5-1-2013

Evaluating the effects of capture and handling time on plasma corticosterone and heterophil/lymphocyte ratios in the Tufted Titmouse (Baeolophus bicolor)

Michael A. Carlo
University of Mary Washington

Evaluating the effects of capture and handling time on plasma corticosterone and heterophil/lymphocyte ratios in the Tufted Titmouse (*Baeolophus bicolor*)

by

Michael A. Carlo

Thesis

Submitted in partial fulfillment of the requirements for Honors in Biology at the University of Mary Washington

Fredericksburg, Virginia

May 1st, 2013

This Thesis by Michael A. Carlo is accepted in its present form as satisfying the thesis requirement for Honors in Biology.

Date:

Approved:

_____

_____
Andrew S. Dolby, Ph. D.
Professor of Biology
Chairman of the Honors Committee

_____

_____
Deborah A. O'Dell, Ph. D.
Associate Professor of Biology

_____

_____
Rosemary Barra, Ph. D.
Professor of Biology

Curriculum Vitae

Michael A. Carlo
DOB: December 4th, 1984
Beaufort, South Carolina

Education

Graduated May 11th, 2013 from the University of Mary Washington with honors, Summa Cum Laude, with a Bachelor of Science in Biology.

Honoraries: Chi Beta Phi Science and Mathematics Honor Society
Alpha Phi Sigma Honor Society

Awards: William B. Castle Award for outstanding graduate in Biology
Departmental Honors in Biology
Biology Scholarship 2012-2013
John Cope '83 Memorial Scholarship 2012-2013

Graduated May 13th, 2011 from Piedmont Virginia Community College with honors, Magna Cum Laude, with an Associate of Science in Science and a concentration in Biology.

Honoraries: Phi Theta Kappa Honor Society

Research

Undergraduate research in Biology: Evaluating the effects of capture and handling time on three physiological stress indicators in the Tufted Titmouse (*Baeolophus bicolor*)

Honors research in Biology: Evaluating the effects of capture and handling on plasma corticosterone levels and heterophil/lymphocyte ratios in the Tufted Titmouse (*Baeolophus bicolor*)

Employment

The Kenmore Inn & Restaurant, Fredericksburg, Virginia (2011-2013)
St. Maarten Café, Inc., Charlottesville, Virginia (2005-2011)

Publications and presentations

Carlo, Michael and Abigail Kimmitt. 2013. Evaluating the effects of capture and handling time on three physiological stress indicators in the Tufted Titmouse (*Baeolophus bicolor*). April 2013, UMW Student Research and Creativity Day. University of Mary Washington. Poster presentation.

Carlo, Michael. 2013. Evaluating the effects of capture and handling time on three physiological stress indicators in the Tufted Titmouse (*Baeolophus bicolor*). April 2013, UMW Donor Appreciation Luncheon: Student Showcase. University of Mary Washington. Poster presentation.

Carlo, Michael, Andrew Dolby, Abigail Kimmitt and Deborah O'Dell. 2013. Evaluating the effects of capture and handling time on three physiological stress indicators in the Tufted Titmouse (*Baeolophus bicolor*). March 2013, 125th Wilson Ornithological Society Conference. College of William & Mary. Poster presentation.

Carlo, Michael and Abigail Kimmitt. 2012. Flight efficiency and physiological stress indicators in the Tufted Titmouse (*Baeolophus bicolor*). October 2012, Virginia Academy of Science Fall Undergraduate Research Meeting. J. Sargeant Reynolds Community College. Poster Presentation.

Acknowledgements

First, I would like to thank my advisor and research mentor Dr. Andrew Dolby for all of his help with my literature searches and readings and my research design, for encouraging me to attend conferences and share our research, for teaching me techniques for field work, and for all of the guidance and advice he has given me in my time here at UMW. Dr. Dolby has not only taught me skills to use in graduate school and beyond into my career, he has inspired me as a scientist, and I will always be grateful for that. I would also like to thank Dr. Deborah O'Dell and Dr. Rosemary Barra for serving on my honors committee and for all of their help and advice. Dr. O'Dell, thank you for all of the guidance through laboratory protocols and through my honors research process. Dr. Barra, thank you for all of the guidance through my honors research process and during my time here at UMW. Your door was always open to me, and for that I will always be grateful. I would like to thank Abby Kimmitt and Diana Gutierrez for all of their time spent in the field and in the lab. For all of the mornings mist netting and the countless hours in the lab running assays and staring at blood smears, thank you. I would like to thank Jim Hazzard and Sally Knight, Gerry Weinberger, Larry Valade, Mike and Joella Killian, Jack and Merrie Morrison, Mike Lott and Dr. Jackie Gallagher for welcoming us to their homes. Thank you for letting us to do our work on your property and thank you for the coffee! I would like to thank the Department of Biological sciences and the University of Mary Washington for the support and the use of space and equipment. Additional thanks to the Thyra V. Valade Memorial Fund and the Virginia Society of Ornithology for funding this research, and a very special thank you to Larry Valade for establishing the Thyra V. Valade fund and inviting us to mist net at his home. Finally, I would like to thank my family and my wonderful fiancée Amy for their love and support.

Abstract

Birds have a protective physiological response to stress, which consists of three main components: the hormonal response, the immune response and the intracellular response. This study evaluates the effects of acute distress due to capture and handling on plasma corticosterone (CORT) and heterophil/lymphocyte ratios (H/L) , physiological indicators of the hormonal and immune components of the stress response in the Tufted Titmouse (*Baeolophus bicolor*), as well as the relationship between the two biomarkers. These stress indicators are currently used in both basic and applied avian research. But their relationship with each other and their responses to specific stressors such as capture and handling have been little studied outside of research limited to work with poultry. The time between the initial moment of capture and blood collection was varied to evaluate the effect of handling time on CORT and H/L. CORT was positively correlated with handling time, and no correlation was found between H/L and handling time. No correlation was found for the within-subject relationship between CORT and H/L after the variation of CORT values owing to the effect of handling was removed. The variation of CORT due to capture and handling corresponds with previous research demonstrating that CORT values obtained more than 2 or 3 minutes after capture are no longer reliable baseline measurements indicative of environmental stress as opposed to handling stress. The resistance of H/L to the effect of capture and handling within handling times typical of avian field research supports it as a more useful tool in measuring chronic stress in birds.

