological condition of birds and point to possible environmental perturbations.

Biomarkers such as the glucocorticoid corticosterone (CORT), heterophil/lymphocyte ratios (H/L), and heat shock proteins (HSPs) have all been used as tools to assess chronic or long-term stress in wild populations. These markers may be predictably regulated according to environmental conditions and various biological challenges (Gross and Siegel 1983; Sapolsky et al. 2000; Vleck et al. 2000; Moreno et al. 2002; Martinez-Padilla et al. 2004; Tomas et al. 2004; Davis 2005; Herring and Gawlik 2007; Busch and Hayward 2009; Cockrem et al. 2009; Krams et al. 2010; Herring et al. 2011), and thus can provide researchers with consistent methods of detecting stress experienced by birds in their natural habitats. However, they may also be affected to varying degrees by acute stress caused by capture and handling leading up to blood sample collection. Therefore, drawing inferences about birds' long-term stress status prior to their capture by researchers may be problematic since the relationships between the different measures used have not been adequately studied.

Quantifying glucocorticoids, such as CORT, is presently the most frequently applied method of assessing individual stress in birds (Sapolsky et al. 2000; Tomas et al. 2004; Herring and Gawlik 2007). However, the release of CORT is highly influenced by acute stress associated with capture and handling during field research which may confound results. Capture and handling can rapidly mobilize CORT, which can make the interpretation of CORT measurements difficult under some circumstances (Sapolsky et al. 2000; Romero and Reed 2005; Fridinger et al. 2007; Herring and Gawlik 2007; Busch and Hayward 2009; Cockrem et al. 2009). When handling time before sampling lasts for more than 2 or 3 minutes, CORT levels may no longer accurately reflect birds' physiological status before their capture (Romero and Reed 2005; Cockrem et al. 2009). In studies involving free-living birds, field conditions may prevent sufficiently prompt blood collection, leading to less reliable measures of chronic stress. Additionally, fecal CORT, can degrade over time in frozen samples (Herring et al. 2007) which reduces reliability of measurements. Herring and Gawlick (2007) compared the use of CORT with that of HSPs as ways of measuring stress associated with allostatic overload (when the energy requirements needed to maintain homeostasis in the body exceed the capacity of the animal) and concluded that HSPs have some advantages over CORT and may represent a viable supplementary or even alternative indicator of chronic stress.

Circulating H/L ratios have also been used to measure chronic stress in birds. These ratios are used because the avian immune response to stress takes significantly longer to initiate, by hours to days, than the rapid CORT response, and changes in leukocyte numbers last longer than changes in CORT levels (Davis et al. 2008). Their slower response to stress and longer endurance indicate that H/L ratios may be informative, especially in obtaining baseline stress measurements. However H/L levels are impacted by disease and infection and may not reflect true levels of stress to external stressors. While studies on Adélie penguins (Vleck et al. 2000) and house finches (Davis 2005)

indicate that H/L ratios are resistant to handling up to 1 hour, a study investigating wintering male great tits (Cirule et al. 2012) found that acute stress due to capture and handling caused an increase in heterophil counts between 30 and 60 minutes and a decline in lymphocyte counts between 60 and 120 minutes after capture. Therefore, H/L changes may be species specific and may change more rapidly than originally thought, which may limit the reliability of results in a way similar to CORT.

A different cellular response to stress is mediated by HSPs. HSPs are a family of proteins whose expression is increased when cells are exposed to both cellular stressors such as parasites (Merino et al. 1998; Martinez-Padilla et al. 2004; Arriero et al. 2008; del Cerro et al. 2010), limited food availability (Zulkifli et al. 2002; Herring et al. 2011), and sibling competition (Martinez-Padilla et al. 2004; Merino et al. 2006) as well as psychosocial stressors such as crating in birds (Zulkifli et al. 2009), fear (Al-Aqil et al. 2013) and social interactions in fish (Currie et al. 2009). They have been found to exist in almost all organisms, including bacteria, plants, and animals (Feder and Hofmann 1999). HSPs are a special class of proteins referred to as molecular chaperones which protect proteins from degradation and correct damage caused by stress-induced instability (Merino et al. 1998; Feder and Hofmann 1999; Tomas et al. 2004; del Cerro et al. 2010). Essentially, they serve to restore and maintain cellular homeostasis during times of increased stress (Tomas et al. 2004; del Cerro et al. 2010).

