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Patterns of variation in leucocyte counts of female tree swallows, *Tachycineta bicolor*: Repeatability over time and relationships with condition and costs of reproduction

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ABSTRACT

The heterophil to lymphocyte (HL) ratio in the peripheral blood is increasingly being recognized as a reliable indicator of stress in birds. In this study we examined whether HL ratio, as well as the proportion of heterophils and lymphocytes, varied systematically with sampling time, date, age, climate conditions or with measures of maternal investment in female tree swallows (*Tachycineta bicolor*) over three breeding seasons. We found that HL ratios showed significant annual variation, which appeared to be driven by annual changes in the proportion of heterophils. HL ratios were higher among those females laying larger clutches, suggesting a potential cost of reproduction. Variation in body condition also appeared to affect stress levels of females, as decreases in body condition were associated with elevated HL ratios. Among females that we sampled over multiple breeding seasons, we were unable to detect significant repeatability for both HL ratios and proportion of heterophils, although proportion of lymphocytes showed low but significant repeatability within individuals. We therefore suggest that caution should be exercised in using these measures for illustrating the inherent quality or health of individuals over time frames beyond the current breeding attempt, or as predictors of future reproductive potential.

1. Introduction

A central goal in many studies of wild animals is to determine phenotypic characters that are able to predict the fitness potential of individuals, or their past, present or future state of health and condition (Nolan et al., 1998; Brawner et al., 2000; Bortolotti et al., 2002; Dawson and Bortolotti, 2006; Saks et al., 2003; Hill and Farmer, 2005). Measurements of stress are increasingly being recognized as a suitable method to analyze the phenotypic quality of birds (Hõrak et al., 1998a; Ots et al., 1998). Hormones such as corticosterone, for example, may provide an accurate description of physiological stress in many species (Wingfield et al., 1998); however, hormones have a short half-life, and this may complicate the collection and interpretation of data. Levels of circulating hormones are also highly responsive to stressors (Gross and Siegel, 1983), such as those induced by researchers during the capture and handling of animals. Leucocyte counts, specifically the heterophil to lymphocyte (HL) ratio in peripheral blood, are also recognized as good indicators of stress in birds (Gross and Siegel, 1983). Changes in circulating levels of both types of blood cells are much slower in comparison to hormonal changes (Dein, 1986; Maxwell, 1993), making the HL ratio relatively insensitive to observer-induced biases.

Heterophils are non-specific, phagocytosing cells that are early modulators of inflammatory responses, and are known to increase in response to abnormalities related to diet, stress, trauma and chronic bacterial infections (Maxwell and Robertson, 1998). In contrast, lymphocytes are highly specific and consist of two basic types of cells: T lymphocytes which are involved in regulation of the immune response and in eliminating antigens, and B lymphocytes which synthesize and secrete immunoglobulins, and are involved with immunologic memory (Goldsby et al., 2000). Circulating lymphocytes become more numerous in the blood as a consequence of parasitic infection (Bonier et al., 2007) and in response to immunological challenges (e.g., Eeva et al., 2005). Low levels of lymophocytes are often interpreted as evidence for immunosuppression (Hõrak et al., 1998b), although this may also be indicative of low levels of parasitic challenge (Ots and Hõrak, 1996). The HL ratio increases in response to food or water deficiency, infectious diseases as well as social stresses, and has been used commonly in studies of poultry (Gross and Siegel, 1983). HL ratios also lend themselves well to ecological studies because of the ease in which samples can be collected and the relatively high precision that cells can be identified (Ots et al., 1998). In addition, HL ratios often show marked variation among individuals, which may help explain, for example, the occurrence of different reproductive decisions within a population (Ots et al., 1998). Indeed, counts of heterophils and lymphocytes, and HL ratios, have been used as measures of stress in studies of both captive and wild avian species, and have been associated with disease status and immunocompetence (Hawkey et al., 1985; Sanz et al., 2004), costs of

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reproduction (Hõrak et al., 1998b; Shutler et al., 2004), plumage development (Bortolotti et al., 2006), habitat quality (Suorsa et al., 2004; Owen et al., 2005) and growth of offspring (Moreno et al., 2002).

