J. Field Ornithol. 76(4):334–338, 2005

Effect of handling time and repeated sampling on avian white blood cell counts

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Received 13 September 2004; accepted 7 February 2005

ABSTRACT. The practice of obtaining white blood cell (leukocyte) profiles and heterophil/lymphocyte (H/L) ratios from avian blood smears has become increasingly popular to assess immune function in wild birds. I captured 28 House Finches (*Carpodacus mexicanus*) and made blood smears from samples obtained from them at 3, 30, and 60 min after capture to evaluate the effect of routine handling time on leukocyte profiles and H/L ratios. Total leukocyte counts decreased significantly with time, but the proportions of each leukocyte type remained the same over the 1-h time period. There was a nonsignificant increase in H/L ratios over time, but comparison with a group of birds held for 1 h before bleeding suggested that this was the result of the repeated bleedings, not handling time. I conclude that researchers should make every effort to obtain blood samples for making smears as soon as possible after capturing birds to ensure an accurate assessment of total leukocyte counts, but that routine handling times under 1 h do not affect H/L ratios.

SINOPSIS. **Efecto del tiempo de manipulación en muestras repetidas en el conteo de celulas blancas en aves**

La práctica de obtener un perfil de células blancas y la tasa de heterófilos/linfocitos (H/L) en la sangre de aves se ha convertido en una práctica popular entre los ornitólogos que quieren determinar la función inmunológica en aves silvestres. Capture 28 individuos de *Carpodacus mexicanus* e hice frotis de muestras obtenidas a 3, 30, y 60 minutos, luego de haber sido capturada el ave para evaluar el efecto del tiempo de manipulación en el perfil de la tasa de H/L. El conteo total de leucocitos disminuyó significativamente con el tiempo, pero la proporción de leucocitos permaneció sin cambio a lo largo de una hora. No hubo un aumento significativo en la tasa de H/L a través de los periodos de tiempo, pero al comparar los datos obtenidos con aves que fueron mantenidas en cautiverio por una hora, previo a ser sangradas sugieren que el resultado se debe a la toma repetitiva de muestras en vez de a la manipulación. Concluyo que los investigadores deben hacer el mejor esfuerzo de tomar muestras de sangre tan pronto capturen al ave para asegurarse de obtener unos datos correctos con respecto al contaje de leucocitos, pero que el tiempo que las aves se mantengan en cautiverio no afecta la tasa de H/L.

Key words: *Carpodacus mexicanus*, handling time, heterophil lymphocyte ratio, House Finch, white blood cell

An increasing practice among ornithologists is the use of blood smears to assess innate immune function in birds. By counting the numbers and proportions of white blood cells (leukocytes) on blood smears, a leukocyte profile (or differential) can be obtained for the individual, giving insight into its immune function at the time of capture (Davis et al. 2004). Further, the ratio of two leukocyte types, heterophils and lymphocytes (H/L ratio), has been increasingly used by ornithologists to monitor immune function, as it appears to increase with decreasing territory quality (Mazerolle and Hobson 2002), disease (Davis et al. 2004), injury (Vleck et al. 2000), and urbanization (Ruiz et al. 2002). Finally, H/L ratios have been shown to increase in several species following a 1–3 h transport (Parga et al. 2001; Scope et al. 2002; Groombridge et al. 2004). This fact has important implications for researchers, because it suggests that leukocyte profiles in birds can change within the small time intervals associated with general handling or transport of the birds.

Given that leukocyte profiles may be affected by the stress associated with transportation, an important question is whether leukocyte profiles in birds change during the time associated with routine handling and data collection (up to 1 h). With the widely used corticosterone response, it is well-known that basal hormone levels must be sampled within 3 min of capture (Romero and Romero 2002). The same might be true for leukocyte profiles. To answer this question I captured House Finches (*Carpodacus mexicanus*) and made blood smears from samples obtained at 3, 30, and 60 min after capture to evaluate the effect of handling time on leu-

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kocyte profiles. Further, to determine if the act of repeatedly bleeding the birds could influence their leukocyte numbers, I also examined smears made from a second set of 10 House Finches that were held for 1 h before blood sampling.

METHODS

The birds used in this study were trapped as part of a long-term study of mycoplasmal conjunctivitis in House Finches in Atlanta, Georgia (Altizer et al. 2004), but for this study, only House Finches with no visible signs of conjunctivitis were used. Details of the trapping methods are provided elsewhere (Altizer et al. 2004; Davis et al. 2004). I trapped 28 House Finches consisting of a range of age and sexes: 14 adults, four juveniles, and 10 of unknown age, while 17 were males, and 11 were females. All birds were captured from January through March 2003. Trapping the finches involved either mist nets set up next to bird feeders or metal walk-in cage traps (Hill 2002). The cage traps were constructed of hardware mesh encircling a standard bird feeder. Two holes on either side of the cage allowed birds to walk into the trap, whereby they usually would sit at the feeder inside and eat normally (A. K. Davis, pers. obs.).