These molecules may be reliable indicators of chronic stress because they are maintained at high levels for longer periods after stressors are applied, and some research has shown that handling stress does not cause their rapid up-regulation (Martinez-Padilla et al. 2004; Herring and Gawlik 2007; Herring et al. 2011). However, the resistance of HSPs to acute stress triggered by capture and handling has not been systematically compared to those of CORT and H/L ratios in the same study.

The tufted titmouse *(Baeolophus bicolor)* is a good model species to expand research on the relationships between these three stress indicators and their relative sensitivities to handling time. Close relatives of the tufted titmouse, the blue tit and the great tit, have been used in past studies examining both the intracellular and hormonal stress responses (Arriero et al. 2008; Cockrem et al. 2009; del Cerro et al. 2010). The tufted titmouse is a canopy-dwelling, omnivorous permanent resident species of eastern North American deciduous forests (Grubb and Pravosudov 1994). During the winter, they spend time in flocks of about 2 to 5 individuals. Caching food during cold weather, they commonly frequent feeders and carry 1 seed at a time to store within 40 meters of the feeders, allowing them to be easily captured with mist nets (Grubb and Pravosudov 1994).

This study compared the sensitivity of HSP60, CORT, and H/L to acute stress induced by the capture and handling of tufted titmice to evaluate their reliability as tools in avian stress research. The relationships between CORT, H/L, and HSP60 and the time elapsed between capture of subjects and blood collection, as well as the relationships among these bioindicators, were analyzed. We predicted that CORT levels, but not H/L ratios or HSP60 levels, would be positively correlated with handling times longer than 2 minutes after capture of subjects. Finally, we examined whether CORT, H/L, and HSP values were correlated within subjects and related to body condition.

MATERIALS AND METHODS

Capture, Handling, Blood Sampling and Slide Formation

Tufted titmice were captured using mist nets set around feeders at sites in Spotsylvania and Stafford counties in Virginia. A total of 41 subjects were captured between December 2011 and February 2013. Of these, 24 were used to assay H/L ratios while 12 were used for CORT and HSP60 assays.

Blood samples were drawn after varying handling times after capture. Handling time was defined as the elapsed time in seconds from when a subject flew into the mist net to the start of blood sampling. Handling time was intentionally varied between 120 and 915 seconds (2-15 minutes) to simulate the range of handling times typical of this type of field research.

A 26-gauge needle was used to puncture the left brachial vein of each subject. A capillary tube was used to collect the blood and transfer it to an individually labeled 5.0 mL centrifuge tube. The blood samples were kept cool with a freezer pack in a small cooler for transport from the sampling site to the laboratory. Immediately upon return to the laboratory (1-4 hours after capture and sampling), the blood samples were centrifuged at 500 X G for 10 minutes to separate the plasma supernatant from the hematocrit. The plasma and hematocrit samples were kept frozen, separately, in individually labeled 0.5 mL centrifuge tubes in a subzero (-80 ˚C) freezer.

Preparation of Slides

At the time of blood sampling, a small drop of blood collected from each bird was used to create a blood smear on an individually marked microscope slide using the two-slide wedge method (Houwen 2000). The blood smears were air dried before being transported from the sampling site to the laboratory. Immediately upon return to the laboratory (1-4 hours after capture and sampling), blood smears were fixed in methanol for 30 seconds then stained using the Wright-Giemsa stain procedure. Slides were analyzed using a Leica compound light microscope.

Body Condition Measures

Right wing chord length was measured to the nearest 0.5 mm and mass to the nearest 0.5 g for each subject before its release. A body condition index (BCI) was calculated for each subject as a value obtained from the equation $\frac{mass}{winglength} \times 10^6$, a modification of the condition index used by Owen and Cook (1977).

Heterophil to Lymphocyte Ratios

Stained blood smears were examined under a compound microscope with 1,000x magnification using oil immersion. Areas of each slide with a monolayer of erythrocytes were searched for leukocytes. Identification of leukocytes was based on information and images found in the *Atlas of Clinical Avian Hematology* (Clark et al. 2009).

For each individual slide, only fields of view with similar erythrocyte densities and those containing countable leukocytes were examined. If a field of view did not contain any leukocytes, it was skipped as the slide was systematically searched from field to field. For each leukocyte-containing field of view, counts were recorded for numbers

of heterophils, lymphocytes, total leukocytes, and total erythrocytes. Counting was arrested when at least 100 leukocytes were found on a slide.