In this paper, we use female tree swallows (*Tachycineta bicolor*) as models to identify factors that influence variability in HL ratios and proportions of heterophils and lymphocytes. Tree swallows are insectivorous secondary cavity nesting passerines that readily accept artificial nest boxes (Robertson et al., 1992). Specifically, we tested whether these measures varied systematically with sampling time or date, climatic conditions or with the relative age of the bird. We also tested whether they would reflect stresses that are associated with the costs of reproduction. For measures of individual quality, such as HL ratios, to have utility in reflecting the state of an individual over long time spans, it is necessary that they are relatively consistent and repeatable over time. To evaluate the repeatability of these measures, and therefore their ability to reveal information about the inherent quality of individual birds, we also sampled individuals over two or three consecutive breeding seasons. Hõrak et al. (2002) suggested that if these measures were relatively consistent within individuals, any among-individual differences might be a reflection of differences in levels of chronic stress. Alternatively, inconsistency of these measures within individuals may suggest that levels of stress vary over time (Vleck et al., 2000).

2. Methods

We studied tree swallows (*T. bicolor*) from May to August, 2002–2004, approximately 25 km south of Prince George, British Columbia, Canada (53° N, 122° W). The study site consisted mainly of pasture and idle hayfields with a number of small wetlands, surrounded by second-growth forest of various ages. Tree swallows arrived on the site in early May and began laying eggs in late-May and early June. We visited nest boxes every 1–3 days beginning in mid-May and subsequently documented the date that the first egg was laid, clutch size, date of hatching and number of eggs hatched. Nests were visited periodically during the brood-rearing period and again after young had left the nest to determine number of offspring fledging.

We captured female tree swallows with box traps immediately after all of the eggs in the clutch had hatched. Females were ringed with a standard aluminum leg band, and classified as either in their second year (SY) or after-second year (ASY) based on plumage coloration (Hussell, 1983). Each female was weighed to the nearest 0.25 g with a spring scale, and the length of their ninth primary flight feather was measured to the nearest 0.5 mm with a ruler. A drop of blood was collected from the brachial vein of each individual, and immediately smeared onto a slide. Smears were fixed in 100% ethanol, stained with Wright Giemsa, and scanned for leucocytes using a compound microscope with 10× ocular and 100× oil immersion lenses. A minimum of 100 leucocytes were identified from each slide, noting frequencies of heterophils, eosinophils, basophils and lymphocytes, using standard avian guidelines (Campbell, 1988). We calculated the HL ratio, and scaled counts of heterophils and lymphocytes to number per 100 leucocytes. A subsample of smears ($n=27$) were scanned twice, and repeatability analyses (details below) showed that there was good agreement between these two scans within individuals (heterophils: $r=0.94$, $F_{26,27}=31.89$, $P<0.001$; lymphocytes: $r=0.81$, $F_{26,27}=9.54$, $P<0.001$; HL ratio: $r=0.95$, $F_{26,27}=41.02$, $P<0.001$).

2.1. Data analysis

We analyzed data from 61 blood smears collected over the three field seasons, which consisted of 23 birds that were sampled once, 16 birds sampled in two successive years, and two birds that were sampled in three successive years. We took two approaches to analyzing our data. First, we examined sources of variation among individuals. For birds that were sampled more than once, we chose the first sample for inclusion in this data set. We tested for effects of female age and annual variation on HL ratios, and on the proportion of heterophils and lymphocytes per 100 leucocytes, using analysis of variance (ANOVA). HL ratios were not normally distributed so we used a square-root transformation prior to analysis. Given our results (see below) we then standardized (mean=0, SD=1) HL ratios and proportion of heterophils and lymphocytes separately for each year (see Hõrak et al., 2002). We performed stepwise linear regressions, with probability of 0.10 for entry and 0.20 for removal, to examine whether standardized HL ratios, proportion of heterophils or proportion of lymphocytes could be predicted by Julian date of capture, time of day of sampling, body size (estimated by length of ninth primary feather), body mass, body condition, clutch size, Julian date of clutch initiation and average temperature and total rainfall during the 20 days preceding sample collection as possible explanatory variables. An index of body condition was derived using the residuals from a regression between mass and length of the ninth primary flight feather ($F_{1,36}=3.21$, $P=0.08$). Data for temperature and rainfall were obtained from a weather station located 15 km north of the study area. We chose to use data from 20 days prior to sampling because this would approximately encompass the period when females are laying eggs and incubating, and so subjected to reproductive stresses. Prior to analysis, we standardized measures of clutch initiation date and clutch size for each year in the same manner as we did for HL ratios and proportions of heterophils and lymphocytes (mean=0, SD=1). Spearman's correlations were also used to test whether standardized HL ratios, or proportion of heterophils or lymphocytes could predict the ability of females to raise and fledge offspring.

