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PHYSIOLOGIC PARAMETERS AND THEIR RESPONSE TO HANDLING STRESS IN A NEOTROPICAL MIGRATORY SHOREBIRD DURING THE NONBREEDING SEASON

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ABSTRACT: Physiologic traits are promising indicators of population health in the face of rapidly changing environments. We obtained values of diverse physiologic parameters for Two-banded Plovers (*Charadrius falklandicus*) in coastal sites in Patagonia, Argentina, with the objectives of determining the timeline in which these parameters become affected by the stress of capture and handling and of obtaining reference values for future monitoring of these populations. We analyzed packed cell volume, white blood cell profile, heterophil/lymphocyte ratio, bacterial agglutination titer, and total protein, glucose, triglyceride, and cholesterol levels in apparently healthy birds. Glucose, total white blood cell count, lymphocytes, and eosinophil levels showed changes with handling times >60 min after capture. The remaining parameters did not manifest significant alterations in response to capture and handling of up to 232 min (average=105.2, SD=56.7). Therefore, although researchers should attempt to obtain blood samples as soon as possible after capture, inclusion of physiologic parameters in monitoring studies of species not easily sampled in a few minutes, such as Two-banded Plovers and other shorebird species during migration, should not be discouraged. Here we provide a physiologic report for the species that can be considered as reference values during the nonbreeding season at Patagonian coastal sites.
Key words: Biochemistry, hematology, immunity, Patagonia, Two-banded Plovers.

INTRODUCTION

The use of physiologic indices in ecology and conservation biology is becoming increasingly common due to the importance of monitoring wildlife populations in the face of rapidly changing environments, which has given rise to the relatively new discipline of conservation physiology (Cooke et al. 2013; Madliger and Love 2015). Physiologic traits at the individual level are promising indicators of population health and can signal a problem before demographic consequences are observed (Carey 2005; Wikelski and Cooke 2006). An obstacle to using physiologic parameters is separating the effects of stress caused by the environmental factors being studied from the stress effects of capture and handling. This is particularly important for animals that cannot be easily sampled within a few minutes of capture, such as shorebird species captured in large flocks during migration or winter (Buehler et al. 2008). For these taxa, it is essential to understand the timeline under which some physiologic parameters (e.g., immune, nutritional, hormonal, general body condition indices) become affected by the stress of capture and handling. Some parameters can be highly sensitive, changing within minutes of capture, while others might not show significant alterations for hours (Buehler et al. 2008; Davis et al. 2008).

Stress response in vertebrates is mediated by glucocorticoids (e.g., cortisol, corticosterone), which rapidly increase in the bloodstream upon capture and handling (Romero 2004; Davis et al. 2008). For birds and mammals, this increase generally occurs within 3 min of capture (Romero and Romero 2002; Romero and Reed 2005), whereas times are more variable and tend to be longer for fish, amphibians, and reptiles (Romero and

Romero 2002; for a review see Davis et al. 2008). In turn, glucocorticoids affect other physiologic parameters (Ellis et al. 2012), but the timeline of such effects has been less studied. Stress and immune parameters are linked through complex interactions between the neuroendocrine and immune axes (McEwen et al. 1997), with stress responses suppressing some forms of immunity while enhancing others (Apanius 1998; Martin 2009). Lowered packed cell volume (also called hematocrit), lower hemoglobin concentrations, and poor body condition have also been linked to stress in some species (Wingfield and Kitaysky 2002; Lindström et al. 2005). Similarly, handling stress can affect some blood biochemical parameters such as glucose, uric acid, and triglyceride levels in birds (Dietz et al. 2009; Davies et al. 2013).

We investigated the timeline in which the stress of capture and handling affects diverse blood physiologic parameters related to health, nutrition, and immune function in Two-banded Plovers (*Charadrius falklandicus*), a short-distance migratory shorebird endemic to southern South America. Migratory shorebirds constitute an ideal model system for our study because many species are showing population declines and thus their monitoring has been intensified in recent years (Wetlands International 2016). The inclusion of physiologic parameters in conservation programs can help the identification of potential causes for observed declines (Carey 2005; Wikelski and Cooke 2006). Capture of migratory shorebirds, especially during migration or in the nonreproductive season, usually involves the use of cannon nets (Kasprzyk and Harrington 1989). This capture method can trap many individuals simultaneously (dozens to hundreds). This can be ideal for banding programs but presents a challenge for physiologic monitoring, because birds need to be kept in shaded cages until sampled, sometimes for hours after capture. Thus, knowledge of the timeline during which the stress of capture and handling affects diverse physiologic parameters can help identify those parameters that can be used in monitoring programs involving the use of cannon nets and those that might need an alternative capture method to provide reliable information.