To account for variability among cell counts owing to uneven distribution of cells in the blood smears, each slide was evaluated in duplicate by separate researchers and the resulting counts were averaged. H/L was calculated as the number of heterophils divided by the number of lymphocytes. To standardize the counts for comparison, the total number of leukocytes to the total number of erythrocytes counted on each slide was converted to the number of leukocytes per 10,000 erythrocytes. An H/L index was calculated for each slide by multiplying the H/L by the number of leukocytes per 10,000 erythrocytes.

Corticosterone Levels

Frozen plasma samples were brought back to room temperature and analyzed using a Corticosterone Enzyme Immunoassay (EIA) kit from Enzo Life Sciences International, Inc. The assay buffers and reagents were prepared according to the instructions in the kit. Corticosterone standards were prepared in 5 labeled tubes with concentrations of 20,000, 4,000, 800, 160, and 32 pg/mL. Plasma samples were diluted 1:30 with assay buffer to produce a 100 µl sample as previously determined to provide the best results with this assay (Dolby et al. 2011). Samples were assayed in duplicate in a 96-well microwell plate according to the layout sheet provided in the EIA kit.

Following the EIA protocol, a 100 µL of standard diluent, standards, and diluted plasma samples and 50 µL of assay buffer, conjugate, and corticosterone antibody were dispensed into wells of the plate. The plate was incubated at room temperature on a plate shaker for 2 hours at 500 rpm, washed 3 times using the prepared wash buffer, and then aspirated to remove remaining wash buffer. After washing, 5 µL of conjugate and 200 µL of pNpp substrate solution were added to the wells. The plate was incubated at room temperature for 1 hour without shaking. Then 50 µL of stop solution was added to every well to stop the reaction. The plate was immediately read at 405 nm optical density using an Eon microplate reader and Gen5 microplate reader software from BioTek Instruments, Inc.

Using the optical densities bound as reported by the software, the average net optical density bound for each standard and sample was calculated and the binding of each pair of standard wells was calculated as a percentage of the maximum binding wells. These calculated values were used to create a standard curve with which the concentration of corticosterone in each sample could be determined in pg/mL diluted serum. The concentrations calculated according to this procedure were recalculated to adjust for the initial dilution of the plasma samples, and the resulting concentrations were recorded for each sample in ng/mL serum.

HSP60 Concentrations

Frozen hematocrit samples were thawed and washed by adding 1.0 M PBS (BioRad) using gentle pipetting to disperse the cells. The samples were centrifuged at 500 X G for 10 minutes, and the supernatant removed and discarded. The cells were lysed by adding 100 µl of extraction buffer (Enzo Life Sciences) containing 1 µl of protease inhibitor cocktail (Thermo Scientific) to each sample. The cells were vortexed for 5 minutes, sonicated for 1 minute, and then centrifuged at 2500 X G for 15 minutes. Using the BioRAD DC Protein Kit, protein concentrations of the supernatent for each

sample was measured in duplicate along with standards of BSA (bovine serum albumin, BioRad). Sample absorbance was measured using an Eon microplate reader and Gen5 microplate reader software (BioTek Instruments, Inc.) at 750 nm. Protein levels for each sample trial were calculated from the generated standard curve, and the protein levels for the 2 trials for each sample were averaged to give total protein in mg/ml for each sample.

An Enzyme Linked Immunoassay (ELISA) kit for Human HSP60 (Enzo Life Sciences) was used to determine the concentration of HSP60 in each sample. This kit was previously shown to detect avian HSP60 in our lab (Humayon et al. 2010). The protocol from the kit was followed. HSP60 protein standards ranging from 50 ng/mL to 1.56 ng/mL were produced by dilution of the supplied protein standard with sample buffer. For the assay, samples were applied at 200 µg of total protein/well, and each sample was assayed in duplicate. Sample buffer was added to each sample to give a final volume of 100 µl. The samples, standards, and negative control (buffer only) were applied to the assay plate using 100 µL total volume per well, and the plate was incubated for 1 hour at room temperature. The plate was washed with a 1X Wash Buffer to remove unbound antigens, followed by the addition of 100 µL of biotinylated anti-HSP60 (Enzo) to each well. The plate was incubated for a further 30 minutes at room temperature, and washed again with wash buffer. Addition of 100 µL of HRP conjugate (Enzo) to each well, was followed by incubation for 10 minutes at room temperature. After washing, 100 µL of TMB substrate (Enzo) was added, and the reaction was allowed to proceed for 5-10 minutes producing a detectable color change. Stop solution was added, and the absorbance was measured using an Eon microplate reader and Gen5 microplate reader software (BioTek Instruments, Inc.) at 450 nm. The total mass (ng) of HSP60 in each sample was calculated based on absorbance relative to the standard curve. This value was then divided by 200 µg to express the results as ng of HSP60/µg of total hematocrit protein for each sample.