Next, we examined variation in HL ratios and proportion of heterophils and lymphocytes within those individuals that were sampled over more than one breeding season. To assess repeatability within individuals, we used the methods of Lessells and Boag (1987) to calculate the interclass correlation coefficient for standardized HL ratios and proportion of heterophils and lymphocytes. Repeatability provides an estimate of the upper limit of heritability of a particular trait (Falconer, 1981). We also tested whether the changes in standardized HL ratios or proportions of heterophils and lymphocytes between two breeding season could be explained by changes in sampling date, sampling time, body condition, date of clutch initiation or clutch size using a series of stepwise multiple regressions (P to enter =0.10, P to exit =0.20). In cases where we had 3 observations from an individual bird, we used the first two samples.

All analyses were performed with SPSS (Norušis, 2000). Means are presented ± 1 SE and we considered results significant at the 0.05 level.

3. Results

3.1. Variation among individuals

Using a single observation from each female tree swallow in our sample, ANOVA revealed significant annual variation in HL ratios ($F_{2,38}=3.55$, $P=0.04$), and Bonferroni-corrected post-hoc comparisons showed that ratios were higher in 2002 than in 2003 ($P=0.04$; Fig. 1). Variation in HL ratios appeared to be driven primarily by annual differences in proportion of heterophils ($F_{2,38}=5.93$, $P<0.01$), although in this case post-hoc comparisons showed that proportions of heterophils were significantly higher in 2002 than in both 2003 ($P<0.01$) and 2004 ($P=0.02$; Fig. 1). In contrast, numbers of lymphocytes did not vary among the 3 years of our study ($F_{2,38}=0.94$, $P=0.40$; Fig. 1). What is ultimately driving annual variation in HL ratios and proportion of heterophils of tree swallows is not clear. While some variables that are expected to affect HL ratios and proportion of heterophils also showed significant differences

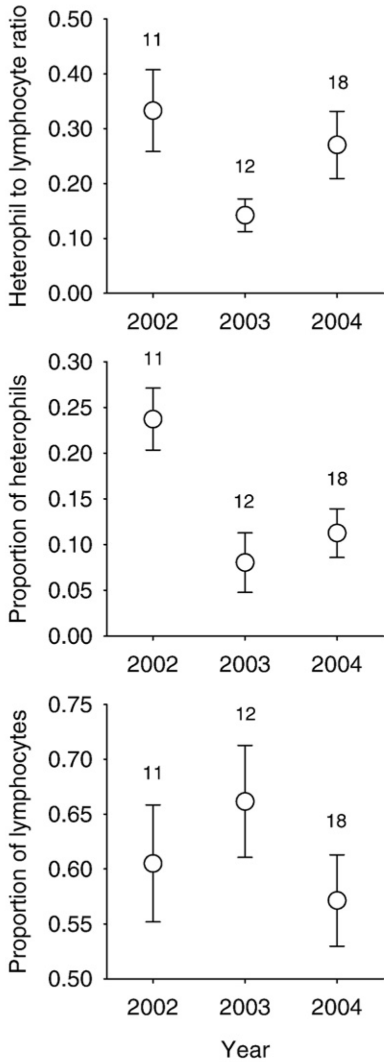

Fig. 1. Annual variation in heterophil to lymphocyte ratios, and proportion of heterophils and lymphocytes per 100 leucocytes in female tree swallows sampled during the brood-rearing period near Prince George, BC, Canada. Error bars are ±1 SE, and sample sizes are indicated above bars.

among years, the patterns of variation in these variables were not the same. For example, clutch sizes in our population varied annually ($F_{2,183}$=4.09, P=0.02); however, clutches were smallest in 2002 when HL ratios were highest and intermediate in 2003 when HL ratios were lowest (Fig. 1). Age of females was not a significant predictor in any of the above analyses.