Table of Contents

INTRODUCTION

Stress in Birds

Stress is a part of daily life for all organisms as they face biological challenges. All free-living organisms face a variety of internal and external sources of stress, which may affect physiological function and reduce fitness. Acute stress results from a specific novel event that elevates the stress response, such as a predation event or capture and handling by researchers. Chronic stress results from prolonged exposure to biologically challenging conditions, such as exposure to extreme temperatures, periods of limited food availability, and anthropogenic pressures such as pollution and habitat disturbance. To cope with such stressors, birds and other animals have a protective 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 and compromising growth and reproduction.

Detecting the stress experienced by birds is useful to researchers in areas such as conservation and bird management because stress levels can indicate the general physiological condition of birds and point to possible environmental perturbations. The avian stress response consists of several different measurable components including the hormonal response, the intracellular response, and the immune response. Measures of these different components of the stress response can be used as physiological indicators of stress, especially chronic stress. Researchers utilize biomarkers such as the glucocorticoid corticosterone (CORT), heat shock proteins (HSPs), and heterophil/lymphocyte ratios (H/L) as tools to

assess chronic or long-term stress in wild populations because they respond to physiological distress and are measurable in blood samples.

In avian ecology, researchers are interested in physiological stress indicators such as CORT, H/L, and HSPs because they 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). Such indicators can provide researchers with consistent methods of detecting stress experienced by birds in their natural habitats, thus they may be important contributors to conservation biology and basic behavioral ecology research.

Physiological Stress Indicators

The concentration of glucocorticoids obtained from plasma or feces is a commonly used measure of chronic stress in birds. Glucocorticoids are part of the vertebrate hypothalamic-pituitary-adrenal stress response in which hormones such as CORT are released into the circulatory system in response to environmental stressors (Davis et al. 2000; Sapolsky et al. 2000; Herring and Gawlik 2007; Busch and Hayward 2009; Cockrem et al. 2009; Herring et al. 2011). Under normal conditions, plasma CORT is present at low levels to regulate energy reserves. An increased concentration of CORT serves to combat the negative effects of stress by breaking down and mobilizing stored energy reserves through

the redistribution of stored glucose and the catabolism of protein (Sapolsky et al. 2000; Busch and Hayward 2009; Herring et al. 2011).

Though specific CORT levels can vary in a species-specific manner, CORT has been a useful measure of stress in avian ecology research (Sapolsky et al. 2000; Cockrem et al. 2009; Herring et al. 2011). Davis et al. (2000) demonstrated that stress due to long-term caging and reduced food supply can lead to increased CORT levels. Herring et al. (2011) used plasma and fecal CORT to demonstrate that White Ibis nestlings can be physiologically affected by food availability. Cockrem et al. (2009) used research on individual variation and repeatability of CORT responses in Adélie penguins in combination with previous research involving Great Tits and chickens to propose a system using coefficients of variation in CORT responses and power analyses, suggesting the use of such a system in future research on CORT responses in birds may provide more consistent and acceptable statistical results for analysis.

Another measure of chronic stress in birds is the concentration of HSPs in the blood. Since their discovery in the 1960s (Ritossa 1962), HSPs have been increasingly utilized as another indicator of physiological stress in organisms. HSPs are a family of proteins whose expression is increased when cells are exposed to stressors (Merino et al. 1998; Feder and Hofmann 1999; Tomas et al. 2004, del Cerro et al. 2010). 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. During times of increased stress, they 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 stress (Tomas et al. 2004, del Cerro et al. 2010).

Although HSPs were first discovered as a response to stress induced by heat shock (Ritossa 1962), they are now known to respond to many different environmental stressors. Research has shown that 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) can stimulate increased HSP production. Merino et al. (2006) further demonstrated in brood size manipulation experiments that parental effort as well as sibling competition can influence HSP levels in Blue Tits. Tomas et al. (2004) utilized their research on HSP levels in Great Tit blood samples to simplify future research on HSPs in wild bird populations by showing that there are no significant changes in levels of HSPs as long as blood samples obtained in the field are frozen within eight hours of collection. Demonstrating that samples could be safely kept cool for extended periods of time, often necessary in field research, provided further support for the use of HSPs in avian research.

A third indicator of chronic stress in birds is the ratio of heterophils to lymphocytes, which has been used in research since Davison et al. (1983) and Gross and Siegel (1983) applied this ratio as a measure of stress in domestic fowl (Moreno 2002). Since then, H/L has been commonly used in assessing the conditions of chickens reared under different conditions, and it has gradually become more commonly used as a stress indicator in other avian studies (Davis et al. 2008). Heterophils and lymphocytes are two types of leukocytes that can be observed in blood samples. Leukocytes, also known as white blood cells, are immune system cells that primarily function to protect against foreign pathogens.

Of the leukocytes found in avian blood, heterophils and lymphocytes are the most abundant (Campbell 1995; Davis et al. 2008; Moreno et al. 2002). Heterophils enter tissues during the non-specific inflammatory response, whereas lymphocytes are part of the specific immune response (Moreno et al. 2002; Krams et al. 2010). The difference in the cellular responses of heterophils and lymphocytes to stressful conditions allows for the use of H/L as a quantifiable indicator of stress, such that higher H/L may be indicative of increased stress (Zulkifli et al. 2000; Davis et al. 2008; Cirule et al. 2012).