Values of physiologic traits are scarce in the literature for wild migratory shorebird species in South America (D'Amico et al. 2010). Thus, another objective of this study was to provide physiologic reference values that are important as baseline values for studies investigating the multiple threats that migratory shorebirds can face in the diverse areas they use during their annual cycles (e.g., Klaassen et al. 2012). The population of Two-banded Plovers is estimated at between 25,000 and 100,000 individuals (BirdLife International 2016). Trends in abundance in Patagonia, Argentina, are unknown, with just a few reports for some local populations (Hevia 2013). Beaches of northern Patagonia are used by some Two-banded Plovers as breeding sites from October to December (Hevia 2013) and they can reach as far north as Rio de Janeiro, Brazil, during their short northward migration (Woods and Woods 1997). To our knowledge, this is the first study reporting data on nonbreeding Two-banded Plovers captured at feeding sites in northern Patagonia.

MATERIALS AND METHODS

We sampled birds at two feeding sites, Bahía San Antonio (Río Negro) and Peninsula Valdés (Chubut), covering a range between 40°S, 65°W and 42°S, 64°W during the nonbreeding season in northern Patagonia, Argentina (Fig. 1). Sampling took place in April, once in 2014 and twice in 2015 at Bahía San Antonio and once in March 2015 in Peninsula Valdés. Individuals were captured using a cannon net launched over a flock of Two-banded Plovers resting on the beach during the high tide following standard protocols (Breese et al. 2010). All captured individuals were kept in shaded cages placed on the sand that were continuously wetted to avoid overheating until sampled (Breese et al. 2010). Birds were weighed with an analytical balance (±0.01 g); bill length was measured with a caliper (±0.1 mm) and wing length was measured with a ruler to the nearest millimeter. Blood samples (0.3–0.35 mL) were obtained from the brachial vein using 27-gauge needles and collected into heparinized microcapillary tubes (Tecnon, Buenos Aires, Argentina) that were stored at 4 C until analysis. Thin blood smears were prepared with a drop of fresh blood, air dried, fixed with absolute ethanol for 3 min, and

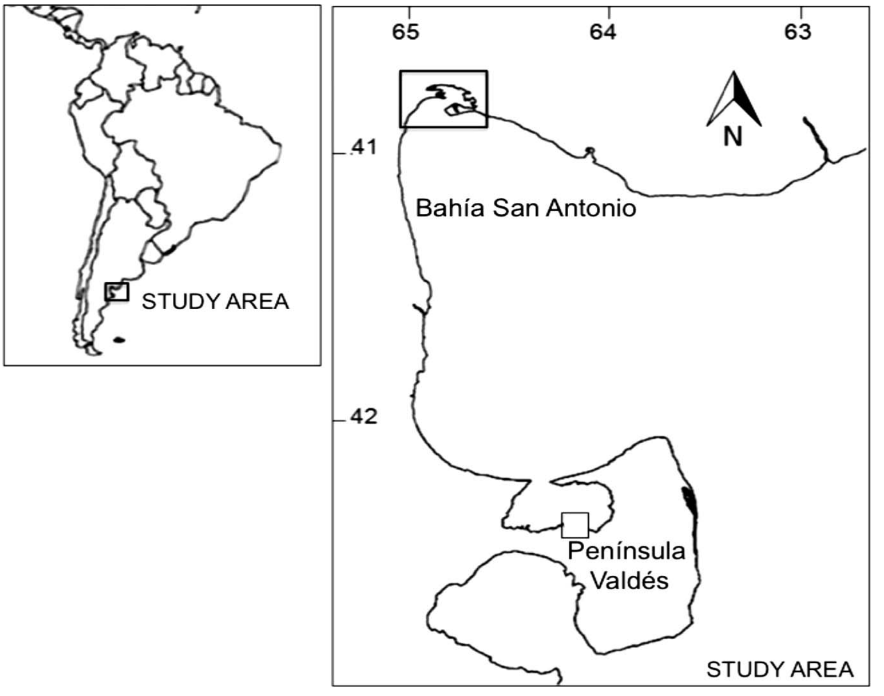

FIGURE 1. Study sites (boxes) in Bahía San Antonio and Península Valdés, in northern Patagonia, Argentina, where sampling of Two-banded Plovers (*Charadrius falklandicus*) occurred in 2014–15.