To ensure that data collected for each year were directly comparable in subsequent analyses, we standardized HL ratio and proportions of heterophils and lymphocytes for each year separately (mean=0, SD=1). Stepwise multiple regression ($F_{3,34}$=6.92, P<0.001) suggested that HL ratios increased significantly with the standardized clutch size (t=3.12, P<0.01), the time of day that the blood sample was collected (t=3.32, P<0.01) and as body condition of females decreased (t=−3.63, P<0.001; Fig. 2). These relationships were driven by variability in proportions of both heterophils and lymphocytes. Heterophils became more numerous with increasing clutch sizes (t=3.67, P<0.001), later time of sampling (t=2.92, P<0.01) and as female body condition decreased (t=−3.52, P<0.001), while proportion of lymphocytes showed the opposite pattern (clutch size: t= −2.38, P=0.02; sampling time: t=−2.79, P<0.01; body condition: t=2.77, P<0.01). No other variables contributed significantly these multiple regression models. Spearman's correlations failed to show any significant relationships between number of offspring fledged and HL ratio (r_s=−0.07, n=41, P=0.65), proportion of heterophils (r_s= −0.04, n=41, P=0.78) or proportion of lymphocytes (r_s=0.12, n=41, P=0.46).

3.2. Repeatability within individuals and influences on variability

Using data from birds captured over multiple breeding seasons, neither HL ratio (r=−0.03, $F_{17,20}$=0.94, P=0.55) nor proportion of heterophils (r=−0.06, $F_{17,20}$=0.88, P=0.60) showed significant repeatability within individuals. In contrast, proportion of lymphocytes showed weak but significant repeatability across breeding seasons (r=0.37, $F_{17,20}$=2.22, P=0.045). Changes in HL ratio or proportion of heterophils between years were not influenced by changes in any of the independent variables that we used in multiple regression analyses (all Ps>0.28). Changes in lymphocyte counts were negatively related to changes in sampling time between years ($F_{1,16}$=6.26, P=0.02: Fig. 3). This analysis suggests that when the second sample

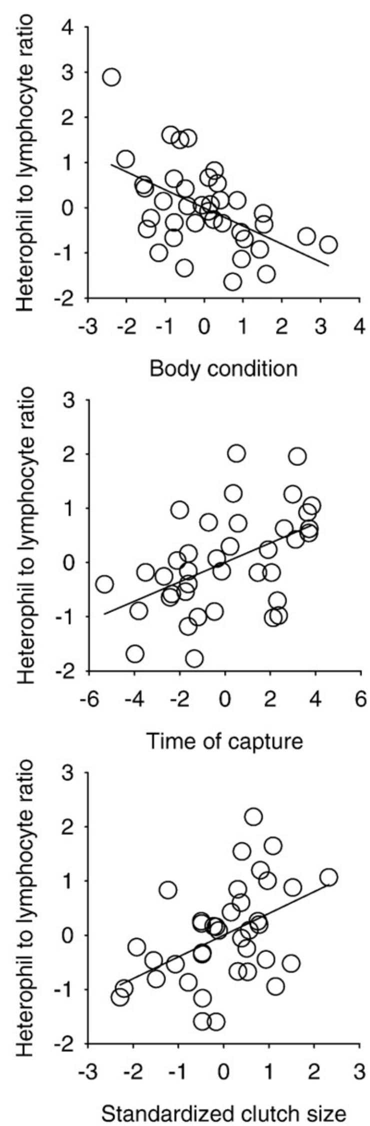

Fig. 2. Partial residual plots for the relationships between heterophil to lymphocyte ratios of female tree swallows and body condition, time of day that birds were sampled, and clutch size, 2002–2004. Body condition was calculated as the residuals from a regression between mass and length of ninth primary flight feather, and clutch sizes were standardized for each year separately (mean=0, SD=1) prior to analysis.