Certain stressors can cause an increase in heterophil counts and a reduction in lymphocyte counts, resulting in an increase of the H/L. Gross and Siegel (1983) found that in chickens, H/L increased in response to increasing levels of CORT in the chicken feed and to various stressors such as social stress, blood sampling, and the administration of inoculations. Zulkifli et al. (2000) evaluated the effect of pre-slaughter handling on H/L in broiler chickens and surprisingly found no significant difference in H/L response between roughly and gently handled chickens. However, the chickens were treated with ascorbic acid to experimentally modify fear-related behavior, which may have affected the H/L results. As a result of the study, Zulkifli et al. (2000) suggested that supplemental ascorbic acid may alleviate fearfulness and enhance the welfare of broiler chickens, in which case the observed H/L response may have been inhibited by such effects. Ewenson et al. (2001) compared H/L between wild, captured, and aviary-bred zebra finches, concluding that the avian immune system may respond to stressors independently of body condition. Moreno et al. (2002) reported a positive correlation between H/L and nutritional stress resulting in reduced growth in nestling Pied Flycatchers. In that same study, Moreno et al. (2002) also demonstrated a possible aspect of the relationship between the responses of H/L and HSP60 to stress caused

by limited food availability. According to their conclusions, H/L was likely related to the mean effects of malnutrition within the broods, while HSP60 likely reflected stress imposed by asymmetric sibling competition brought on by food restriction. Al-Murrani et al. (2006) used the magnitude and direction of the change in H/L in response to infection as an indicator of resistance to stress, finding that selection for stress resistance based on measurement of H/L is positively correlated with some reproduction and fitness traits in chickens. Krams et al. (2010) found a positive correlation between forest management and physiological stress in Siberian Tits, further demonstrating H/L as a reliable stress indicator.

Effects of Capture and Handling

Glucocorticoids, such as CORT, are 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, this method is often unreliable, as release of CORT can be induced by acute distress associated with capture and handling during field research. Being caught and handled is an acutely distressful experience for birds that can rapidly mobilize CORT, which makes the accuracy of CORT measurements questionable (Sapolsky et al. 2000; Romero and Reed 2005; Fridinger et al. 2007; Herring and Gawlik 2007; Busch and Hayward 2009; Cockrem et al. 2009). Fridinger et al. (2007) showed that Black-legged Kittiwake nestlings exhibit increased levels of CORT in response to handling stress and to the removal of nest mates by researchers. When handling time before sampling lasts for more than two or three minutes, CORT levels may no longer accurately reflect birds' physiological

status before their capture (Romero and Reed 2005; Cockrem et al. 2009). Also, CORT, especially fecal corticosterone, can degrade over time in frozen samples (Herring et al. 2007).

HSPs may be a more useful indicator of chronic stress because they are maintained at high levels for longer periods of time than CORT, and some research has shown that handling distress does not cause their rapid upregulation (Martinez-Padilla et al. 2004; Herring and Gawlik 2007; Herring et al. 2011). HSPs are assumed to be more resistant to acute distress by capture and handling, but this assumption has not been systematically examined as only limited research has been done to demonstrate the reliability of HSPs in avian research. The effects of capture and handling on HSPs were evaluated in a companion study by this research group but were not reported for the purposes of this thesis study.

Furthermore, the avian immune response to stress takes significantly longer to begin 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). The slower stress response and longer endurance of increased H/L indicate it may be a more reliable measure than the more commonly used CORT, especially in obtaining baseline measurements. Vleck et al. (2000) compared the responses of CORT and H/L in Adélie penguins to different stressors, including handling time and repeated sampling. Vleck et al. (2000) found no increase in H/L or CORT in penguins repeatedly handled and sampled with an average interval of 13.4 days between samples. They observed no significant increase in H/L or CORT in samples taken with handling times less than 5 minutes, but CORT did increase in samples taken more than 5 minutes after capture. Davis (2005) examined the effects of handling time and repeated sampling on H/L in House finches and found that H/L did not increase significantly in response to handling times of up to one hour.

More recently, Cirule et al. (2012) compared the use of circulating glucocorticoid levels to H/L as physiological stress indicators in a study of wintering male Great Tits, concluding that H/L is a useful alternative method for measuring stress in birds. Though in that same study, Cirule et al. (2012) found that acute distress 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 may change more rapidly than originally thought due to acute distress, which may limit the reliability of results in a way similar to CORT. This concern justifies further study of H/L in response to acute distress in comparison to other well-studied methods of measuring physiological stress, such as CORT.

Evaluating Reliability and Accuracy

The reliability of these stress assessment tools is paramount to the success of avian research concerning bird management and conservation. CORT and H/L may not be equally consistent stress assessment tools for avian research since their values can be affected in different ways and over different time periods by the acute distress experienced by birds upon their capture and handling (Sapolsky et al. 2000; Romero and Reed 2005; Fridinger et al. 2007; Herring and Gawlik 2007; Busch and Hayward 2009; Cockrem et al. 2009; Cirule et al. 2012). The significantly slower H/L response time and longer endurance of the H/L response demonstrate that H/L may be a much more reliable measurement of long-term stress in birds. However, the relative utility of H/L as a stress assessment tool has been little studied outside of research concerning domestic fowl. Much research has been done to refine

the use of glucocorticoids and H/L as stress assessment tools, but limited research has been done to compare the sensitivities of both CORT and H/L to handling stress. With the exception of researchers studying chickens, few studies have compared CORT and H/L in blood samples taken from the same subjects at the same time, and very few have compared the biomarkers in such samples taken from free-ranging birds. More research is needed to evaluate the causal relationships between stress due to capture and handling and these biomarkers of the avian stress response.

This study compared the sensitivity of CORT and H/L to acute distress induced by the capture and handling of Tufted Titmice to evaluate their use as reliable tools in avian research. Previous research has demonstrated the sensitivity of CORT to handling stress (Romero and Reed 2005; Fridinger et al. 2007; Herring and Gawlik 2007; Busch and Hayward 2009; Cockrem et al. 2009), but the relationship between H/L and handling stress has not been as well studied. To further evaluate H/L as a reliable indicator of chronic moderate physiological distress, the relationships between CORT and H/L to the time elapsed from capture to blood sampling and the relationships between the two bioindicators were analyzed. It was predicted that a positive correlation would be found between CORT and handling times longer than 2 minutes after capture of subjects. It was predicted that no correlation would be found between H/L and handling time in samples taken more than 2 minutes after capture. It was also predicted that no correlation would be found between values of CORT and H/L within subjects.