Statistical Analysis

Blood samples were collected from a total of 41 birds. After obtaining the CORT and HSP60 concentrations and H/L for each sample, only samples with less than a 10 percent difference between duplicates were included in further analysis. Additionally, birds with missing data were excluded from analysis, which reduced the sample size to 12 subjects for CORT and HSP60 analysis. Samples from 24 birds were used for analysis of H/L ratios. IBM SPSS Statistics software was used to evaluate the relationships between the following variables: HSP60, CORT, H/L, H/L Index, body mass (Mass), BCI, and handling time (Time). Linear regression and analysis of variance (ANOVA), were used to describe the following relationships between variables in determining correlations: CORT vs Time, H/L vs Time, H/L index vs Time, H/L vs Mass, H/L Index vs Mass, H/L vs BCI, H/L Index vs BCI, HSP vs Time, HSP vs BCI, and HSP vs Mass.

Since the relationship between CORT, HSP60, and H/L may depend on the time elapsed between initial capture and sampling, the analysis was also performed using the residuals of CORT vs Time (CORT residuals) to account for the variation in CORT values owing to differences in handling time. Linear regression and ANOVA were performed on H/L vs CORT residuals, H/L Index vs CORT residuals, and HSP60 vs CORT residuals. Regressions were also run to determine if body condition or body

Table 1. Summary of descriptive statistics of data obtained from field measurements, corticosterone assay, HSP 60 assay, and stained-slide cell counts.

Variable	N	Mean	StDev	Range
CORT[a]	12	16.42	14.56	1.13-44.41
CORT residuals[b]	12	0.385	7.97	-14.69-11.89
H/L[c]	24	0.157	0.082	0.047-0.362
H/L Index[d]	24	95.869	79.878	9.37-320.4
HSP60[e]	12	0.076	0.028	0.032-0.125
Mass (g)	12	21	1.11	19-22
BCI[f]	12	39.86	2.3	35.8-45.1
Time (s)[g]	12	400.25	265	120-915

[a] Corticosterone concentration in ng/mL serum
[b] Standard residuals of CORT vs. Time linear regression
[c] Heterophil to lymphocyte ratio
[d] H/L multiplied by number of leukocytes per 10,000 erythrocytes
[e] Heat Shock Protein 60 concentration in ng/μg total hematocrit protein
[f] Body condition index calculated from mass (g) divided by wing length cubed (mm^3)
[g] Handling time, defined as the time elapsed between capture and blood sampling

mass was correlated with CORT (CORT residuals vs BCI and CORT residuals vs Mass) and HSP60 (HSP60 vs BCI and HSP60 vs Mass).

The SPSS software was also used to calculate Spearman's rho, a nonparametric correlation coefficient, to compare within-subject values of H/L and CORT residuals, and CORT residuals and HSP60. Results were determined to be significant when $p \leq 0.05$.