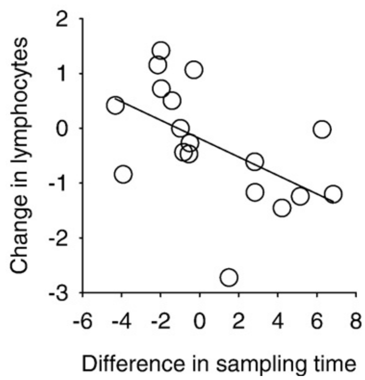

Fig. 3. Changes in proportion of lymphocytes of female tree swallows sampled over two consecutive breeding seasons in relation to the differences in the time of day that the samples were collected.

was taken later in the day than the first sample, lymphocytes were more likely to decrease in number.

4. Discussion

Our study of breeding female tree swallows has revealed that HL ratios show significant annual variation, and that these changes across breeding seasons are the consequence of varying proportions of heterophils (Fig. 1). Multiple regression analysis also showed that HL ratios of female tree swallows increased with the time of day that the sample was collected, with the number of eggs that females had laid, and with decreases in their body condition (Fig. 2). In contrast to annual variation, the increase in HL ratios in this case was due to concurrent increases in heterophils and decreases in lymphocytes.

The underlying processes that are ultimately driving the annual variation in HL ratios and proportion of heterophils are unclear. While there was also annual variation in some of the variables that are expected to affect HL ratios, the patterns seen were different than we found for HL ratio and proportion of heterophils. Similarly, there was no obvious indication that incidence of disease or injury, both of which can affect leucocyte numbers (Hawkey et al., 1985; Vleck et al., 2000; Sanz et al., 2004), was more pronounced in 1 year over others. Horak et al. (2002) observed a higher proportion of lymphocytes in greenfinches infected with blood parasites, whereas Figuerola et al. (1999) found that parasitized cirl buntings (*Emberiza cirlus*) showed a higher proportion of lymphocytes and a lower proportion of heterophils when infected with blood parasites. Although these factors would contribute to a low HL ratio, our results show that lymphocytes were consistent among individuals across years, suggesting that parasitism may not have been a major contributor to annual variation in HL ratios. One possibility for the variation in HL ratios among years, which has not been explored in previous studies, is that stressful conditions experienced in wintering areas were still detectable during the following breeding season. Measures of large-scale climatic variation, such as the North Atlantic, Pacific Decadal and Southern Oscillations, are being used increasingly to investigate how birds may be responding to changing climates. We averaged the Pacific Decadal Oscillation (PDO) index for the period from November to February preceding each breeding season when sampling occurred (data courtesy of N. Mantua, University of Washington). Although it is not possible to make robust inferences, the pattern of variation in HL ratios of breeding female tree swallows showed considerable similarity to the patterns in the PDO index during months when birds are on wintering areas, such that HL ratios were high in years where winters had low PDO indices (Fig. 4). The PDO is a pattern of climate variability that is associated with changes in surface water temperatures in the Pacific, and like El Niño and La Niña events, can cause climatic anomalies in North America, although these are usually not as extreme (Mantua, 2001). While we have little knowledge of the wintering areas of our population of tree swallows, there is some evidence from banding recoveries that some of our birds winter in Texas (Dawson, unpublished data). During cool phases of the PDO, the southern United States and northern Mexico receive below average precipitation (Mantua, 2001); drier conditions on wintering areas could potentially affect food resources for wintering swallows by influencing the abundance of aquatic insects, which in turn may be reflected in elevated HL ratios of birds during the following breeding season. Regardless, more research is needed to determine how prevailing conditions on wintering areas affect stress levels of birds, and the indices we use to measure them, during the breeding season.