stained with Tinción 15 (Biopur S.R.L., Rosario, Argentina). Time in minutes between capture (firing of cannon net) and blood draw was recorded for all individuals and varied depending on the number of birds simultaneously trapped (20–64), the number of field assistants available for setting up the keeping cages and getting birds out of the net (2–10), and the number of researchers available for bleeding (1–2). All captured individuals were considered adults based on the two distinguishable breast bands (Narosky and Yzurieta 2010) and body mass (Wiersma et al. 2016). No signs of illness or poor health were seen based upon plumage brightness and absence of both ectoparasites and feather damage. Birds did not present evidence of molting. All were released at the site of capture after sampling.

Blood was centrifuged at 12,000 × G for 12 min (Cavour VT 1224, Buenos Aires, Argentina) and packed cell volume was measured with a microhematocrit ruler (J. P. Selecta, Barcelona, Spain). Packed cell volume is an index of general condition (Fair et al. 2007) and provides an estimate of aerobic capacity (Beldomenico et al. 2008). Smears were examined under a light microscope scanning monolayer fields with similar densities of erythrocytes for all individuals to obtain white blood cell counts (Campbell 1995). Total white blood cell count per 10,000 erythrocytes was estimated by counting the number of erythrocytes in one microscopic visual field and multiplying it by the number of microscopic visual fields that were scanned until reaching 100 leukocytes (Lobato et al. 2005). The proportion of each leukocyte type was obtained from a sample of 100 leukocytes under 1,000× magnification (oil immersion) classified into basophils, heterophils, eosinophils, lymphocytes, and monocytes (Campbell 1995). Total counts for each leukocyte were obtained by multiplying the total leukocyte count and the respective percentage. Heterophil/lymphocyte ratio (H/L) as a measure of stress (Davis et al. 2008), was calculated from the corresponding leukocyte counts.

To determine total protein (g/dL), glucose (mg/dL), triglyceride (mg/dL), and cholesterol (mg/dL) levels, plasma was analyzed by colorimetric and enzymatic methods and processed on a spectrophotometer (Metrolab 1600 Plus, UV-Vis, Buenos Aires, Argentina). Quality control was based on Levy-Jennings plots of the average value of dispersion for both methods: Biuret reaction for total proteins and enzymatic for lipids and carbohydrates. These biochemical parameters contribute to the assessment of body condition and nutritional status of birds (D'Amico et al. 2010).

Agglutination of *Escherichia coli* (ATCC 8739) by plasma components, an index of constitutive humoral innate immunity, was measured following a protocol (Sahoo et al. 2008) that we adapted for use in shorebirds. Briefly, bacteria were grown in tryptic soy broth and fixed in 1% formalin overnight at 4 C. Fixed bacteria were washed three times with phosphate buffered saline (PBS) and adjusted to a concentration of approximately 1×10^9 bacteria/mL. Plasma samples (15 μL) were added to the first column of a 96-well plate and serially diluted twofold with PBS. A negative control (PBS) was included in each plate and 15 μL of fixed bacteria were added to all wells. Plates were vortexed and incubated at 40 C overnight. Agglutination titers were determined as $-\log_2$ of the highest dilution showing bacterial agglutination. Interplate variation, calculated as the coefficient of variation, was 4.6%.

Two-banded Plovers are not sexually dimorphic, so sex was molecularly determined for a random subset of individuals for which a portion of blood had been preserved in ethanol (n=51) following the protocol of Fridolfsson and Ellegren (1999). Briefly, avian sex DNA marker amplification was performed using PCR-based methods (Bioer Life Express Thermal Cycler, Hangzhou Bori Technology, Hanzhou, China) using specific oligonucleotide primers 2550F and 2718R (Invitrogen Life Technologies, Carlsbad, California, USA). The PCR products were compared to a 100-base pair (bp) DNA ladder. Males were identified as displaying one PCR product (from CHD 1Z, 600 bp) while females showed two products (from CHD 1W, 450 bp and from CHD 1Z; Fridolfsson and Ellegren 1999).

