The relationship between measures of stress and condition in nestling European starlings

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Abstract

Maintaining an effective immune system is energetically costly. During times of stress, the immune system and an individual's physical condition can become compromised. Elevated white blood cell (WBC) counts, and high heterophil/lymphocyte (H/L; types of WBCs) ratios are two reliable indicators of stress in birds. The objective of this study was to determine whether nestling condition was negatively correlated with stress in European starlings (*Sturnus vulgaris*). I predicted that nestlings in poor condition would have higher stress and therefore higher overall WBC counts and H/L ratios than nestlings in good condition. The best and worst condition nestlings were chosen from each nest when 14 -15 days of age (n = 16 nests), as measured by regressing mass against tarsus length. Blood smears were made for each of these nestlings with a small blood sample. Smears were then fixed in methanol, stained using Hema III and examined with microscopy to estimate WBC count and H/L ratio per 10,000 erythrocytes. Condition differed significantly between the two nestling groups (best and worst condition). Mean WBC counts per brood tended to be positively correlated with mean H/L ratios per brood. However, counter to my prediction, no correlation existed between mean WBC count and mean nestling condition per brood or mean nestling H/L ratio and mean condition per brood. Differences in H/L ratio, however, were positively correlated with differences in nestling condition, suggesting that nestlings in similar condition are similarly stressed. The lack of significant relationship between H/L ratio and condition found in nestlings contrasts with results from another study done on adult starlings in this same population. Nestlings at 14-15 days of age are likely still developing their immune response, and so WBC counts and H/L ratios are not good measures of stress.

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Introduction

The fields of ecophysiology and conservation are interested in examining stress levels and reactions of organisms in response to stressors and changes in the environment, such as habitat degradation, climate change and human interference (Banbura et al. 2013). As climate changes and the increasing human population become more pressing concerns, these fields are more interested in examining stress and the organism's ability to cope to the changing environment (Muller et al. 2011; Banbura et al. 2013). Long-term research into stress levels of different species becomes increasingly important to monitor the impact of these global trends on wildlife (Liker et al. 2008).

The physiological stress response is activated through two methods that act independently of one another; one is the well-known fight or flight response, also called the sympathetic-adrenal-medulla axis that is activated within seconds, and the other is the hypothalamic-pituitary-adrenal (HPA) axis that is activated over minutes to hours (de Bruijn & Romero 2013). The HPA axis is the focus of this study; it releases the stress hormone corticosterone, which in turn affects the number and type of circulating white blood cells (Masello et al. 2009; de Bruijn & Romero 2013). The magnitude of the stress response elicited by an organism when confronted by a stressor is specific to the type of stressor and the environment within which the stressor is experienced (de Bruijn & Romero 2013). Stress is important to study because repeated and long-term exposure is detrimental to the health of the organism. Consistently high corticosterone levels may cause dysregulation of homeostasis, immunosuppression, and even cognitive impairment of the individual (de Bruijn & Romero 2013; Fiske et al. 2013). Fiske et al. (2013) noted that stress-response sensitivities are species-specific and can vary with development.

Davis (2009) also observed that different species had different baseline average and percentages of white blood cell types circulating in the blood stream. He speculated that the demonstrated high variance in proportions of leukocytes across species is due to differing stress levels and the effects of stress on white blood cells.

The immune system consists of two main components, adaptive immunity and innate immunity (Pihlaja et al. 2006; Brzek & Konarzewski 2007). Adaptive immunity involves acquired immune responses, or B- and T-cell-mediated immunity, involving lymphocytes in highly specific responses that are more effective against pathogens (Saino et al. 1997; Pihlaja et al. 2006; Masello et al. 2009). Acquired immune responses are slow and costly to develop and are resource and nutritionally demanding, but are relatively low-cost to use once developed (Pihlaja et al. 2006; Brzek & Konarzewski 2007). Innate immunity involves the responses of macrophages and phagocytic cells, such as monocytes (Pihlaja et al. 2006; Mitchell & Johns 2008). Innate immunity is inexpensive to develop and maintain, but the costs of the system increase with use and after the development of adaptive immunity because adaptive immunity is more effective against many pathogens (Pihlaja et al. 2006; Brzek & Konarzewski 2007; Masello et al. 2009).

The immune response enhances an organism's ability to survive (Parejo et al. 2007) and influences fledging success and fledgling survival (e.g. Naguib et al. 2004; Quillfeldt et al. 2008; Masello et al. 2009; Tilgar et al. 2009; Tilgar et al. 2010). An individual's current stress level can therefore be a predictor of one's future stress and survival (Naguib et al. 2004; Lendvai et al. 2009). Immunity is negatively related to the stress levels of the individual (Loiseau et al. 2008; Muller et al. 2011). For example, Muller et al. (2011) found that an increase in the stress hormone corticosterone was

correlated with a decrease in a measure of immunity called phytohaemagglutinin (PHA)-induced swelling response. PHA is a novel antigen that is injected sub-cutaneously to assess immune response, measured by the amount of swelling (Brzek & Konarzewski 2007; Muller et al. 2011). This response is ambiguous, however, because it involves reactions from all components of the immune system, but it does permit researchers to make broad conclusions about the costs of immunity (Brzek & Konarzweski 2007).