MATERIALS AND METHODS

Research Subjects

This study was conducted using the Tufted Titmouse (*Baeolophus bicolor*), a permanent-resident passerine bird that is commonly found throughout eastern North America, including Virginia (Pravosudova et al. 2001). Winter is a critical time for the survival of permanent-resident birds, such as the Tufted Titmouse, primarily due to food scarcity and temperature extremes (Pravosudova et al. 2001). Since the Tufted Titmouse supplements its diet by caching food such as seeds during cold winter weather, it commonly frequents bird feeders.

Previous stress research utilizing CORT and H/L as physiological stress indicators has been performed using species of passerine birds that are close relatives of the Tufted Titmouse, such as the Great Tit and the Siberian Tit (Davis 2005; Krams et al. 2010; Cirule et al. 2012). The use of related species in stress research can better serve as a basis of comparison for data collected to evaluate stress indicators.

Capture and Handling

Mist nets were set around established feeders to capture Tufted Titmouse subjects at sites in Spotsylvania and Stafford counties in Virginia within 30 miles of the University of Mary Washington campus. All subjects were captured during the field portion of this study, which was conducted from December 2012 through February 2013. Ambient conditions were

recorded as the average overnight air temperature (˚C) during the night before capture and air temperature (˚C) at the time of capture for each subject.

Upon capture with the mist net, subjects were carried in small canvas bags a short distance to the blood sampling site. As a consequence of the complications of capturing and handling birds in a mist net, blood samples were drawn at varying 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 942 seconds to mimic the range of handling times typical to this type of field research. Subjects were individually marked with metal leg bands engraved with unique identification numbers.

Physical Measurements and Blood Sampling

Right wing chord length was measured to the nearest .5 mm and mass to the nearest .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 $\frac{\text{mass}}{\text{wing length}^3} \cdot 10^6$, a modification of the condition index used by Owen and Cook (1977). This BCI can be used in evaluating whether birds are over- or underweight in relation to their body structural size.

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 mL centrifuge tube. The blood samples were kept cool with a freezer pack in a small styrofoam cooler in transition 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 G for 10 minutes to separate the plasma supernatant from the hematocrit. The separated plasma and hematocrit samples were kept frozen in individually labeled 5 mL centrifuge tubes in a subzero (-80 ˚C) freezer.

Corticosterone Levels

After the conclusion of field work, the frozen plasma samples were brought back to room temperature and analyzed using a Corticosterone Enzyme Immunoassay (EIA) kit from Enzo Life Sciences International, Inc. Prior to the procedure, the assay buffer was prepared by diluting 10 mL of assay buffer concentrate in 90 mL of deionized water, and the wash buffer was prepared by diluting 5 mL of wash buffer concentrate in 95 mL of deionized water. Corticosterone standards were prepared in five labeled tubes with concentrations of 20,000, 4,000, 800, 160 and 32 pg/mL. Volumes of plasma obtained for several of the samples were insufficient for the EIA protocol. Therefore, plasma samples were diluted 1:30 by mixing 3.3 µL of the plasma sample into 96.7 µL of assay buffer. According to the EIA protocol, samples were assayed in duplicate in a 96-well microwell plate according to the layout sheet provided in the EIA kit (Figure 1). The total number of samples assayed was 28, though only 26 were run in duplicate because two of the diluted plasma samples did not have adequate volume to be run in duplicate. The data points from those two samples were excluded from analysis.

Following the EIA protocol, a micropipetter was used to dispense 100 µL of standard diluent, standards and diluted plasma samples and 50 µL of assay buffer, conjugate and

corticosterone antibody into wells as instructed in the EIA protocol. The plate was incubated at room temperature on a plate shaker for two hours at 500 rpm, washed three times using the prepared wash buffer, and then aspirated to remove remaining wash buffer. After washing, a micropipetter was used to add 5 µL of conjugate and 200 µL of pNpp substrate solution to wells as instructed in the EIA protocol. The plate was incubated at room temperature for one hour without shaking. A micropipetter was then used to stop the reaction by adding 50 µL of stop solution to every well. The plate was immediately read at 405 nm optical density using an Eon microplate reader and Gen5 microplate reader software from BioTek Instruments, Inc.

A1 Blank	A2 Std 1	A3 Std 5	A4 U4	A5 U8	A6 U12	A7 U16	A8 U20	A9 U24	A10 U28	A11 Blank	A12 Blank
B1 Blank	B2 Std 1	B3 Std 5	B4 U4	B5 U8	B6 U12	B7 U16	B8 U20	B9 U24	B10 U28	B11 Blank	B12 Blank
C1 TA	C2 Std 2	C3 U1	C4 U5	C5 U9	C6 U13	C7 U17	C8 U21	C9 U25	C10 Blank	C11 Blank	C12 Blank
D1 TA	D2 Std 2	D3 U1	D4 U5	D5 U9	D6 U13	D7 U17	D8 U21	D9 U25	D10 Blank	D11 Blank	D12 Blank
E1 NSB	E2 Std 3	E3 U2	E4 U6	E5 U10	E6 U14	E7 U18	E8 U22	E9 U26	E10 Blank	E11 Blank	E12 Blank
F1 NSB	F2 Std 3	F3 U2	F4 U6	F5 U10	F6 U14	F7 U18	F8 U22	F9 U26	F10 Blank	F11 Blank	F12 Blank
G1 B_0	G2 Std 3	G3 U3	G4 U7	G5 U11	G6 U15	G7 U19	G8 U23	G9 U27	G10 Blank	G11 Blank	G12 Blank
H1 B_0	H2 Std 4	H3 U3	H4 U7	H5 U11	H6 U15	H7 U19	H8 U23	H9 U27	H10 Blank	H11 Blank	H12 Blank

Figure 1. Corticosterone plate layout. The alphanumeric sequences in the upper left corners of the cells indicate the arrangement of the wells on the microwell plate. The centered labels indicate the type of treatment for each well. Blank: background absorbance caused by reagent, subtracted from absorbance readings of other wells. TA: Total Activity, total enzymatic activity of tracer. NSB: Non-Specific Binding, non-immunological binding of tracer to well in absence of antibody. B_0: Maximum Binding, maximum amount of tracer antibody can bind. Std: Standard. U: Unknown, prepared sample run in duplicate.