Data from the four captures were pooled for statistical analyses. The effects of handling time, body mass, and body condition on physiologic parameters were evaluated using Spearman rank correlations that included data for all individuals captured (males, females, and individuals of unknown sex). For variables affected by handling time, we then analyzed the changes over time following protocols of Buehler et al. (2008) and Cïrule et al. (2012). For this, we examined the response of parameters at 30-min intervals from the time of capture (0–30, 31–60, 61–90, 91–120, 121–150, and >150 min) and tested for differences among intervals using Dunn's multiple comparisons post hoc tests (Sokal and Rohlf 2012). Mann-Whitney U-tests were used to compare parameters between the sexes in the subset of known-sex birds. Body condition was calculated as the residuals of body mass on wing length. Because results using the body condition index and body mass were the same, only the latter are presented. Sample sizes differ among measured parameters because blood volume was insufficient for all measurements in all individuals. We provide descriptive statistics for all parameters as reference values for the species discriminated by sex. All analyses were performed using STATISTICA 7.0 (StatSoft Inc., Tulsa, Oklahona, USA) and significance is reported using an alpha of 0.05.

RESULTS

We captured 137 individuals and bled 119. Time between capture and blood draw ranged from 10 min to 232 min with an average of 105.2 (SD=56.7) min. Reference values of the parameters considered for all Two-banded Plovers captured are presented in Table 1, whereas parameters on the subset of individuals discriminated by sex (n=51) are shown in Table 2. Sex ratio in the latter group of birds was near 1:1 (51% males; 49% females). Body mass was slightly greater for males than for females (U=220, P=0.046; Table 2); bill and wing length did not vary between sexes. Glucose level was the only physiologic parameter correlated with body mass (r=0.27, $P<0.0063$) and was greater for males than for females (U=58.5, P=0.042; Table 2). Sexes did not differ in handling time or in any of the remaining physiologic parameters measured (Mann-Whitney, all $P>0.05$); caution is required nevertheless given the relatively small sample sizes available for some variables (Table 2).

Packed cell volume and levels of total protein, cholesterol, and triglycerides did not change with handling time in the range of times tested (i.e., 10–232 min, all $P>0.05$). Glucose levels increased with handling time (r=0.35, P=0.0004, n=101), with significant changes detected after 150 min of capture of birds (Fig. 2A). Total white blood cell counts decreased after 60 min of capture of birds (r=–0.30, P=0.002, n=100; Fig. 2B). Total counts of lymphocytes also showed changes with handling time (r=–0.26, P=0.008, n=100); decreased values were manifested at two points, at the 61–90 min interval and at 121–150 min of capture (Fig. 2C). Total and percentage of eosinophils showed changes with handling time (r=–0.56, $P<0.0001$, n=100 and r=–0.50, $P<0.001$, n=100, respec-

TABLE 1. Reference values for morphologic and physiologic parameters of adult Two-banded Plovers (*Charadrius falklandicus*) captured in northern Patagonia, Argentina, during the nonbreeding season 2014–15. Sample sizes differ among measured parameters because blood volume was insufficient for all measurements in all captured birds.

	n	Mean±SE	Median (minimum–maximum)
Body mass (g)	137	63±0.3	63 (53–73)
Wing length (mm)	112	126±0.03	127 (116–134)
Bill length (mm)	112	18.6±0.07	18.5 (17.09–21.2)
Packed cell volume (%)	111	50.9±0.3	51 (37–62)
Glucose (mg/dL)	101	280±5.4	272 (155–461)
Triglycerides (mg/dL)	74	96.9±5.3	80 (65–352)
Cholesterol (mg/dL)	45	215.4±6	211 (145–305)
Total protein (g/dL)	94	5.7±0.05	5.7 (4.5–7)
Total white blood cells	100	31.9±1.5	28 (11–97)
Total lymphocytes	100	15.3±0.6	13.3 (3.3–36.2)
Total heterophils	100	9.3±0.6	7.8 (1.6–44.9)
Total eosinophils	100	4.5±0.3	3.1 (0.2–16.9)
Total monocytes	100	2.2±0.2	1.7 (0–24)
Total basophils	100	0.3±0.04	0.2 (0–2.1)
% Lymphocytes	100	49.7±1.1	50 (26–78)
% Heterophils	100	28.5±1.02	27.5 (6–57)
% Eosinophils	100	13.7±0.8	12 (1–37)
% Monocytes	100	6.9±0.3	6 (0–25)
% Basophils	100	1.2±0.1	1 (0–6)
Heterophil/lymphocyte ratio	100	0.6±0.03	0.6 (0.1–1.6)
Bacterial agglutination titer	15	5.8±0.2	5.5 (3.6–8.5)