Maintaining an immune system is energetically costly (Masello et al. 2009). These costs can be amplified during a period of stress due to the activation of other costly physiological processes and subsequent energy demands (Parejo et al. 2007). A trade-off between immunity and condition has been suggested by Bourgeon et al. (2011) that is particularly true of animals who are in the process of growing – a very energetically demanding process. Therefore, although the two processes are occurring simultaneously, they each have their own energy demands and requirements. When an individual is in a high stress environment, where energy requirements for all processes are not being met, the individual reduces the energy allotted to one or more of these processes (Brzek & Konarzewski 2007; Doeschl-Wilson et al. 2009; Moreno-Rueda & Redondo 2011). Several studies on adult and nestling birds have found a significant negative relationship between stress and condition (e.g. Loiseau et al. 2008; Lendvai et al. 2009), a significant negative relationship between stress and immunity (e.g. Almasi et al. 2010; Tilgar et al. 2010), and significant positive relationships between immunity and condition (e.g. Naguib et al. 2004; Soler et al. 2007).

The total white blood cell (WBC) count and the leukocyte profile (the different types of WBCs) are important components of the immune response (Davis et al.

2008). WBC counts estimate the relative abundance of leukocytes circulating within the peripheral blood when estimated through blood smears. A high level of circulating leukocytes is a symptom of stress or inflammation (Ots et al. 1998; Parejo et al. 2007). Leukocyte profiles examine the abundance of each of the five leukocyte types, typically by estimating their proportions out of 100 leukocytes (Davis 2005). Heterophils are involved in the inflammatory response (Mitchell & Johns 2008), and also in the presence of infection, poor diet, trauma or stress (Gross & Siegel 1983; Davis et al. 2008). Lymphocytes are part of the acquired immune system (Masello et al. 2009), and are important in specific immune responses requiring B-cell-mediation (Grasman 2002; Pihlaja et al. 2006) and immunoglobulin production (Campbell 1996). Heterophils and lymphocytes are the two most abundant leukocyte cell types in avian species (Grasman 2002). Monocytes, another type of leukocyte, are large phagocytic cells involved in defending the individual from invading infections and bacteria (Mitchell & Johns 2008). Eosinophils, a fourth leukocyte type, are involved in fighting parasites (Grasman 2002). Finally, basophils are thought to be involved in inflammation, but their exact function is largely unknown (Davis et al. 2008; Mitchell & Johns 2008; Vinkler et al. 2010).

The heterophil to lymphocyte (H/L) ratio is determined through leukocyte profiles to measure chronic stress levels in birds (Davis et al. 2008). It is regulated by stress hormones that increase the proportion of circulating heterophils while reducing the numbers of circulating lymphocytes, resulting in H/L ratios being positively associated with stress (Parejo et al. 2007; Davis et al. 2008; Masello et al. 2009). The H/L ratio is a better indicator of chronic stress than the level of the stress hormone corticosterone or the PHA-induced swelling immune response test, which are better indicators of acute

stress (Vleck et al. 2000; Davis et al. 2008). Chronic stress is more reliably measured with H/L ratios because of the longer time period required to influence the abundance of leukocytes. The H/L ratio is unaffected by handling time provided blood is sampled within the hour (Davis 2005), compared to the spike in corticosterone levels that occurs within minutes (Davis 2005; de Bruijn & Romero 2013). H/L ratios require only a single drop of blood (Masello et al. 2009) and are therefore an easy and relatively non-invasive way to assess the stress level of an individual.

Davis et al. (2008) proposed an important challenge that occurs when looking at leukocyte profiles: how does one distinguish stress from inflammation and disease when looking at a high H/L ratio? Heterophils are often involved in fighting disease and causing inflammation (Davis et al. 2008; Masello et al. 2009), so it is difficult to discriminate whether H/L ratios are high due to stress or disease. Davis et al. (2008) resolved this conflict by proposing that an increase in the number of monocytes (fighting infections and bacteria) indicates an infection whereas a reduction in the number of eosinophils (parasite fighters) in the peripheral blood is an indication of high stress. Therefore, when examining leukocyte profiles, it is important to examine the relationships between condition and: a) the overall number of WBCs, b) the H/L ratio and c) the number of monocytes and eosinophils (Davis et al. 2008; Masello et al. 2009). Baseline numbers of leukocytes vary across species but known accumulated data for passerines shows that the average number of these cells found within the leukocyte profile is 16.2% heterophils and 74% lymphocytes, compared to an average 2.7% for monocytes and 3.9% for eosinophils (Davis 2009).