RESULTS

The average handling time before blood sampling was 400 seconds (N = 12, Mean = 400.25 ± 265, Range 120 to 915) (Table 1) or 6.6 minutes. CORT was positively and significantly correlated with handling time (R^2 = .707, N = 12, P < .001) (Fig. 1, Table 2). No other relationships were significant (Table 2), including H/L and CORT residuals (Table 2), H/L and handling time (Fig. 2), HSP60 and handling time (Fig. 3), or HSP60 and CORT (Fig. 4).
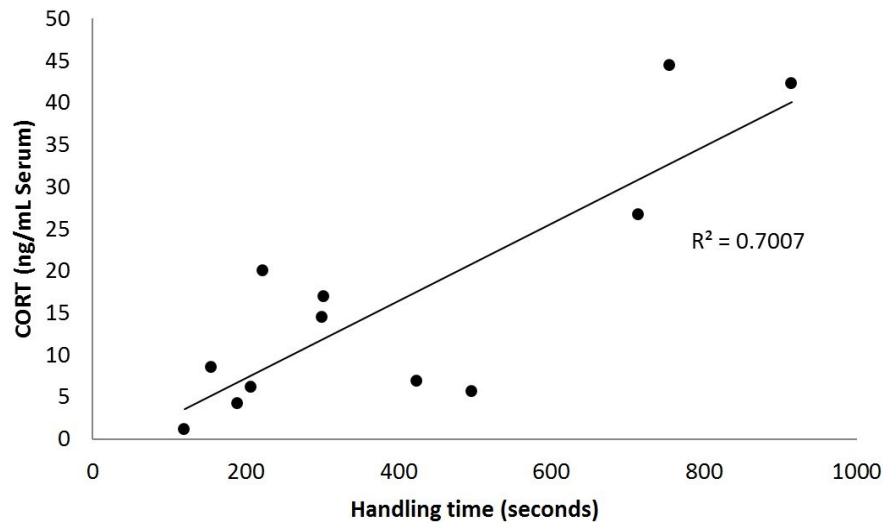
FIGURE 1. Corticosterone (CORT) concentration (ng/mL serum) is significantly correlated with handling time (seconds). CORT levels increase as handling time after capture increases ($R^2 = 0.7007$, N = 12, p < 0.001).

DISCUSSION

Corticosterone and H/L comparison

CORT was positively and significantly correlated with handling time, as expected based on the results of previous research (Romero and Reed 2005; Fridinger et al. 2007; Herring and Gawlik 2007; Herring et al. 2011; Busch and Hayward 2009; Cockrem et al. 2009). Also as expected, no correlation was found between H/L and handling time, or between H/L Index and handling time. These results support the hypothesis that H/L is less sensitive than CORT to handling time in samples taken 2-8 minutes after capture and supports previous studies which show that H/L ratios are not as responsive to acute stress such as in handling (Vleck et al. 2000). This supports the conclusion that the use of H/L may be preferable for measuring chronic stress levels in free living birds when unavoidably prolonged handling after capture for blood sampling may affect CORT levels.

The results for heterophil counts, lymphocyte counts, and H/L in this study are similar to those for Siberian tits in managed habitats (Krams et al. 2010) and for wintering male great tits (Cirule et al. 2012) and were within ranges reported by Davis (2005). The ranges of leukocyte counts and H/L in this study are consistently lower than the those reported in several studies on chickens (Gross and Siegel 1983; Davis et al. 2000; Zulkifli et al. 2000; Al-Murrani et al. 2006), Adélie penguins (Vleck et al. 2000), pied flycatcher nestlings (Moreno et al. 2002), and Eurasian kestrel nestlings (Martinez-Padilla et al. 2004). The disparities among these studies may reflect species-specific differences in leukocyte profiles.

Table 2. Summary of results from statistical analysis. For each relationship listed, SPSS software was used to perform linear regression and ANOVA[a]. Relationships were determined to be significant when P<0.05.

Variable	df	F	R^2	p
CORT[b] vs. Time[c] (s)	11	23.409	0.707	< .001
H/L[d] vs. Time (s)	23	1.21	0.053	0.28
H/L Index[e] vs. Time (s)	23	1.782	0.075	1.19
H/L vs. CORT residuals[f]	11	1.2	0.114	0.28
H/L Index vs. CORT residuals	11	0.975	0.088	0.34
H/L vs. Mass (g)*	21	0.022	0.001	0.88
H/L Index vs. Mass (g)*	21	0.142	0.007	0.71
H/L vs. BCI[g]*	21	0.612	0.030	0.45
H/L Index vs. BCI*	21	0.708	0.034	0.41
HSP60[h] vs Time (s)	11	0.517	0.49	0.48
HSP60 vs Mass(g)	11	0.210	0.020	0.65
HSP60 vs BCI	11	0.188	0.185	0.67
HSP vs CORT	11	0.207	0.020	0.66
HSP vs CORT residuals	11	0.048	0.004	0.83

[a]Analysis of variance
[b]Corticosterone concentration in ng/mL serum
[c]Handling time, defined as the time elapsed between capture and blood sampling
[d]Heterophil to lymphocyte ratio
[e]H/L multiplied by number of leukocytes per 10,000 erythrocytes
[f]Standard residuals of CORT vs. Time linear regression
[g]Body condition index calculated from mass divided by wing length cubed (mm^3)
[h] Heat Shock Protein 60 concentration in ng/μg total hematocrit protein concentration
*Mass was only obtained from 22 birds for these analyses

