Running tittle: Physiology and handling stress in a migrant shorebird

Physiological parameters and their response to handling stress in a neotropical migratory shorebird during the non-breeding season

Verónica L. D'Amico [1, 5], María G. Palacios [1], Allan J. Baker [2], Patricia M. González [3], Enrique Madrid [4] and Marcelo Bertellotti [1]

[1] CESIMAR, Centro Nacional Patagónico, Bvrd. Brown 2915, Puerto Madryn CP 9120, Chubut, Argentina. [2] Royal Ontario Museum, Toronto, Canada. [3] Fundación Inalafquen, San Antonio Oeste, Río Negro, Argentina. [4] Laboratorio de Vertebrados y Laboratorio de Genética, Universidad Nacional de Mar del Plata, Argentina. [5] Corresponding author: E-mail: damico@cenpat-conicet.gob.ar

Word count (from Introduction to Discussion): 2906

Abstract:

Physiological traits are promising indicators of population health in the face of rapid changing environments. We obtained values of diverse physiological parameters for Two-banded Plovers (*Charadrius falklandicus*) in coastal sites in Patagonia, Argentina, with the objective 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 patagonian 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 minutes after capture. The remaining parameters did not manifest significant alterations in response to capture and handling of up to 232 minutes (average = 105.2 ± 56.7). Therefore, although researchers should attempt to obtain blood samples as soon as possible after capture, inclusion of physiological parameters in monitoring studies of species not easily sampled in a few minutes, as Two-banded Plovers and other shorebird species during migration, should not be discouraged. Data presented here constitute the first physiological report for the species and can be considered as reference values during the non-breeding season at Patagonian coastal sites.

Key words: BIOCHEMISTRY; HEMATOLOGY; IMMUNITY; PATAGONIA; TWO-BANDED PLOVERS

Introduction

The use of physiological 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). Physiological traits at the individual level are promising indicators of population health and can signal a problem even before demographic consequences can be observed (Carey 2005; Wikelski and Cooke 2006). An obstacle to using physiological 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 some shorebird species captured in large flocks during migration or winter (Buehler et al. 2008). For these taxa, it is essential to understand the timeline in which different physiological parameters (e.g., immune, nutritional, hormonal, general body condition indices) become affected by the stress of capture and handling, as some parameters can be highly sensitive, changing within minutes of capture, while others might not show significant alterations for hours (Buehler et al. 2008, Davies 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 minutes 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 2002, for a review see Davis et al. 2008). In turn, glucocorticoids affect other physiological 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 physiological 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 2015). The inclusion of physiological 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 non-reproductive season, usually involves the use of cannon nets (Kasprzyk and Harrington 1989). This capture method can trap a large number of individuals simultaneously (dozens to hundreds), which can be ideal for banding programs but presents a challenge for physiological monitoring, given that birds need to be kept in shaded cages until sampled, sometimes for hours after capture. Thus, knowledge on the timeline in which the stress of capture and handling affects diverse physiological parameters can help identify those 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 physiological 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 physiological reference values that are important as baseline 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 in between 25,000 and 100,000 individuals (BirdLife International 2012). Trends in abundance in Patagonia, Argentina, are currently unknown, with just a few reports for some local populations (Gonzalez 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 of non-breeding Two-banded Plovers captured at feeding sites in northern Patagonia.

Materials and Methods

Birds were sampled at two feeding sites, Bahía San Antonio (Río Negro) and Peninsula Valdés (Chubut), covering a range between 40ºS, 65ºW- 42ºS, 64ºW during the non-breeding 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 (Kasprzyk and Harrington 1989). All captured individuals were kept in shaded cages placed on the sand that were continuously wetted to avoid overheating until sampled (Kasprzyk and Harrington 1989). Birds were weighed with an analytical balance (+/- 0.01 g), bill length was measured with a caliper (+/- 0.1 mm) and wing length with a ruler to the nearest mm. Blood samples (0.3 to 0.35 ml) were obtained from the brachial vein using 27G needles and collected into heparinized microcapillary tubes (Tecnon, Argentina) that were stored at 4ºC until analyses. Thin blood smears were prepared with a drop of fresh blood, air dried, fixed with absolute ethanol for 3 min and stained with *Tinción 15* (Biopur S.R.L., Rosario, Argentina). Time expressed as 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 following Narosky and Yzurieta (2010) and their body mass (Wiersma et al. 2016). No signs of illness or poor health were noticed based upon plumage brightness and absence of both ectoparasites and feather damage. Individuals did not present evidence of molting. All birds were released at the site of capture after sampling.

Blood was centrifuged at 12,000 g for 12 min (Cavour VT 1224, Argentina) and packed-cell volume was measured with a microhematocrit ruler (J.P. Selecta, Spain). Packed-cell volume is considered 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 the 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 1000x (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 measure of stress (Davis et al. 2008) was calculated from the corresponding leukocyte counts.

In order 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, Argentina). Quality control