A negative correlation between the H/L ratio (level of stress) and condition

has been demonstrated in some adult birds (Quillfeldt et al. 2008; Copan 2013). However, the development of the immune system in avian species is not well studied (Pihlaja et al. 2006; Fiske et al. 2013). The relationships among stress, condition and leukocyte profiles may be different in nestlings compared to adults. For example, although a negative correlation was found between the H/L ratio and condition in adult thin-billed prions, *Pachyptila belcheri* (Quillfeldt et al. 2008), and European starlings, *Sturnus vulgaris* (Copan 2013), a positive correlation was found between the H/L ratio and body condition in burrowing parrot nestlings, *Cyanoliseus patagonus* (Masello et al. 2009). This adjusted relationship of stress and condition within nestlings could be species-specific based on the differences in development and energy requirements among different species of nestlings (Tilgar et al. 2009).

By establishing a relative relationship between nestling condition and H/L ratio, we will be better able to understand the development of immunity through comparing various measures of it in nestlings at multiple points in development. Some studies have found that H/L ratios are not age-related, such as in nestling burrowing parrots where H/L ratios were measured every 5 days and did not change with age (e.g. Masello et al. 2009). Other studies have found the opposite; the H/L ratio increased with age, as found in thin-billed prions (Quillfledt et al. 2008) and several species of bustards (*Chlamydotis undulate macqueenii*, *Eupodotis ruficrista gindiana* and *Eupodotis senegalensis*) (Howlett et al. 2002). Tilgar et al. (2009) clearly demonstrated that these increases in H/L ratio are developmental rather than stress-related because they found that baseline corticosterone levels as well as corticosterone levels under stress both rose slightly with age.

Nestling stress levels could be influenced by a number of factors, but because nestlings of altricial species are confined to the nest, one important consideration is the influence of their parents (Banbura et al. 2013). There is some evidence that stress is, in part, heritable (Naguib et al. 2011). The condition and stress levels of the parents would also impact their offspring (Parejo et al. 2007; Banbura et al. 2013). Adult stress levels, assessed through corticosterone levels, have been shown to negatively impact the rate of nestling provisioning (Lendvai & Chastel 2010). Stress levels in adults (measured through H/L ratio) have also been negatively correlated with parental condition (e.g. Quillfeldt et al. 2008; Copan 2013). If stressed parents are not able to maintain high offspring feeding rates, their nestlings may develop chronic stress and also be in poor condition. Previous studies have shown that intense begging in nestlings is correlated with increased stress levels and decreased immune response (Kitaysky et al. 2001; Loiseau et al. 2008; Moreno-Rueda & Redondo 2011) and that measures of nestling stress are related to the nutritional condition of the nestlings (Pihlaja et al. 2006; Brzek & Konarzewski 2007; Soler et al. 2007).

European starlings were introduced to North America in 1890 and 1891 (Kessel 1957), and since their introduction in New York City, they have become well established throughout North America (Cabe 1999). As European starlings are generalists and well adapted to urban environments (Feare 1984; Bruun & Smith 2003), they become an important species to study in order to understand the detrimental affects of climate change and urbanization on bird populations (Moussus et al. 2011). With urbanization comes a decrease in open, grassy spaces in densely populated areas (Bruun & Smith 2003; Liker et al. 2008). This habitat degradation affects the foraging behaviours of

European starlings that prefer to forage in green areas such as local parks (Mennechez & Clergeau 2001). If these stressors impact European starlings, it is an indication for concern of other species commonly found in these environments.

The purpose of my study is to examine stress in relation to condition in European starling nestlings. The objectives of this study are to: 1) examine the numbers and proportions of leukocytes in the peripheral blood as an indicator of stress in European starling nestlings by examining all aspects of the leukocyte profile (i.e. heterophils, lymphocytes, monocytes, eosinophils and basophils) 2) determine if these measures of stress are related to nestling condition and 3) examine differences in stress and condition among broods. By examining the nestlings in best and worst condition from each nest, I predict that WBC count and H/L ratio will be negatively correlated with nestling condition since higher WBC counts and H/L ratios indicate increased levels of stress.

Methods

This study was conducted on a population of wild breeding European starlings that occupied nestboxes on Saint Mary's University campus, Halifax, Nova Scotia, Canada (44º 37' 54.07" N, 63º 34' 47.09" W) from May through July of 2012. European starlings typically lay 3-7 eggs per clutch (Feare 1984) and nestlings fledge 21-24 days after hatch (Higgins et al. 2006). Nestboxes were monitored during mid to late April for egg laying and were checked daily in the time leading up to the predicted hatch date of the eggs. Hatch day was defined as day 0. Nestlings were handled when 5/6 days, 11/12 days and 14/15 days of age. European starlings typically have two broods over the breeding season (Serra et al. 2012). In 2012, first broods hatched between May 5th

and May 15th while second broods hatched between June 19th and June 22nd. Fledging success is often lower in second broods than in first broods (e.g. Serra et al. 2012).