The positive relationship between number of eggs laid and levels of stress in female tree swallows (Fig. 2) may represent a cost of reproduction. Life-history theory suggests that organisms cannot increase investment in one trait or activity without a concomitant decrease in investment in other processes that are competing for the same resources or energy (Stearns, 1992). The trade-off operating in this case may be that females are allocating energy to reproduction at a cost to their immune defense. Hõrak et al. (1998b) found that great tits (*Parus major*) breeding in a rural area had elevated HL ratios when raising enlarged broods. This appeared to be due to numbers of lymphocytes decreasing, which suggests immunosuppression resulted from increased reproductive effort (Hõrak et al., 1998b). Alternatively, Ardia et al. (2003) observed a stronger secondary immune response in female tree swallows laying larger clutches (but see Deerenberg et al., 1997). Female tree swallows lay an average of about 6 eggs in our population, with an average mass of 1.72 g each (O'Brien and Dawson, 2005); therefore, an average clutch of eggs represents close to half of a female's body mass. Stresses associated with aerial foraging to procure energy and nutrients for formation of large clutches in the spring when food resources are relatively scarce may partly explain elevated HL ratios in our birds, as numbers of lymphocytes can decrease after strenuous physical activity (Hoffman-Goetz and Pedersen, 1994), while heterophils are known to increase in number during stressful situations (Maxwell and Robertson, 1998).

While the idea of a cost of reproduction is an important component in theories of life-history evolution, the detection of such costs in tree swallows has remained elusive. Previous studies have failed to detect any costs of reproduction for this species when raising enlarged broods (De Steven, 1980; Wheelwright et al., 1991; Wiggins, 1990; Murphy et al., 2000; Shutler et al., 2006), and Shutler et al. (2006) suggested that time constraints associated with producing extra eggs

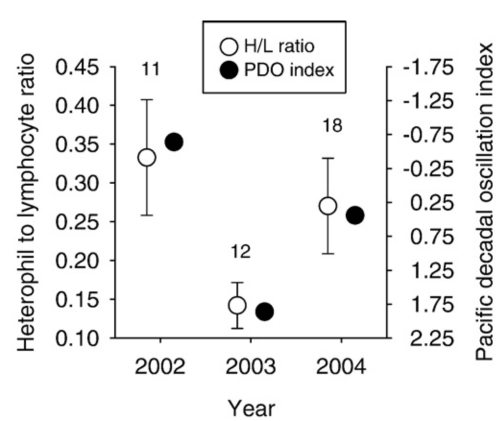

Fig. 4. Heterophil to lymphocyte ratios (±1 SE) of female tree swallows during the breeding seasons of 2002–2004 near Prince George, BC, Canada (sample sizes above error bars), and the average Pacific decadal oscillation (PDO) index for the period from November to February prior to each breeding season. Note that to facilitate comparisons, the *y*-axis for the PDO index is plotted showing values largest to smallest.

was probably more important in determining reproductive decisions of tree swallows than were costs of reproduction. However, Ardia (2005) observed that some female tree swallows mounted weaker secondary immune responses when raising enlarged broods, suggesting a trade-off between immunological health and reproduction. Although Shutler et al. (2004) also showed that HL ratios increased in adult tree swallows raising enlarged broods, whether the increase detected in their study, as well as our own, represents a real cost of reproduction is unclear. Great tits with low HL ratios were more likely to return to breed than those with high HL ratios, suggesting that birds with high levels of stress during breeding were less likely to survive (Kilgas et al., 2006b). Unfortunately, we lack sufficient data to test in a rigorous way whether there are associations between survival and HL ratios in our population.

HL ratios of female tree swallows were higher in samples that were collected later in the day (Fig. 2). Both Ots et al. (1998) and Hõrak et al. (1998a) could detect no relationship between time of day and HL ratios in great tits; however, both studies found counts of heterophils and lymphocytes to be higher at night than during the day. It was suggested that such changes were due to birds allocating energy to cell proliferation at night that during the day would have been used for other processes, or possibly the migration of cells from peripheral blood to tissues during the day (Ots et al., 1998). The patterns in HL ratios that we detected were due to heterophils increasing with time of day while lymphocytes decreased. Changes in heterophils with sampling time may be a reflection of the accumulation of stress during the day (Maxwell and Robertson, 1998), while decreases in lymphocytes may be a response to the physical exertion of provisioning nestlings (Hoffman-Goetz and Pedersen, 1994).