No correlation between CORT and any leukocyte variable after removing the variation in CORT owing to the effect of handling time was detected. These findings agree with Gross and Siegel (1983), who found no correlation between H/L and CORT
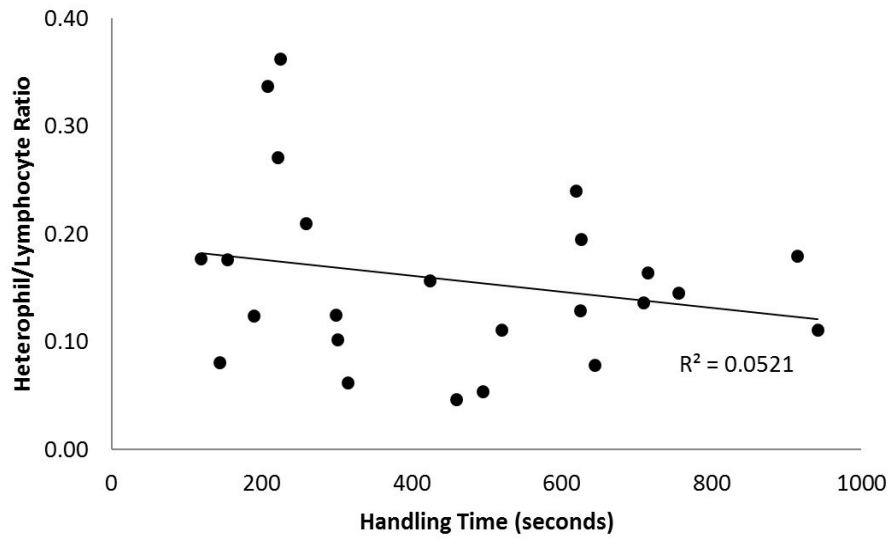
FIGURE 2. Heterophil/lymphocyte ratios (H/L) are not significantly correlated with increases in handling time (seconds) (N = 24, R^2 = 0.0521, p = 0.39).
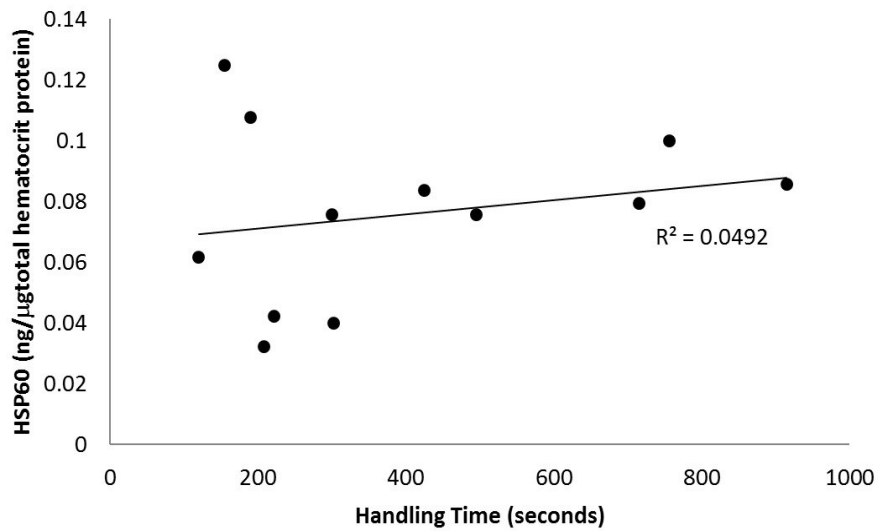