From each nest having at least three nestlings, two nestlings were selected for leucocyte analysis based on their condition (best and worst). Nests with mortality before day 14/15 were excluded from leucocyte analysis. The sample size was 32 nestlings from 16 nests (11 nests from the first broods and 5 nests from second broods). Intermediate broods (n = 3) were excluded, and fewer second broods were used, due to the abundance of nests with a) only one or two nestlings and b) nestlings dying before day 14/15 of age.

At day 5/6, nestlings were removed from the nest usually in groups of two, depending on clutch size, to ensure there were always nestlings present when parents returned (so parents would not abandon the nest). Each nestling was banded with a different coloured plastic band to track his/her identity throughout the nestling period. Measurements (tarsus and mass), as well as blood samples, were taken on day 5/6. Tarsus was measured three to five times with digital callipers to the nearest 0.1 mm and an average was taken for each individual. Mass for every nestling was measured to the nearest 0.5 g using a spring scale. Blood samples were taken for DNA analysis from the medial metatarsal vein with a 50μL capillary tube and stored in 1mL 95% ethanol; these samples were used for other studies. During this procedure, two blood smears were made for each individual nestling to ensure at least one good slide for analysis (these slides for day 5/6 were not examined for this study). Each smear was made using one small drop of blood (Masello et al. 2009). The edge of another new slide was used to push the blood gently across the slide to evenly distribute the blood cells for the blood smears (Walberg 2001). All smears were air-dried and then fixed in methanol within 48 hours and later

stained using Hema III staining solution (Cole 1943). Before doing leucocyte counts, coverslips were placed on slides by adding three small drops of permount solution to the centre of each coverslip, before placing it carefully on the slide. Slides were left overnight to dry so the coverslips would set.

At day 11/12, nestlings were revisited and banded with a Canadian Wildlife Service (CWS) band. Their mass and tarsus measurements were taken again and were used to assess nestling condition for this study. Nestling condition was determined at day 11/12 by regressing mass against mean tarsus length. The resulting residuals were used as an index of phenotypic condition.

These nestlings were handled for the last time when 14/15 days of age (2/3 of the way through their nestling period) when a small drop of blood was taken for the blood smears analyzed in this study. At this point in the nestling development, the skin (including the skin on the tarsus) is no longer transparent and so blood was sampled instead from the brachial vein located underneath the wing, using the same procedure as for day 5/6. This vein is easy to find and access at this age during the nestling period, much like the medial metatarsal vein at day 5/6, because the feathers have not yet grown in to hide it. Nestlings were not approached after 15 days of age due to the risk of premature fledging.

Leucocyte counts were done by counting the number of red blood cells in the field of view of a compound microscope (Leitz Laborlux D microscope) at 400 times magnification in an area where the blood cells were distributed evenly in a single layer. Subsequently the number of WBCs within the field of view was also counted. This procedure was repeated until the total number of leukocytes within an estimated total

of 10 000 red blood cells was recorded (WBC count). The first 100 leukocytes encountered were identified as to type – heterophils, lymphocytes, monocytes, eosinophils and basophils – at 1000 times magnification under oil immersion. Heterophils have a pale cytoplasm and multiple large, dark rounded granules within the cytoplasm (Mitchell & Johns 2008). Lymphocytes vary in size, but are round with a large round nucleus surrounded by only a small amount of cytoplasm (Claver & Quaglia 2009). Monocytes are often identified with a pink stained kidney-shaped nucleus surrounded by bluish cytoplasm (Claver & Quaglia 2009). Eosinophils are approximately the same size as heterophils, but have a bluer cytoplasm with rod-shaped granules (Mitchell & Johns 2008). Basophils typically appear to have smaller granules than eosinophils and are more reddish in colour (Claver & Quaglia 2009). The number of heterophils to lymphocytes (H/L ratio) was calculated for each nestling using their proportions within the 100 leukocytes counted.

GraphPad Prism software (version 5.04 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com) was used to analyze all data. Data were tested for normality using a D'Agostino & Pearson omnibus normality test. Paired t-tests were done to compare the differences between the good and poor condition nestlings from each nest for condition, WBC, H/L ratio, and relative monocyte, eosinophil and basophil abundances. To examine relationships between the measures of stress and condition, Pearson correlations were done between 1) mean WBC count and mean condition per brood, 2) mean H/L ratio and mean condition per brood and 3) mean H/L ratio and mean WBC count per brood. Means per brood were calculated using the best and worst condition nestlings from each nest to avoid pseudo-replication. Correlations were also

done between differences in WBC count and differences in condition as well as differences in H/L ratio and differences in condition. These differences were calculated by subtracting the worst condition nestling from the best condition nestling for each nest. Paired t-test comparisons were also done to determine differences between WBC counts and H/L ratios. Condition of the 2012 nestlings used for this study was also compared to previous data on 2011 nestling condition through a t-test to examine long-term variation in nestling condition. Comparing nestling condition between years was done using mean condition per brood. All results were considered significant when $P \leq 0.05$ and means are reported ± SE.