tively), decreasing significantly after 90 min and after 150 min of capture, respectively (Fig. 2D, E). The remaining immune parameters did not change with handling time (all $P>0.05$; sample sizes in Table 1).

DISCUSSION

Body mass of Two-banded Plovers in this study had a wider range (53–73 g) than previously reported for adults in this species (62–72 g, Wiersma et al. 2016). Males averaged slightly heavier than females, but body mass ranges overlapped. In addition, sexes did not differ in bill and wing length and, together with the lack of plumage differences between males and females, these factors highlight the need to use molecular data to determine sex. Body mass and glucose levels were the only two parameters that showed slight but significant differences between sexes in the subset of birds with sex determined using DNA; both were higher in males than in females. However, future studies should increase sample sizes as some of the physiologic variables (particularly cholesterol and agglutination titer) were very small.

Packed cell volume ranged from 43% to 62% (Table 1), in accordance with values reported for other shorebird species (Piersma and Everaarts 1996; Jenni et al. 2006) and in the range reported for healthy birds in general (40–60%; Campbell 1995). No reports on biochemical parameters were found for short-distance migratory shorebird species. However, ranges for values of total protein, cholesterol, triglycerides, and glucose were wider for Two-banded Plovers than for a long-distance migrant, the Red Knot (*Calidris canutus rufa*), sampled at the same feeding area (D'Amico et al. 2010). Lymphocytes were

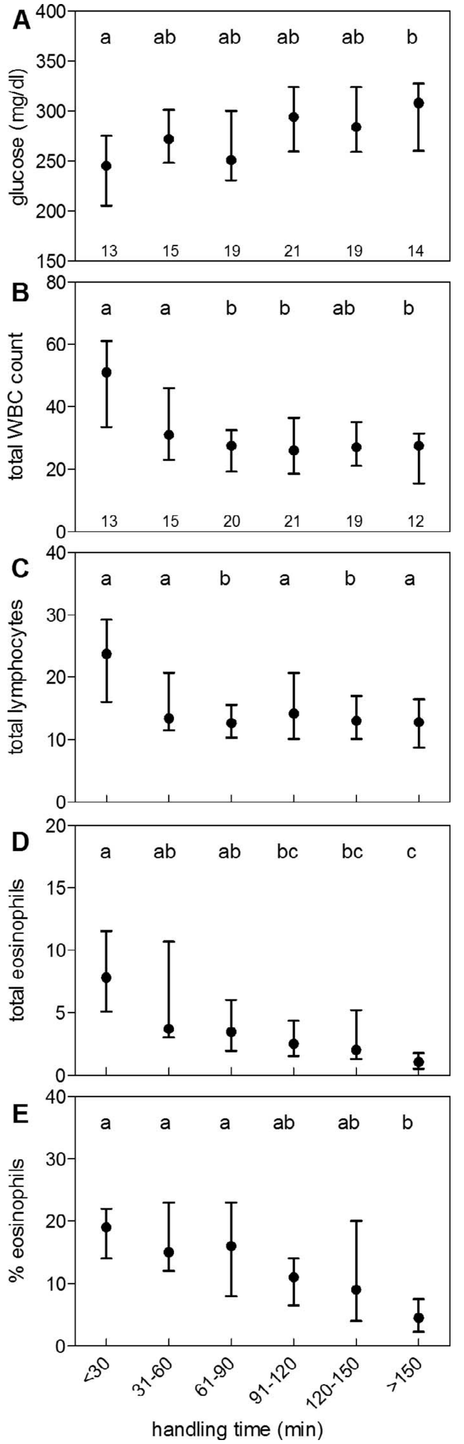

FIGURE 2. Physiologic parameters of Two-banded Plovers (*Charadrius falklandicus*) that showed significant changes as a function of handling times ranging from 10 min to 232 min. Timeline was divided into six intervals of 30 min from capture. Graphs depict medians (dots) and interquartile ranges (whiskers). Letters indicate results of post-hoc tests for multiple comparisons. Intervals not sharing letters are significantly different from each other ($P<0.05$). Sample sizes are shown below each time interval for glucose levels (panel A) and for the four leukocyte parameters (panel B). WBC=white blood cells.