FIGURE 3. Heat shock protein 60 (HSP60) concentrations (ng/µg total hematocrit protein) are not significantly correlated with handling time (seconds) (N = 12, R^2 = 0.0492, p = 0.48).
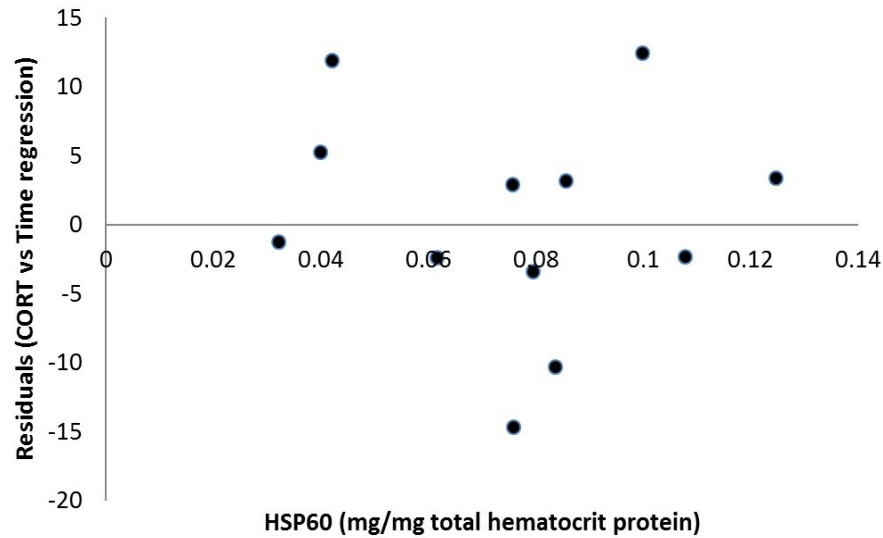
FIGURE 4. Heat shock protein 60 (HSP60) levels (expressed as a fraction of total hematocrit protein concentration) measured in tufted titmouse blood samples were not correlated with the residuals found from the CORT (ng/mL serum) vs. time in seconds regression (N = 12).

in response to various stressors. The only situation in which Gross and Siegel (1983) found a correlation between H/L and CORT was in well-socialized groups of chickens in low-stress environments with stable social hierarchies before any experimental stressors were applied. The absence of a correlation between H/L and CORT in our study does contradict the results of more recent research. Prolonged increases in levels of glucocorticoids have been shown to have both an immunosuppressive effect resulting in reductions of circulating leukocytes (Sapolsky et al. 2000), although studies using exogenous administration of CORT can also increase levels of H/L (Müller et al. 2011) in free living birds. According to Sapolsky et al. (2000), immune activation in response to stressors contributes to the subsequent release of glucocorticoids such as CORT by synthesizing molecules similar to adrenocorticotropic hormone and cytokines that stimulate the adrenocortical axis. Thus decreases in leukocyte counts in response to prolonged increases in glucocorticoids may not be due to the depletion of leukocytes, but to the diversion of leukocytes to local areas of need in response to specific stressors. Therefore, the overall leukocyte proportions in a subject's system should not be significantly affected by variations in CORT levels, a scenario which would be supported by the findings of this study. Leukocyte counts, however, may vary in such a case, depending where in the system leukocytes are being diverted as well as where and how samples are taken. Based on our methods, we would not have detected this. The results of a study by Muller et al. (2011) using free-living Eurasian kestrals subjected to increased CORT using exogenous implants demonstrated that H/L levels can be increased by prolonged CORT elevation. However, their study also

demonstrated that CORT and H/L will normally respond to different stressors, indicating that the body will respond to different types of stress using different mechanisms. Additionally, El-Lethey et al. (2003) have noted that there are both stress resistant and stress susceptible antigen responses leading to immune suppression, which can further confound results. Therefore, when attempting to measure chronic stress in birds, multiple measures of stress should be used.

Because H/L did not change with differences in handling time, H/L may be a useful indicator of chronic stress in field research. It is relatively resistant to the effects of acute stress and would, therefore, allow researchers more time to obtain blood samples in the field than CORT. In addition to decreasing the pressure on researchers to minimize handling times, the use of H/L as a stress measurement tool offers other benefits that the use of CORT may not. First, the volume of blood necessary to prepare a blood smear is much smaller than that required for CORT assays. A blood smear requires 1-2 µl of blood whereas typical assays to measure CORT concentrations require at least 10 µl of blood to get an appropriate sufficient amount of plasma. The average tufted titmouse body mass in this study was just over 21 g, and the smallest bird sampled was 19 g. A smaller blood sample might produce fewer negative effects on small songbirds than larger blood samples (Brown and Brown 2009). Second, the relative costs of obtaining H/L and CORT measurements for research are substantially different. CORT assay kits are expensive, and analysis of samples requires the use of sensitive and expensive equipment and specialized software. The method for measuring H/L requires only stain, microscope slides, a microscope, and immersion oil.