Results

Mean WBC count for nestling European starlings was 125.4 ± 5.46. The mean percentage of heterophils was 33.19 ± 1.53, lymphocytes was 53.47 ± 1.69, monocytes was 3.81 ± 0.46, eosinophils was 5.59 ± 0.78, and basophils was 4.03 ± 0.37. The residuals for condition (mass against mean tarsus length) were highly variable ranging from -14.449 to 14.762 (n = 63 nestlings from 16 nests) (Figure 1). Nestlings from 2011 were in significantly better condition than those in 2012 (Mann-Whitney U = 288.0, P = 0.02; Figure 2).

A significant difference in condition existed between nestlings deemed to be the best and worst condition nestlings from each nest (best: 3.06 ± 1.58, worst: −3.61 ± 1.21; paired t = 7.293, df = 15, P < 0.0001; Figure 3). However, there were no significant differences between the two groups for any leukocyte parameters (Table 1).

There was no significant correlation between mean brood WBC counts and

mean brood condition ($r = -0.005427$, $n = 16$, $P = 0.98$) or mean brood H/L ratio and mean brood condition ($r = 0.2472$, $n = 16$, $P = 0.36$). There was a tendency towards a significant positive relationship between mean brood H/L ratio and mean brood WBC count ($r = 0.4529$, $n = 16$, $P = 0.08$; Figure 4).

The difference between best and worst condition nestling was taken for condition residuals, WBC count and H/L ratios. There was a significant positive correlation between difference in H/L ratio and difference in condition ($r = 0.5155$, $n = 16$, $P = 0.04$). However, there was no significant correlation between difference in WBC count and difference in condition ($r = 0.3104$, $n = 16$, $P = 0.24$). Differences in condition were considered small when the difference was below 6.87 and deemed large when above 6.87. A comparison between small and large differences in condition for each of WBC count and H/L ratio showed no significant differences for either (WBC: Mann-Whitney $U = 30.00$, $P = 0.87$; H/L: Mann-Whitney $U = 24.50$, $P = 0.46$).

Table 1. Leukocyte parameters of nestlings in the best and worst condition within a brood (n = 16 broods).

Parameter	Best ± SE	Worst ± SE	Paired t (15 df)	P value
WBC count	123.3±7.98	127.6±7.69	0.7569	0.46
H/L ratio	0.71±0.08	0.62±0.06	1.188	0.25
Monocyte abundance	3.75±0.69	3.88±0.64	0.1421	0.89
Eosinophil abundance	4.50±0.66	6.69±1.39	1.424	0.17
Basophil abundance	3.94±0.45	4.00±0.60	0.1306	0.90

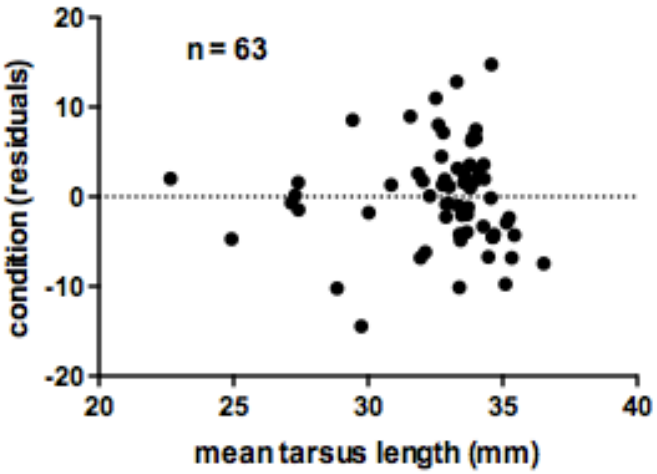

Figure 1. The condition of nestlings derived from the residuals of mass against mean tarsus length taken at 11/12 days of age.

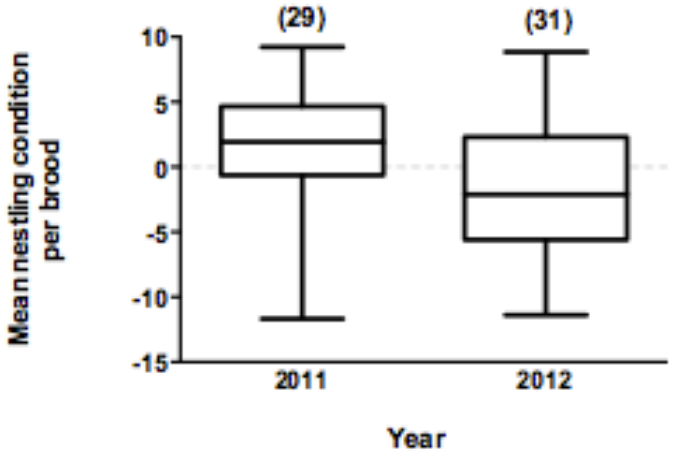

Figure 2. The difference in mean nestling condition per brood between the years 2011 and 2012. Sample size is in brackets.