Both trapping methods are effective in capturing House Finches, but because they operate differently to capture birds, they often sample different subsets of the finch population (Davis 2005). These methods also induce different reactions by the birds upon capture. Mist-netted birds tend to thrash in the net as soon as they are caught. House Finches in the cage traps usually did not realize they were trapped until I approached the trap (A. K. Davis, pers. obs.). Thus, any stress response was probably initiated upon capture with mist nets, but not until I approached the cage traps to remove the birds. Because of this difference, I used a different procedure for blood sampling depending on capture method. When using mist nets, I immediately extracted any House Finch captured and collected a blood sample from the bird within 3 min of it hitting the net. When cage traps were used, I did not begin timing until I approached the cage trap (traps were checked every 15 min). I then removed the finches from the trap and obtained a blood sample within 3 min. All House Finches were placed in cloth bags and brought to the lab, where each bird was bled again at 30 and 60 min after capture. Birds were bled by puncturing the brachial vein with a 25-gauge needle and siphoning approximately 20 microliters of blood from the puncture with a standard microhematocrit capillary tube. Blood smears were made immediately after drawing blood using the two-slide wedge method (Houwen 2000). After air drying, slides were fixed in methanol and later stained with Wright-Giemsa Quik stain. For 10 additional House Finches captured to examine the effect of handling time only, all were handled and treated identically, except that they were only bled once at 60 min after capture.

All smears were examined under 1000× following Davis et al. (2004). I counted all white blood cells up to a total of 100, while keeping track of the number of fields of view. Only fields of view with adequate numbers of red blood cells were examined, and the number of red blood cells in each field of view was estimated. From these data I calculated the total number of leukoctyes per 10,000 erythrocytes, as well as the proportion of each leukocyte type. To calculate the numbers of each leukocyte type (per 10,000 erythrocytes), the proportions were multiplied by the total number of leukocytes per 10,000 erythrocytes.

Data analysis. To test for effects of handling time on leukocyte parameters, I used repeated measures ANOVA on each of the following independent variables: total leukocyte count, heterophil/lymphocyte ratio, and numbers and proportions of heterophils, lymphocytes, eosinophils, monocytes, and basophils. Time from initial capture (3, 30, and 60 min) was the categorical factor. Further, I also compared each of these same variables in the 3-min sampling of the 28 birds (multiple-sample group) to that of the 60-min sampling of the 10 birds (one-sample group) using two-sample *t*-tests. To meet assumptions of normality, all leukocyte counts (i.e., numbers per 10,000 erythrocytes) were log-transformed, and H/L ratio and all leukocyte proportions were arcsine square-root transformed prior to analyses. All tests were performed using SPSS software (SPSS 2002), and all tests were considered significant when $P < 0.05$.

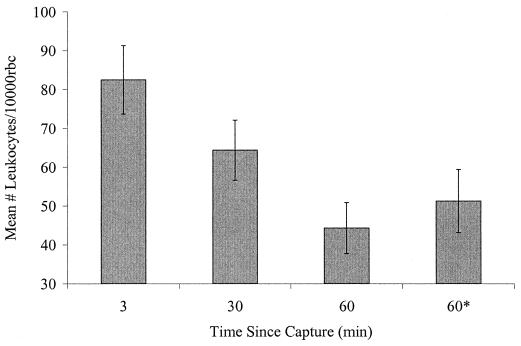

Fig. 1. Mean number of leukocytes per 10,000 red blood cells of 26 House Finches sampled at 3, 30, and 60 min after capture. Also shown (60*) is the mean for 10 birds that were sampled only once, 60 min after capture. Error bars represent standard errors.

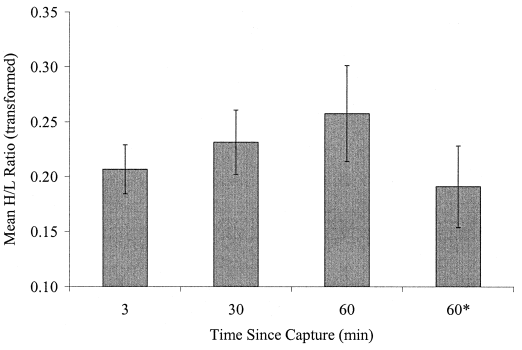

Fig. 2. Mean heterophil/lymphocyte ratio (arcsine square-root transformed) of 28 House Finches at 3, 30, and 60 min after capture. Also shown (60*) is the mean for 10 birds that were sampled only once, 60 min after capture. Error bars represent standard errors.

RESULTS

There was a clear negative effect of time on total leukocyte numbers in the multiple-sample group ($F_1 = 17.4$, $P < 0.001$; Fig. 1). The majority of this decline occurred between the 30- and 60-min intervals. Comparisons of groups using paired *t*-tests showed no significant difference in total leukocyte numbers between the 3- and 30-min samples ($t_{23} = 1.28$, $P = 0.21$), but significant differences between 30 and 60 min ($t_{23} = 4.52$, $P < 0.001$) and between 3 and 60 min ($t_{24} = 4.67$, $P < 0.001$). The total leukocyte counts of the one-sample group were significantly lower than the initial sample of the multiple-sample group (two-sample *t*-test, $t_{36} = 2.09$, $P = 0.04$), indicating that this effect was driven by time since capture, not repeated bleeding.