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 per 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 per mL serum.

Heterophil to Lymphocyte Ratios

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.

The 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, also known as red blood cells, were examined in search of leukocytes. For each individual slide, only fields of view with similar erythrocyte densities as 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. Identification of leukocytes was based on information and images found in the *Atlas of Clinical Avian Hematology* (Clark et al. 2009).

In order to account for the variability of 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. The number of leukocytes per 10,000 erythrocytes was calculated and recorded, and an H/L index was calculated for each slide as the H/L multiplied by the number of leukocytes per 10,000 erythrocytes.

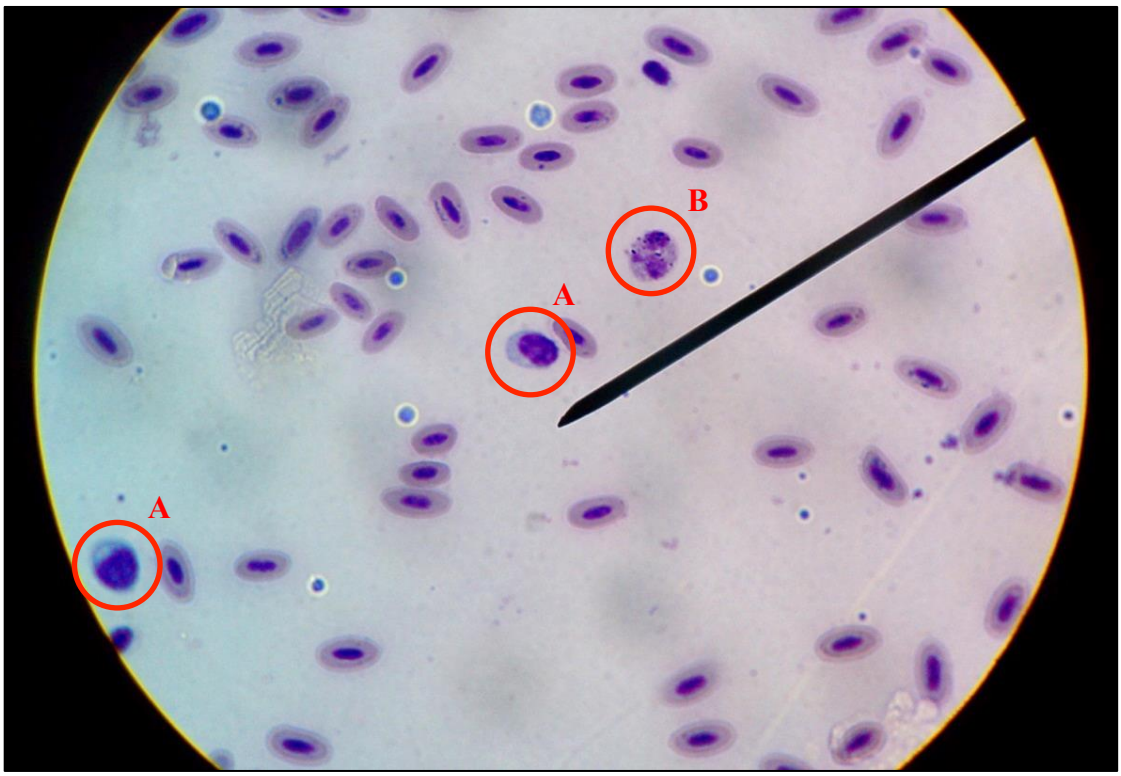


Figure 2. A field of view showing a stained blood smear under a compound microscope with 1,000x magnification using oil immersion. The circles labeled A indicate lymphocytes. The circle labeled B indicates a heterophil.

Statistical Analysis

After obtaining the CORT concentration and H/L for each sample, IBM SPSS Statistics software was used to evaluate the relationships between the variables CORT, H/L, H/L Index, body mass (Mass), BCI, and handling time (Time). Linear regression and analysis of variance (ANOVA), a multiple comparison test of significance, were used to describe the following relationships between variables in determining correlations: CORT versus Time, H/L versus Time, H/L index versus Time, H/L versus Mass, H/L Index versus Mass, H/L versus BCI, and H/L Index versus BCI. All tests performed using the SPSS software were considered significant when $P < 0.05$.

Since the relationship between CORT and H/L may depend on the time elapsed between initial capture and sampling, the analysis was also performed using the residuals of CORT versus Time (CORT Resids) to account for the variation in CORT values owing to capture and handling. Linear regression and ANOVA were performed on H/L versus CORT Resids and H/L Index versus CORT Resids. 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 versus Time.

RESULTS

After all stained blood smears were examined, some slides remained with blood smears that were too thick to obtain reliable counts and were therefore excluded. After the analysis of plasma samples for CORT concentration, some results were excluded due to relative error >10% between the duplicate runs of the samples in the assay. After excluding

such results, leukocyte data was obtained for 24 slides, and CORT values remained for 14 samples with coinciding leukocyte data and physical measurements (Table 1). Due to complications in the field that prevented body mass from being measured on two subjects, out of the 24 slides with leukocyte data only 22 had coinciding physical measurement (Table 1).

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

Variable	N	Mean	S.D.	Range
CORT[a]	14	17.538	13.772	1.13 to 44.41
CORT Resids[b]	14	0.000	0.961	-1.879 to 1.594
Heterophils[c]	24	5.667	3.299	2.0 to 16.0
Lymphocytes[d]	24	37.438	12.255	15.5 to 62.0
H/L[e]	24	0.157	0.082	0.047 to 0.362
H/L Index[f]	24	95.869	79.878	9.367 to 320.4
Mass (g)	22	21.273	1.193	19.0 to 24.5
BCI[g]	22	40.695	2.668	35.8 to 46.4
Time[h] (s)	24	454.000	250.390	120 to 942

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

The average handling time before blood sampling was 454 seconds (N = 24, Mean = 454 ± 250.39, Range 120 to 942), or just under 8 minutes (Table 1). CORT was positively and significantly correlated with handling time (R^2 = .702, N = 14, P < .001). No other relationship examined resulted in a significant correlation (Table 2).