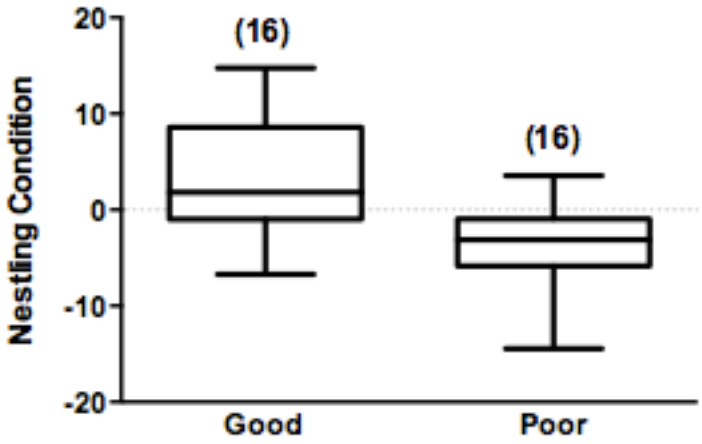

Figure 3. The difference in condition between best and worst condition nestlings selected from each nest. Sample size is in brackets.

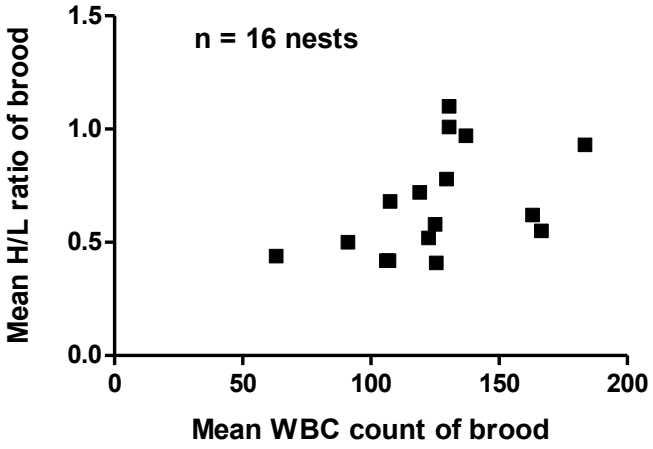

Figure 4. Relationship between mean H/L ratio and WBC count per brood (best and worst condition nestling) for nestlings at 14 days of age.

Discussion

Accumulated data on baseline leukocyte profiles for passerines indicate the average proportions of these cells found within 100 leukocytes is 0.27 for H/L ratios (16.2% heterophils and 74% lymphocytes), 2.7% for monocytes, 3.9% for eosinophils and 3.3% for basophils (Davis 2009). Other research on parrots gives comparable ranges of these measures in the Psittacidae family; 40-75% heterophils, 20-50% lymphocytes, 0-3% monocytes, 0-2% eosinophils, and 0-5% basophils (McDonald 1996). Research in this field has determined these measures are largely species specific, and this is demonstrated here by the average passerine percentages falling outside of the psittacidae ranges. However, Vinkler et al. (2010) studied leukocyte profiles in Scarlet rosefinch, *Carpodacus erythrinus*. They found different percentage ranges for the types of leukocytes in the adult and nestlings of a passerine species. The ranges they give for nestlings are 0-25.86% for heterophils, 4.59-41.38% for lymphocytes, 0-10.74% for monocytes, 0.83-33.88% for eosinophils and 7.76-90.99% for basophils (Vinker et al. 2010). The European starling nestling data do not compare well to any of the described data from previous research, emphasizing the differences across species.

Nestling condition in 2012 (this study) was significantly worse than that in 2011. It is possible that nestling condition in 2012 was so poor overall that any significant

differences in the stress levels (WBC count and H/L ratio) were not detectable due to low variation. As the environment fluctuates over time, it is important to monitor nestling condition and stress to determine how the environment affects them. Previous research has shown that variation in climatic conditions and changes in the weather induced stress responses in avian species, such as European starlings (e.g. de Bruijn & Romero 2011; de Bruijn & Romero 2013). De Bruijn & Romero (2011; 2013) found that increased rainfall and decreased temperatures acted as independent stressors on European starling adults with inclement weather inhibiting foraging activity. A study looking at the effects of stress on provisioning in male house sparrows, *Passer domesticus*, shows that males decrease provisioning after stress (Lendvai & Chastel 2010). Therefore, inclement weather would affect nestling provisioning by the parents, resulting in decreased condition of the entire brood. It would be beneficial to replicate this study over multiple years to examine variation across years.

The finding that condition differed significantly between nestlings in best and worst condition within a nest demonstrates they were effectively separated into these two groups. Despite the significant difference in condition between these two groups, no differences in stress level, as ascertained through leukocyte numbers and types were detected, contrary to my predictions. These findings suggest that nestlings within a nest are similarly stressed due to their dependence on their parents (Almasi et al. 2010). European starling nestlings are altricial, and so are confined to the nest, interacting almost exclusively with their parents and their siblings (Almasi et al. 2010), and therefore experience stressors indirectly through their parents (Banbura et al. 2013). These nestling stressors would consist of familial interactions, including parental feeding rates, and

degree of sibling competition (brood size) (Brzek & Konarzewski 2007). As such, all nestlings within a nest would be exposed to similar stressors during the nestling period, which could explain why condition was unrelated to WBC count of H/L ratio within a nest.