the most abundant leukocyte followed by heterophils (Table 1), as it has been reported for birds in general (Campbell 1995) and as has been observed in other shorebirds (Buehler 2008; D'Amico et al. 2010). Percentages of eosinophils were high (13.7±0.8 SE) compared to those of Red Knots sampled at the same study site (0.95±0.3; D'Amico et al. 2010). Although there is interspecific variation, in general eosinophils, basophils, and monocytes are in low percentages in healthy birds (Campbell 1995). Elevations of eosinophils above normal ranges are usually related to gastrointestinal parasitic infections (Thrall et al. 2012). We did not assess endoparasite infection because the birds were released alive. Future studies are thus needed to determine whether Two-banded Plovers are susceptible to endoparasite infections or if they normally have higher values of eosinophils. Mean H/L ratio was 0.6±0.03 SE (Table 1), which suggests that birds were not exhibiting high levels of chronic stress at the site. For example, studies in gulls (Laridae) reported values of H/L about 0.6 in apparently healthy individuals compared to 2.9 in birds that were oiled, emaciated, injured, or infected by parasites (Averbeck 1992). Similarly, Nisbet et al. (2015) reported an increase of 4.5 times in H/L ratios in terns (Sternidae) exposed to oil spills.

Regarding the potential effects of handling time on physiologic parameters, packed cell volume and blood levels of cholesterol, triglycerides, and total proteins showed no changes over the broad range of handling times (10–232 min) in our study. The only blood biochemical parameter affected by handling time was glucose, which increased significantly after 150 min of capture, meaning an increase of 13.4% (37 mg/dL). Other studies have reported increases in glucose

TABLE 2. Reference values reported for female and male Two–banded Plovers (*Charadrius falklandicus*) captured in northern Patagonia, Argentina, during the nonbreeding season 2014–15. Sample sizes differ among measured parameters because blood volume was insufficient for all measurements in the subset of individuals with known sex.

	Females			Males		
Parameter	*n*	Mean±SE	Median (minimum–maximum)	*n*	Mean±SE	Median (minimum–maximum)
Body mass (g)	25	62.9±0.8	62 (56–73)	26	64.2±0.4	64 (58–69)
Wing length (mm)	20	125±0.1	126 (120–130)	24	126±0.05	126 (120–132)
Bill length (mm)	20	18.4±0.1	18.4 (17.1–19.6)	24	18.7±0.1	18.5 (17.2–20.6)
Packed cell volume (%)	13	49.9±1	50 (43–54)	21	52.4±0.9	52 (46–62)
Glucose (mg/dL)	15	276.3±18.1	259 (211–461)	14	310.9±14.2	312 (244–422)
Triglycerides (mg/dL)	10	90.8±10.3	70 (68–153)	12	88.5±8.1	70 (65–138)
Cholesterol (mg/dL)	3	185.7±11.2	176 (173–208)	4	218±19.2	221 (176–254)
Total protein (g/dL)	11	5.9±0.2	5.9 (4.9–7.0)	14	5.4±0.1	5.5 (4.8–6.2)
Total white blood cells	14	28.6±2	28.5 (17–40)	13	27.3±2.9	27 (11–50)
Total lymphocytes	14	14.9±1.3	14.9 (6.1–22.4)	13	13.1±1.2	12.7 (6.5–20.7)
Total heterophils	14	8.2±0.8	7.9 (1.6–44.1)	13	8.01±1.3	7.7 (1.8–18.5)
Total eosinophils	14	3.1±0.6	2.7 (0–7.8)	13	3.8±1.2	1.8 (0.5–15.2)
Total monocytes	14	2±0.3	2 (0–4.8)	13	1.9±0.4	1.6 (0.2–5.5)
Total basophils	14	0.4±0.1	0.3 (0–1.7)	13	0.4±0.2	0 (0–2.1)
% Lymphocytes	14	52.3±1.2	54 (27–69)	13	50.2±3.6	53 (26–73)
% Heterophils	14	28.6±2.1	27.5 (17–43)	13	29±3.3	30 (7–45)
% Eosinophils	14	10.6±2	10 (1–24)	13	12.5±2.9	8 (3–35)
% Monocytes	14	7.1±1.1	7 (0–13)	13	6.5±0.8	6 (1–13)
% Basophils	14	1.6±0.5	1 (0–6)	13	1.2±0.6	0 (0–6)
Heterophil/lymphocyte ratio	14	0.6±0.1	0.6 (0.1–1.6)	13	0.6±0.1	0.6 (0.1–1.5)
Bacterial agglutination titer	2	5.8±0.3	5.8 (5.5–6)	2	4.3±0.6	4.3 (3.6–5)