We found that HL ratios of female tree swallows were negatively related to their body condition (Fig. 2). There is evidence that birds in poor body condition also have relatively higher levels of stress, which is reflected in elevated HL ratios. For example, common eiders (*Somateria mollisima*) in poor body condition showed increasing HL ratios during incubation, suggesting birds under stress were investing less in immune function, whereas those in good body condition decreased their HL ratios during incubation (Hanssen et al., 2003). Maintaining high levels of immunocompetence while investing in other energy-demanding processes, such as reproduction, may not be possible for birds in poor condition (Totzke et al., 1999; Sanz et al., 2004). Such relationships appear to not be universal, as Vleck et al. (2000) found no relationship between body mass of male Adelie penguins (*Pygoscelis adeliae*) and HL ratios during fasts that occur during courtship and incubation, even though males may lose up to a third of their mass during this period.

Several studies have shown that HL ratios and leucocyte differentials can provide insight into subsequent performance of individuals. For example, mourning doves (*Zenaida macoura*) with high HL ratios were more likely to succumb to experimental lead poisoning than those birds that had lower HL ratios (Schulz et al., 2006). Lobato et al. (2005) suggested that number of heterophils in peripheral blood was a better predictor of recruitment probabilities in pied flycatchers (*Ficedula hypoleuca*) than were more traditional measures such as hatching date or body mass. Nonetheless, HL ratios, as well as proportion of heterophils and lymphocytes, appeared to have little utility in being strong predictors of future reproductive success of female tree swallows, as there was no relationship between these variables and number of offspring fledged. This is perhaps not surprising, because females were sampled at the beginning of the brood-rearing period, and in addition to the inherent quality of the parents, the ability to fledge offspring can be highly dependent on environmental factors that may have occurred after sampling and so would not necessarily be reflected in HL ratios or in proportions of heterophils or lymphocytes. In our study area, for example, two of the most important factors influencing breeding success are predation and weather conditions during the brood-rearing period (Dawson et al., 2005).

Vleck et al. (2000) suggested that HL ratios had merit as relatively long-term indicators of injuries to birds, and past studies have generally found HL ratios to be relatively consistent within individuals over both short and longer time periods. For example, Davis (2005) found consistency in HL ratios of house finches (*Carpodacus mexicanus*) from samples taken up to 1 h apart, while Ots et al. (1998) showed that HL ratios of adult great tits were correlated between days 8 and 15 of the nestling period. Hõrak et al. (2002a) found that while there were systematic differences in HL ratios, and proportions of heterophils and lymphocytes, in captive greenfinches (*Carduelis chloris*) sampled in fall and spring, each of these variables showed significant repeatability over a 4 month period. Although our analyses testing for factors that influence HL ratios among individuals suggested that this measure can reveal information about body condition and potentially costs of reproduction, when we examined samples from individuals across multiple breeding seasons, we found that only proportion of lymphocytes was repeatable, and the interclass correlation coefficient for this variable was low. Both HL ratios and proportion of heterophils did not show significant repeatability across breeding seasons in female tree swallows. There are several potential explanations for the differences in repeatability of HL ratios between tree swallows in our study and other species. Hõrak et al. (2002) sampled greenfinches in captivity where food was not limiting and so their results may represent the potential for leucocyte numbers to be repeatable under relatively benign conditions. Furthermore, the samples from greenfinches were collected outside of the breeding season, and levels of circulating leucocytes can change within individuals not only during the period of breeding (e.g., Kilgas et al., 2006a), but also between the breeding and non-breeding season (e.g., Owen and Moore, 2006). Alternatively, there may be interspecific differences in the degree that individuals maintain a consistent state of health or in levels of stress. Our samples were collected 1 year apart and the health state of individuals may have changed significantly over this relatively long time period.

As we were unable to detect significant repeatability for both HL ratios and proportion of heterophils, we suggest that caution should therefore be exercised in using these measures for illustrating the inherent quality or health of individuals over long time frames. Nonetheless, our results do suggest that HL ratios, as well as proportions of heterophils and lymphocytes, may reveal information about levels of stress resulting from costs of reproduction or decreased body condition, although it is important to test for possible confounding effects such as sampling time (Figs. 2 and 4). Moreover, these measures may be useful in examining how prevailing conditions on wintering areas affect stress levels, and by extension reproductive performance, during the breeding season. Clearly, further work is required to confirm the relationships between levels of stress detected on breeding areas and conditions experienced during the previous winter.

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