There was no significant relationship between time and H/L ratios in the multiple-sample group ($F_1 = 0.76$, $P = 0.39$; Fig. 2). There were no significant differences between 3- and 30-min samples ($t_{23} = -0.50$, $P = 0.62$), 30- and 60-min ($t_{23} = -0.76$, $P = 0.45$), or 3- and 60-min samples ($t_{24} = -0.99$, $P = 0.34$). The H/L ratio of the one-sample group was similar to that of the first sample of the multiple-sample group (two-sample *t*-test, $t_{36} = 0.39$, $P = 0.70$; Fig. 2), suggesting that the repeated bleeding, not time, had caused the H/L increase in the multiple-sample group.

Lymphocytes were the only leukocyte type to show significant differences in numbers (per 10,000 erythrocytes) across time intervals ($F_1 = 21.3$, $P < 0.001$; Table 1). There was an almost significant difference in lymphocyte proportions across time intervals ($F_1 = 3.68$, $P = 0.07$). The trend was negative in each case (Table 1). However, the 10 birds that were held for one hour prior to bleeding did not differ in the number of lymphocytes from the 3-min

Table 1. Mean estimated numbers of all House Finch leukocyte types (per 10,000 erythrocytes) counted at 3, 30 and 60 min after initial capture. Proportions of each leukocyte type are also shown.

	3 min		30 min		60 min		60 min[a]	
	Mean	Proportion	Mean	Proportion	Mean	Proportion	Mean	Proportion
Heterophils	3.3	0.04	3.6	0.05	2.8	0.06	1.6	0.04
Lymphocytes	69.5	0.84	53.0	0.83	33.1	0.78	45.5	0.88
Monocytes	0.8	0.01	0.4	0.01	0.3	0.01	0.7	0.02
Eosinophils	3.3	0.04	1.9	0.03	2.7	0.06	2.5	0.05
Basophils	5.5	0.07	5.4	0.08	5.5	0.09	0.9	0.02

[a] For 10 birds that were sampled only once, 60 min after capture.

sample of the larger group (two-sample *t*-test, $t_{36} = 1.8$, $P = 0.08$), and there was no significant difference in monocyte numbers ($F_1 = 3.14$, $P = 0.09$). No other significant effects were found for other leukocyte types (Table 1). There were no significant differences in other leukocyte types between the first sample of the multi-sample group and the 60-min sample of the one-sample group.

DISCUSSION

The results of this study indicate that the time from initial capture must be considered when interpreting certain leukocyte parameters from blood smears. Most importantly, the total leukocyte counts decreased over the 1-h interval I considered. This result is consistent with both Parga et al. (2001) and Scope et al. (2002), who showed that stressful events such as transportation of wild birds over a 1–3 h period induced a significant decrease in white blood cell numbers. Research with poultry has also shown that overall white blood cell numbers decrease within 1 h after stressful events (Wang et al. 2003). I found that leukocytes declined after an even lesser time interval: after 30 min (although not significantly), but most of all after 60 min from initial capture.

Much of the decline in total leukocyte numbers I found appeared to be attributable to the reduction in lymphocyte numbers over time. Lymphocytes make up the majority of leukocytes in House Finches (Davis et al. 2004), with most healthy individuals having leukocyte profiles of over 70% lymphocytes and an unusually low number of heterophils, approximately 5%. The large proportion of lymphocytes in this species means that any changes in their numbers would no doubt affect the estimated total leukocyte count. It is unclear why lymphocyte numbers would be reduced after capture and handling; however, early work in poultry demonstrated a negative relationship between corticosterone concentration and lymphocyte numbers in blood (Gross and Siegel 1983). Since corticosterone levels increase in wild birds within minutes of capture in response to stress (Romero and Romero 2002), they may indirectly act to reduce the number of lymphocytes, at least in the peripheral blood stream. However, further research into avian immune systems would be needed to clarify this issue.

The lack of significant effects of handling time on H/L ratios suggests that the time frame I considered (one hour) does not affect this measure and that H/L ratios are not affected by routine handling. Further, the similarities between initial samples and those of birds held for one hour before sampling also suggest that H/L ratios are not affected by short time intervals and that the nonsignificant increase I observed was caused by the bleeding procedure itself, not handling time. However, since most researchers need only one blood sample per bird upon capture, any potential increase in H/L ratios caused by prior bleeding, if any, would not apply in most cases. This result contrasts with that found by Groombridge et al. (2004); however, their study examined the simultaneous effects of handling and transport across rugged terrain, which surely would have invoked a higher stress response in their subjects than what I examined here.

I conclude that routine handling times of up to 1 h cause significant changes in the total numbers of leukocytes in wild birds but not in the relative proportions of leukocyte types. Thus, researchers should make every effort to obtain blood samples for making smears as soon as possible after capturing birds to ensure an accurate assessment of total leukocyte counts. Time since capture need not be considered if leukocyte proportions or H/L ratios are used.

ACKNOWLEDGMENTS

Katherine Cook and Sonia Altizer assisted with capturing House Finches. Part of this work was supported by the National Science Foundation (DEB-0094456) and by a University Research Committee Award from Emory University.

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