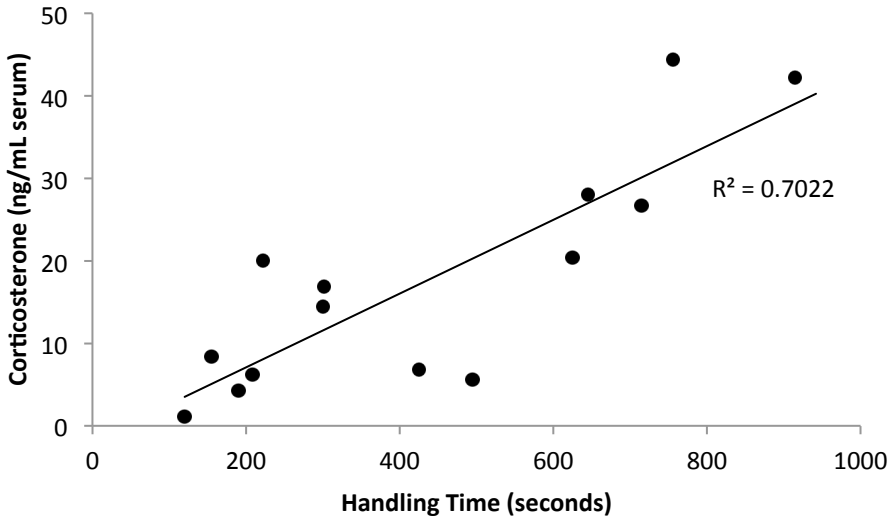
H/L was not significantly correlated with CORT after variation in CORT owing to handling time was removed (Spearman's rho = .241, N = 14, P = .407).

Table 2. Summary of results from statistical analysis. For each relationship listed, SPSS software was used to perform linear regression and ANOVA[a].

Variables	df	F	R^2	P
CORT[b] vs. Time[c] (s)	13	28.295	.702	< .001
H/L[d] vs. Time (s)	23	1.220	.053	.281
H/L Index[e] vs. Time (s)	23	1.782	.075	.196
H/L vs. CORT Resids[f]	13	1.518	.112	.241
H/L Index vs. CORT Resids	13	1.182	.090	.298
H/L vs. Mass (g)	21	0.033	.002	.859
H/L Index vs. Mass (g)	21	0.142	.007	.710
H/L vs. BCI[g]	21	0.612	.030	.443
H/L Index vs. BCI	21	0.708	.034	.410

[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)

(a)



(b)

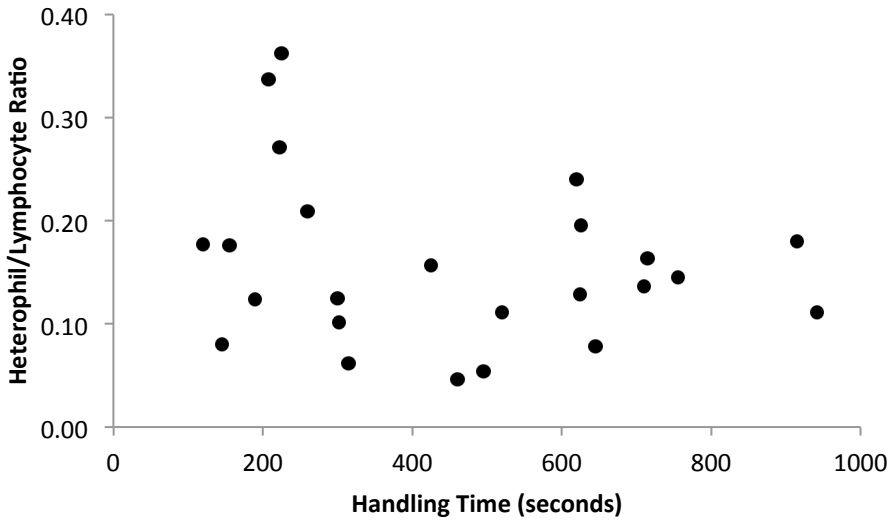


Figure 3a and b. Scatter plots showing (a) corticosterone (CORT) concentration (ng/mL serum) versus handling time (seconds), and (b) heterophil/lymphocyte ratios (H/L) versus handling time (seconds). Linear regression was performed on both data sets using SPSS software. The R-square value for (a) the correlation between CORT and handling time is displayed below the trendline on the plot.

DISCUSSION

After performing linear regression and ANOVA to examine the relationships between the variables, no significant correlations were found between the CORT data, leukocyte data, and data from physical measurements taken in the field. 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; 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 more than 2 minutes after capture, supporting H/L as a better measure of chronic stress for some research applications.

The CORT results in this study were similar to the results of studies on free-ranging Adélie penguins by Vleck et al. (2000) and Cockrem et al. (2009), to the results of Davis et al. (2000) on Single Comb White Leghorn hens, and to the results of Herring et al. (2011) on White Ibis nestlings. CORT results were not consistent with values obtained in the research of Gross and Siegel (1983) on broiler chickens or of Fridinger et al. (2007) on Black-legged Kittiwake nestlings. Herring and Gawlik (2007) reported fecal CORT in ng/g, thus the plasma CORT values in ng/mL reported in this study could not be directly compared.