Copan (2013) also found no relationship between mean WBC count and mean condition per brood within the adult starling population from the same year - 2012. Although increased WBC count is a common symptom of stress (Ots et al. 1998; Lobato et al. 2005; Hylton et al. 2006), it may not have varied enough in this study; the WBC count ranged from 60-192 WBCs in 10 000 erythrocytes across all individuals examined in this study. Corticosterone levels and PHA tests are more commonly used with H/L ratio than WBC count when examining stress responses and the relationship between condition and stress (e.g. Ilmonen et al. 2003; Naguib et al. 2004; Tilgar et al. 2010). Other studies have found that nestlings in better condition had an increased WBC count (Masello et al. 2009). Masello et al. (2009) explain their results in nestling burrowing parrots may be related to development; their nestlings in better condition are better able to devote more resources towards a vigorous immune system reflected in the increased number of leukocytes in the blood. Another study showed a variation between years in the relationship between WBC count and survival. Hylton et al. (2006) found that in a year with higher H/L ratios (stress), WBC count was negatively correlated with survival, but in the previous year WBC count was positively correlated with survival. Hylton et al. (2006) also found in the year with the positive correlation between WBC count and survival, there was a negative relationship between eosinophil counts and survival. These results were explained through the function of WBCs; a high WBC count indicates

fighting an infection but a low WBC count can be an indication of poor immune response (Hylton et al. 2006). The variation between years found in their study emphasizes the importance of examining stress across multiple years.

Contrary to my prediction, mean H/L ratio was not negatively correlated with mean condition per brood. This finding differs from a study by Copan (2013), who found a significant negative correlation between H/L ratio and condition in adults from that same year. The mean nestling H/L ratio (0.66 ± 0.05) was lower than the mean H/L ratio and had less variation than the previously reported findings in the adults (Copan 2013). These differences between nestlings and adults suggest there are likely development-related changes in the proportions of different WBCs circulating in the blood, which would affect the overall ratio of heterophils and lymphocytes, as these nestlings are only two thirds of the way through the nestling period. In studies that have looked at H/L ratios as young birds developed, there has been no consistent pattern across species in H/L ratios over time. For example, Shutler et al. (2010) found that leukocyte profiles of ducklings varied over time (with development), but did not follow a consistent pattern. Their sample size to detect these patterns was relatively small, and a number of other studies have demonstrated variation in age relationships in H/L ratios across species. Two studies have shown that H/L ratio increases with age in nestling birds (Quillfeldt et al. 2008; Tilgar et al. 2010), while another study found no relationship between H/L ratio and age (Masello et al. 2009). Nestling condition has been positively correlated with H/L ratio in studies of other avian families, for example, in burrowing parrots, belonging to the family Psittacidea (Masello et al. 2009), but H/L ratio has been negatively correlated with measures of nestling condition (size and mass) in other passerines, such as

Scarlet rosefinches, at about three quarters of the way through their nestling period (Vinkler et al. 2010).

Both WBC count and H/L ratio are indicators of stress (Davis et al. 2008), which explains the tendency towards a positive correlation between mean H/L ratio and mean WBC count per brood. This interrelatedness suggests that both measures should yield similar results when individually correlated with condition. Parejo et al. (2007) also found a positive correlation between WBC count and H/L ratio in nestling European rollers, *Coracias garrulus*. However, neither my study on nestlings, nor Copan's (2013) study on adult European starlings has found a significant relationship between WBC count and condition, but Copan (2013) did find a relationship between H/L ratio and condition. This is consistent with Parejo et al.'s (2007) findings that H/L ratio was correlated with some measures of condition, but WBC count had no significant relationships with any measures of condition used in their study. However, Masello et al. (2009) found a positive correlation between WBC count and condition as well as H/L ratio and condition. The positive relationship between H/L ratio and WBC count does yield similar results when individually correlated with condition in their study, in contrast with other research discussed here. This suggests species-specific differences in relationships between WBC count and H/L ratio as indicators of stress, as suggested by Tilgar et al. (2009).