There are challenges to the use of H/L, though, as previous researchers such as Davis et al. (2008) have pointed out. First, H/L measured from a single blood smear is only indicative of the relative proportions of leukocytes that were circulating in the blood at the moment and the specific bodily location of sampling. It is, therefore, possible that H/L may not be representative of heterophils or lymphocytes present in other areas of the body. Without taking multiple samples and creating multiple blood smears, there is no way to know if such a difference is present or the magnitude of that difference. Second, H/L is not a direct measure of chronic stress levels, but a relative measure that requires comparison either to other samples or to a reference of some kind. References do not exist for many species which describe typical measures under low-stress and high-stress conditions, which can create issues in interpreting the data. Finally, H/L is influenced by other factors that induce an inflammatory or an antibody response, such as injury or disease (Davison et al. 1983) or prolonged elevations of glucocorticoids (Müller et al. 2011), which could be a response to either physical or psychosocial stress leading to greater confusion in interpretation of data. Therefore, researchers must be cautious in choosing the appropriate stress assessment tool for use in specific research applications, and ideally use several different measures to gain an accurate picture.

Corticosterone and HSP60 level comparison

HSP60 concentrations were not significantly correlated with handling time, while CORT levels in the same subjects were. No correlation between CORT levels and HSP60 levels within individuals was seen. Those subjects with higher CORT levels did not necessarily have higher HSP60 levels. These results support the hypothesis that HSP60 is less sensitive to acute stress caused by handling time than CORT. Therefore,

like H/L ratio, HSP60 may also be a preferable or informative complementary indicator of stress along with CORT when blood samples must be collected under challenging field conditions.

Our HSP60 results cannot be directly compared to past studies because of the methods used. Most of these previous studies used cell fractionation and Western blots to evaluate HSP concentrations (Merino et al. 2002; Barbosa et al. 2007; Arriero et al. 2008; del Cerro et al. 2010; Martinez-de la Puente et al. 2010) whereas we used ELISA. Western Blots report results using optical density measures while our results are expressed in micrograms per milligrams of hematocrit protein although trends in relative amounts may be compared. Herring and Gawlik (2013) evaluated HSP60 concentrations using ELISA. However, they reported their results in nanograms per milliliter, which also makes a direct comparison difficult. Our method of reporting HSP60 levels relative to total protein levels standardizes the HSP measurements and removes variation in results owing to experimental technique and blood composition in subjects.

In a study of the relationship between food availability and both CORT and HSP levels in the White Ibis *(Eudocimus albus)*, HSP60 levels increased in a predictable manner when food was limited (Herring et al. 2011). Plasma corticosterone levels (PCORT) were found to be ambiguously related to nutritional condition, indicating that the PCORT results might have reflected handling stress. Our results are consistent with the results of this study.

Additionally, there was no significant relationship between HSP60 concentrations and either CORT or CORT residuals (Table 2, Fig 4). This shows that HSP60 concentration was not related to the remaining variation in CORT levels not accounted for by handling time, and that there is no relationship between increases in CORT levels and HSP levels due to acute stress caused by handling. CORT is present at low levels under normal conditions. However, when the brain recognizes a stressor, it signals the HPA axis to release more CORT (Herring and Gawlik 2007), and levels may remain high when an individual faces chronic stress. HSPs are also present under normal conditions and are up-regulated during periods of both physical (Feder and Hoffman 1999) and psychosocial stress (Isosaki and Nakashima 1998; Currie et al. 2009; Al-Aquil et al. 2013) though increases in HSP levels would be slower in onset and have a longer duration than CORT. Therefore, their levels may more accurately represent the presence of chronic stress. Since CORT and HSPs function in different responses to stress, they should not necessarily be correlated, which is supported by our results.

Body mass, body condition (Table 2) and standardized mass (data not shown) were not correlated with CORT, H/L or HSP60, indicating that our specimens were either not chronically stressed or that the measures we used are not good predictors of chronic stress in this species. Other measures of condition, such as a rating of the pectoral muscle size, may be a more accurate way to index differences in physical condition attributable to factors other than energetic status given that our subjects were food supplemented.

This study was conducted to determine the effect of acute stress due to capture and handling on levels of plasma CORT, H/L, and HSP60 to determine possible correlations between these physiological stress indicators. As described above, the results of this study demonstrate that both H/L and HSP60 are less sensitive to the

effects of handling time than the more commonly used CORT, and may be preferable stress measures in some instances. This study was intended to help researchers identify which indicators may be more effective for particular research applications. In light of these results, this research encourages more frequent use of H/L and HSP60 as complementary measures of chronic moderate stress in avian field research.

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