The results for heterophil counts, lymphocyte counts, and H/L in this study were similar to those for Siberian Tits in managed habitats (Krams et al. 2010) and for wintering male Great Tits (Cirule et al. 2012). Though the lymphocyte counts and H/L values reported in this study were not similar to those of House Finches (Davis 2005), the heterophils counts

were within the range reported by Davis (2005). The ranges of leukocyte counts and H/L in this study were consistently lower than the those reported in several studies, including the results of Gross and Siegel (1983) and Zulkifli et a. (2000) on broiler chickens, Davis et al. (2000) on Single Comb White Leghorn hens, Vleck et al. (2000) on Adélie penguins, Moreno et al. (2002) on Pied Flycatcher nestlings, Martinez-Padilla et al. (2004) on Eurasian Kestrel nestlings, and Al-Murrani et al. (2006) concerning chickens. Upon review of the leukocyte data in this study compared to the results of previous research on H/L, the disparities of results between these studies could be attributed to the differences between the leukocyte profiles of the different species studied. The values found in the results of this study seem to agree more closely with studies concerning species that are more closely related to the Tufted Titmouse, such as the Great Tit and the Siberian Tit (Davis 2005; Krams et al. 2010; Cirule et al. 2012).

The H/L ratio is a direct measure of a physiological change, whereas the concentration of CORT in the blood may be affected by different factors before physiological changes occur in response to environmental stressors (Gross and Siegel 1983). Numbers of different leukocytes present in the blood change more slowly in response to stress than does CORT concentration, and the changes are longer lasting than CORT (Vleck et al. 2000). H/L was not expected to change in response to stress due to capture and handling within the time range in this study, which the data supports. Owing to such differences between these two bioindicators and the resistance of H/L to acute distress from capture and handling that is supported by this study, the consistency and relative dependability of these two stress measures must be considered in field studies which require the handling of subjects to attain blood samples. The rapid effect of capture and handling on CORT requires that blood

samples be collected immediately after capture in order to obtain baseline measurements indicative of environmental stress as opposed to handling stress. The resistance of H/L to the effect of capture and handling demonstrates that this measure is not affected by routine handling and thus allows for the attainment of baseline measurements indicative of environmental stress instead of stress due to capture and handling. H/L is therefore a more reliable and tool for use in field studies measuring chronic stress.

Spearman's rho was calculated to examine the within-subject relationship between CORT and H/L after removing the variation in CORT owing to the effect of handling time, and again no significant correlation was found between the CORT and leukocyte data. This finding agrees with the results of Gross and Siegel (1983) in which no correlation was found between H/L and CORT 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 this study does contradict the results of more recent research that has supported a possible relationship between H/L and CORT. According to Sapolsky et al. (2000) in a comprehensive review of current research on physiological stress indicators, 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. Prolonged increases in levels of glucocorticoids can have an immunosuppressive effect resulting in reductions of circulating leukocytes, which would alter the H/L measured in samples. But Sapolsky et al. (2000) also suggest that decreases in leukocyte counts in such situations may

not be due to the depletion of leukocytes but the diversion of leukocytes to local areas of need in response to specific stressors. If this were true, glucocorticoids such as CORT would not have the described immunosuppressive effect. 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.

The findings of this study support H/L as a more reliable stress measurement tool for use in field research as well as a more convenient tool. The significantly slower H/L response to stress, especially the acute distress due to capture and handling, allows researchers more time to obtain blood samples in the field with accurate baseline measurements. In addition to decreasing the pressure 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 amount of blood necessary to prepare a blood smear is much smaller than that required for CORT assays. A blood smear requires a single drop of blood whereas typical assays to measure CORT concentrations require several microliters. The average Tufted Titmouse body mass in this study was just over 21 g and the smallest bird sampled was 19 g. When extracting blood samples from birds of such small masses, it would be ideal to use the least invasive method of blood sampling. Second, the relative costs of obtaining H/L and CORT measurements for research are significantly 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. H/L

offers a less expensive and more convenient method for measuring stress that may affect the subjects sampled less.

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 represent the numbers of heterophils or lymphocytes present in other areas of the body from which samples were not taken, which may change if certain stressors affect localized regions within a subject's 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. Since references do not exist for many species describing what would be a typical H/L in low-stress or high-stress conditions, this poses an issue for researchers attempting to interpret the stress data represented by this measure. Finally, as a measure based on the immune response to stress, H/L may be influenced by other factors that induce an inflammatory or an antibody response, such as injury or disease. While these factors could be causes of chronic stress, they do pose a problem to researchers with specific applications in mind exclusive of those kinds of stressors. These challenges do not omit H/L as a useful physiological indicator of chronic stress, but they do warrant consideration in choosing the appropriate stress assessment tool for use in specific research applications.

This study was conducted to determine the effect of acute distress due to capture and handling on levels of plasma CORT and on H/L and to determine a possible correlation

between these two physiological stress indicators. As described above, the results of this study support H/L as a reliable stress indicator that is less sensitive to the effects of handling time than the more commonly used CORT. 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 as a measure of chronic moderate stress in avian field research.

LITERATURE CITED

Al-Murrani, W.K., A.J. Al-Rawi, M.F Al-Hadithi and B. Al-Tikriti. 2006. Association between heterophil/lymphocyte ratio, a marker of 'resistance' to stress, and some production and fitness traits in chickens. *British Poultry Science* 47:443-448.

Arriero, E., J. Moreno, S. Merino and J. Martinez. 2008. Habitat effects on physiological stress response in nestling blue tits are mediated through parasitism. *Physiological and Biochemical Zoology* 81:195-203.

Busch, D.S. and L.S. Hayward. 2009. Stress in a conservation context: A discussion of glucocorticoid actions and how levels change with conservation-relevant variables. *Biological Conservation* 142:2844-2853.

Campbell, T.W. 1995. *Avian Hematology and Cytology*. Iowa State University Press, Iowa. 112pp.

Cirule, D., T. Krama, J. Vrublevska, M.J. Rantala and I. Krams. 2012. A rapid effect of handling on counts of white blood cells in a wintering passerine bird: a more practical measure of stress? *Journal of Ornithology* 153:161-166.

Clark, P., W.S.J. Boardman and S. Raidal. 2009. Atlas of Clinical Avian Hematology. Wiley-Blackwell, United Kingdom. 200pp.

Cockrem, J.F., D.P. Barrett, E.J. Candy and M.A. Potter. 2009. Corticosterone responses in birds: Individual variation and repeatability in Adélie penguins (*Pygoscelis adeliae*) and other species, and the use of power analysis to determine sample sizes. *General and Comparative Endocrinology* 163:158-168.