levels following capture and handling in birds (Scope et al. 2002; Corbel et al. 2010; Davies et al. 2013), with timing of changes ranging from 15 min to >60 min after capture. Thus, our results in Two-banded Plovers suggest that measures of aerobic capacity (indexed by packed cell volume) and nutritional biochemistry (except for glucose levels) are insensitive to capture and handling stress (of up to >2.5 h) and can be informative even if blood samples cannot be obtained within minutes of capture.

Among measured immune parameters, total white blood cell counts, total lymphocyte counts, and total and percentages of eosinophils showed decreases with handling times between 60 min and 150 min of capture (Fig. 2). These results are consistent with other reports. For example, white blood cells decreased in response to handling over 1 h of capture in House Finches (*Carpodacus mexicanus;* Davis 2005). Similarly, total white blood cells decreased within 60–90 min of handling stress in Red Knots (Buehler et al. 2008). Other stressful events such as transport >1 h can induce a decrease in total white blood cells of wild birds (Parga et al. 2001). Decreased lymphocyte and eosinophil counts within 60–120 min of handling have been reported for Great Tits (*Parus major*; Cïrule et al. 2012).

In general, heterophils tend to increase and lymphocytes decrease in response to several stressors, suggesting H/L ratios are a good index of stress in birds and other vertebrates (Davis et al. 2008). The H/L ratio increases in response to a wide variety of stressful situations including long-distance migration (Owen and Moore 2006), parasitic infection (Lobato et al. 2005), and reduced nutrition

(Davis et al. 2000). Nevertheless, changes in H/L ratios do not occur immediately after capture. Davis (2005) found that H/L ratios did not increase significantly within 1 h of capture in House Finches and Cïrule et al. (2012) reported increased heterophil and decreased lymphocyte counts leading to increased H/L ratios 60–120 min after capture of Great Tits. Remarkably, Two-banded Plovers did not show changes in heterophil counts and percentages or in the H/L ratio even with handling times up to 232 min. However, we documented a clear decrease in the percentage and number of eosinophils that became significant 90–150 min postcapture. Apparently eosinophils, in addition to being indicators of macroparasite infections, can serve as indicators of stress in some cases, manifest as decreased levels (Davis et al. 2008). Jain (1986) suggested decreases in eosinophil numbers might be more related to stress than to disease at least in some species. Thus, our results, together with those from previous studies, suggest that immune parameters (i.e., leukocyte profiles) are sensitive to capture and handling stress but can be informative provided blood samples are obtained within 1 h of capture of birds.

In summary, many relevant blood physiologic parameters of health, nutrition, and immune function are not affected by handling times of up to 60 min (and in many cases longer periods). Packed cell volume and blood nutritional parameters (except for glucose levels) appear to be less sensitive to handling stress than leukocyte profiles. Therefore, inclusion of blood physiologic parameters should not be discouraged in studies involving species that cannot easily be sampled in a few minutes. Although researchers should always try to minimize handling times and evaluate their effects on the parameters of interest, our results suggest that useful data can be obtained if blood samples are collected within 1 h of capture. We have provided physiologic parameters related to health, immune function, and general body condition for Two-banded Plovers. Values can be viewed as representing apparently healthy adults during the nonbreeding season and can serve as reference for continued monitoring of these Patagonian populations and for comparison to other populations and shorebird species.

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