The positive relationship between differences in H/L ratio and nestling condition differences indicates that nestlings in similar condition (small differences) experience similar stress levels (small differences in H/L ratios). The H/L ratios ranged from 0.225 to 1.4 for these nestlings. The little variation seen in these H/L ratios indicates similar

stress across the nestlings in this study. The increased differences in stress levels for nestlings with higher differences in condition could also be a reflection of developmental differences. Nestlings in poorer condition would face a higher cost of developing their immune system than better condition nestlings (Pihlaja et al. 2006; Brzek & Konarzewski 2007), their immune system development would be depressed in favour of mass gain to compete with siblings for food, due to trade-offs between immunity and growth when resources are limited (Brzek & Konarzewski 2007). Nestlings in larger broods face more sibling competition than nestlings in smaller broods, and therefore nestlings in poorer condition benefit more by devoting resources towards mass gain and increasing size than nestlings in smaller broods that face an increased risk of infection, who would benefit more by using resources to develop their immune response (Pihlaja et al. 2006). Therefore, nestlings in similar condition are similarly stressed due to differential abilities between nestlings in different condition to grow or to develop immune and stress responses.

Previous research has shown that altricial and semi-altricial nestlings do not experience stress to the same degree as adults and precocial nestlings (Fiske et al. 2013). The developmental hypothesis (Schwabl 1999) proposes that nestlings experience first stress at different ages across different species depending on where nestlings are situated on the precocial-altricial spectrum (Fiske et al. 2013). Nestlings that are altricial experience stress later in development than precocial nestlings because they are confined within the nest and not exposed directly to stressful situations (Sims & Holberton 2000; Fiske et al. 2013). The HPA axis stress responses have been shown to increase with age in altricial species (Quillfeldt et al. 2009; Fiske et al. 2013). Therefore, as European

starling nestlings are altricial, they may show little stress response at this young age in their development, i.e. corticosterone levels would not affect European starling nestlings enough at 14 days of age to produce significant differences in H/L ratio.This hypothesis would explain the lack of correlation found between H/L ratio and condition found in these nestlings. Lobato et al. (2005) examined leukocyte profiles of nestling pied flycatchers, *Ficedula hypoleuca,* an altricial passerine species, a few days before fledging (a later developmental stage than this study on European starlings). They found H/L ratios had a significant negative correlation with body mass in pied flycatcher nestlings and their H/L ratios were higher than adult female H/L ratios. Lobato et al. (2005) hypothesized that the difference between nestling H/L ratios and adult female H/L ratios is due to nestlings at this stage in development have better developed innate immune systems and therefore more heterophils than lymphocytes, i.e. as the specific immune response develops, the H/L ratios decrease. However, another study on pied flycatcher nestlings demonstrated that the stress response in nestlings just before fledging was still weaker than an adult stress response (Tilgar et al. 2009). This study indicates that nestlings are still developing their immune and stress responses at late stages of the nestling period. Their research supports the hypothesis that European starling nestlings, two thirds of the way through the nestling period, have not yet developed enough of a stress response to show a clear relationship between H/L ratio and condition.

The human population continues to increase and urban areas continue to expand (Liker et al. 2008), and so it is critical to monitor the effects that stressors have on common generalists species that are able to thrive in urban environments. Moussus et al. (2011) determined that generalists are better able to cope with a changing

environment than specialists. Their study also determined that the fate of generalists, which are experiencing small population declines with climate change, are important to consider in the context of the larger population decreases seen in specialist species. Liker et al. (2008) showed that generalist, non-native bird species, such as house sparrows, thrive in urban habitats. Their criteria for urban birds also apply to European starlings, which are an introduced, generalist species (Davis 1960; Bruun & Smith 2003). The effects of urbanization are particularly important to study with respect to foraging behaviours because a decrease in open grassy spaces will affect the overall nutritional condition of both adults and nestlings and may have significant dietary consequences for many species (Bruun & Smith 2003; Liker et al. 2008). One study examining the effects of urbanization on house sparrows proposed prey become increasingly unavailable in urbanized areas where vegetation is sparse (Liker et al. 2008).

Due to the lack of knowledge about the development of immunocompetence, stress responses, and immune responses in avian species, future studies should examine leukocyte proliferation and proportions at different nestling stages to look for patterns in the development of immune and stress responses. Other future areas of interest include how nestling stress and condition relate to parental stress and condition over time by examining differences between broods. Studies should also be done to compare data over years in order to look at changes in stress levels with ongoing environmental changes, such as climate change, and to look for a threshold effect in stress responses. It would also be beneficial to compare nestling and adult stress, in a generalist species such as European starlings, across more rural and urban environments.

In conclusion, despite significant differences in condition within a brood,

contrary to my predictions there was no significant correlation between WBC counts or H/L ratios and condition in European starling nestlings. The significant positive correlation between differences in H/L ratio and differences in condition signifies that nestlings in similar condition are similarly stressed. These results indicate that European starling nestlings at 14 days of age are still developing immune and stress responses. The difference in nestling condition between years suggests that more long-term research is necessary to draw further conclusions about the relationships between stress, condition and the environment. This preliminary data of nestling stress levels in European starlings provides grounds for further study into development of stress responses in adults and nestlings in a changing environment. European starlings are an important species to consider during this time as they are an introduced, generalist species in North America that are able to thrive within urban environments across the continent. As climate change becomes an increasing concern over time, it is important to consider how these changes will affect both generalist and specialist species and what this could mean for species diversity in the long-term.

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