Davis, G.S., K.E. Anderson and A.S Carroll. 2000. The effects of long-term caging and molt of single comb white leghorn hens on heterophil to lymphocyte ratios, corticosterone and thyroid hormones. *Poultry Science* 79:514-518.

Davis, A.K. 2005. Effect of handling time and repeated sampling on avian white blood cell counts. *Journal of Field Ornithology* 76:334-338.

Davis, A.K., D.L. Maney and J.C. Maerz. 2008. The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. *Functional Ecology* 22:760-772.

Davison, T.F., J.G. Rowell and J. Rea. 1983. Effects of dietary corticosterone on peripheral blood lymphocytes and granulocyte populations in immature domestic fowl. *Research in Veterinary Science* 34:236-239.

del Cerro, S., S. Merino, J. Martinez-de la Puente, E. Lobato, R. Ruiz-de-Castañeda, J. Rivero-de Aguilar, J. Martinez, J. Morales, G. Tomas and J. Moreno. 2010. Carotenoid-based plumage colouration is associated with blood parasite richness and stress protein levels in blue tits (*Cyanistes caeruleus*). *Oecologia* 162:825-835.

Ewenson, E.L., R.A. Zann and G.R. Flannery. 2001. Body condition and immune response in wild zebra finches: effects of capture, confinement and captive-rearing. *Naturwissenschaften* 88:391-394.

Feder, M.E. and G.E. Hofmann. 1999. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annual Review of Physiology* 61:243-282.

Fridinger, R.W., K.M. O'Reilly, S.D. Kildaw and C.L. Buck. 2007. Removal of a nest-mate elicits an age-dependent increase in plasma corticosterone of nestling black-legged kittiwakes. *Journal of Field Ornithology* 78:93-99.

Gross, W.B. and H.S. Siegel. 1983. Evaluation of the heterophil/lymphocyte ratio as a measure of stress in chickens. *Avian diseases* 27:972-979.

Herring, G. and D.E. Gawlik. 2007. The role of stress proteins in the study of allostatic overload in birds: use and applicability to current studies in avian ecology. *The Scientific World Journal* 7:1596-1602.

Herring, G., M.I. Cook, D.E. Gawlik and E.M. Call. 2011. Food availability is expressed through physiological stress indicators in nestling white ibis: a food supplementation experiment. *Functional Ecology* 25:682-690.

Houwen, B. 2000. Blood film preparation and staining procedures. *Laboratory Hematology* 6:1-7.

Krams, I., D. Cirule, T. Krama, M. Hukkanen, S. Rytkonen, M. Orell, T. Iezhova, M.J. Rantala and L. Tummeleht. 2010. Effects of forest management on haematological parameters, blood parasites, and reproductive success of the Siberian Tit (*Poecile cinctus*) in northern Finland. *Annales Zoologici Fennici* 47:335-346.

Martinez-Padilla, J., J. Martinez, J.A. Dávila, S. Merino, J. Moreno and J. Millán. 2004. Within-brood size differences, sex and parasites determine blood stress protein levels in Eurasian Kestrel nestlings. *Functional Ecology* 18:426-434.

Merino, S., J. Martinez, A. Barbosa, A.P. Moller, F. de Lope, J. Pérez and F. Rodríguez-Caabeiro. 1998. Increase in a heat-shock protein from blood cells in response of nestling house martins (*Delichon urbica*) to parasitism: an experimental approach. *Oecologia* 116:343-347.

Merino, S., J. Moreno, G. Tomas, J. Martinez, J. Morales, J. Martinez-de la Puente and J.L. Osorno. 2006. Effects of parental effort on blood stress protein HSP60 and immunoglobulins in female blue tits: a brood size manipulation experiment. *Journal of Animal Ecology* 75:1147-1153.

Moreno, J., S. Merino, J. Martinez, J.J. Sanz and E. Arriero. 2002. Heterophil/lymphocyte ratios and heat shock protein levels are related to growth in nestling birds. *Ecoscience* 9:434-439.

Owen, M. and W.A. Cook. 1977. Variations in body weight, wing length and condition of Mallard *Anas platyrhynchos platyrhynchos* and their relationship to environmental changes. *Journal of Zoology: Proceedings of the Zoological Society of London* 183:377-395.

Pravosudova, E.V., T.C. Grubb Jr. and P.G. Parker. 2001. The influence of kinship on nutritional condition and aggression levels in winter social groups of tufted titmice. *The Condor* 103:821-828.

Ritossa, F. 1962. A new puffing pattern induced by temperature shock and DNP in *Drosophila. Experientia* 18:571-573.

Romero, L.M. and J.M. Reed. 2005. Collecting baseline corticosterone samples in the field: is under 3 min good enough? *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology* 140:73-79.

Sapolsky, R.M., L.M. Romero and A.U. Munck. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocrine Reviews* 21:55-89.

Sorenson, J.G., T.N. Kristensen and V. Loeschchke. 2003. The evolutionary and ecological role of heat shock proteins. *Ecology Letters* 6:1025-1037.

Tomas, G., J. Martinez and S. Merino. 2004. Collection and analysis of blood samples to detect stress protein in wild birds. *Journal of Field Ornithology* 75:281-287.

Vleck, C.M., N. Vertalino, D. Vleck and T.L. Bucher. 2000. Stress, corticosterone, and heterophil to lymphocyte ratios in free-living Adélie penguins. *The Condor* 102:392-400.

Zulkifli, M.T., C. Norma, C.H. Chong and T.C. Loh. 2000. Heterophil to lymphocyte ratio and tonic immobility reactions to preslaughter handling in broiler chickens treated with ascorbic acid. *Poultry Science* 79:402-406.

Zulkifli, M.T., C. Norma, D.A. Israf and A.R. Omar. 2002. The effect of early-age food restriction on heat shock protein 70 response in heat –stressed female broiler chickens. *British Poultry Science* 43:124-145.