147 was based on Levy-Jennings plots of the average value of dispersion for both methods:
148 Biuret reaction for total proteins and enzymatic for lipids and carbohydrates. These
149 biochemical parameters contribute to the assessment of body condition and nutritional status
150 of birds (D'Amico et al. 2010).
151 Agglutination of *Escherichia coli* (ATCC 8739) by plasma components, an index of
152 constitutive humoral innate immunity, was measured following a protocol by Sahoo et al.
153 (2008) that we adapted for use in shorebirds. Briefly, bacteria were grown in tryptic soy (TS)
154 broth and then fixed in 1% formalin overnight at 4ºC. Fixed bacteria were washed three times
155 with phosphate buffered saline (PBS) and adjusted to a concentration of approximately 1 x
156 10^9 bacteria/ml. Plasma samples (15 µl) were added to the first column of a 96-well plate and
157 serially diluted two-fold with PBS. A negative control (PBS) was included in each plate. Then
158 15 µl of fixed bacteria were added to all wells. Plates were vortexed and incubated at 40ºC
159 overnight. Agglutination titers were determined as –log2 of the highest dilution showing
160 bacterial agglutination. Inter-plate variation, calculated as the coefficient of variation (CV),
161 was 4.6%.
162 Two-banded Plovers are not sexually dimorphic, so sex was molecularly determined for a
163 random subset of individuals for which a portion of blood had been preserved in ethanol (n =
164 51) following the protocol described in Fridolfsson and Ellegren (1999). Briefly, avian sex
165 DNA marker amplification was performed using polymerase chain reaction (PCR)-based
166 methods (Bioer Life Express Thermal Cycler, China) using specific oligonucleotide primer
167 2550F and 2718R (manufactured by Invitrogen life technologies). PCR products were
168 compared to 100 bp DNA ladder. Males were identified as displaying a single PCR product
169 (from CHD 1Z, 600 bp) while females showed two PCR products (from CHD 1W, 450 bp
170 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 physiological parameters were evaluated using Spearman rank correlations that included data for all individuals captured (i.e., males, females, and individuals of unknown sex). For variables affected by handling time, we then analyzed the changes over time following protocols described in 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 (i.e., 0-30, 31-60, 61-90, 91-120, 121-150, and > 150 min) and tested for differences among intervals by using Dunn's multiple comparisons *post hoc* tests (Sokal and Rohlf 2012). Mann-Whitney 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. Since 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 version 7.0 and significance is reported using an alpha of 0.05.

Results

A total of 137 individuals were captured and of these 119 were bled. Time between capture and blood draw ranged from 10 to 232 minutes, with an average of 105.2 ± 56.7 minutes. 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 close to 1:1, consisting of 51 % males and 49 % females. Body mass was slightly greater for males than for females (Mann-Whitney U = 220, P = 0.046, Table 2), whereas bill and wing length did not vary

between sexes. Glucose level was the only physiological parameter that was correlated with body mass (rho= 0.27, $P < 0.0063$) and differed between sexes, being greater for males than for females (Mann-Whitney $U = 58.5$, $P = 0.042$, Table 2). Sexes did not differ in handling time or in any of the remaining physiological 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 to 232 min, all $P > 0.05$). Glucose levels increased with handling time (rho = 0.35, $P = 0.0004$, $n = 101$), with significant changes detected after 150 minutes of capture of birds (Fig. 2A). Total white blood cell counts decreased after 60 min of capture of birds (rho = -0.30, $P = 0.002$, $n = 100$, Fig. 2B). Total counts of lymphocytes also showed changes with handling time (rho = -0.26, $P = 0.008$, $n = 100$); decreased values were manifested at 2 points, one at the 61-90 min interval and the other at 121-150 min of capture of birds (Fig. 2C). Total and percentage of eosinophils showed changes with handling time (rho = -0.56, $P < 0.0001$, $n = 100$ and rho = -0.50, $P < 0.0001$, $n = 100$, respectively), decreasing significantly after 90 and after 150 minutes of capture of birds, respectively (Fig. 2D and 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 the present study showed a wider range (53-73 g) than previously reported for adults in this species (62-72 g, Wiersma et al. 2016). Males were on average slightly heavier than females, but body mass ranges overlapped completely. In addition, sexes did not differ in bill and wing length, and together with the lack of plumage differences between males and females, highlight the need to use molecular data to

discriminate gender. Body mass and glucose levels were the only two parameters that showed slight, but significant differences between sexes in the subset of birds that were sexed using DNA, both being higher in males than females. However, future studies should increase sample sizes as some of the physiological variables (particularly cholesterol level and agglutination titer) were very small.

Packed cell volume ranged between 43 and 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, triglyceride, and glucose levels 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 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) compared to those of Red Knots (subspecies *rufa*) sampled at the same study site (0.95 ± 0.3, D'Amico et al. 2010). Although there is interspecific variation, in general eosinophils, together with 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 in this study 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 (Table 1), which suggests that birds were not exhibiting high levels of chronic stress at the site. For example, studies in gulls 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 exposed to oil spills. Regarding the potential effects of handling time on physiological parameters, packed cell volume and blood levels of cholesterol, triglycerides, and total proteins showed no changes over the broad range of handling times (10 to 232 minutes) in this study. The only blood biochemical parameter affected by handling time was glucose level, which increased significantly after 150 minutes of capture, which means an increase of 37 mg/dl in average. Other studies have reported increases in glucose levels as a consequence of capture and handling in birds (Scope et al. 2002, Corbel et al. 2010, Davies et al. 2013), with timing of changes ranging from 15 to more than 60 minutes 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 fairly insensitive to capture and handling stress (of up to more than 2.5 hours) and can thus be informative even if blood samples cannot be obtained within minutes of capture.

Among the measured immune parameters, total white blood cell counts, total lymphocyte counts and total and percentages of eosinophils showed decreases with handling time between 60-150 minutes since capture (Fig. 2). These results are consistent with other reports. For instance, white blood cells decreased in response to handling over 1h of capture in House Finches (*Carpodacus mexicanus,* Davis 2005). Similarly, total white blood cells decreased within 60-90 min of handling stress in another shorebird species, the Red Knot (*Calidris canutus,* Buehler et al. 2008). Other stressful events such as transportation over 1h can also induce a decrease in total white blood cells of wild birds (Parga et al. 2001). Decreased lymphocyte and eosinophil counts as a result of handling stress between 60 and 120 minutes have been reported for Great Tits (*Parus major,* Cïrule et al. 2012).

In general, values of heterophils tend to increase and lymphocytes decrease in response to several stressors, constituting thus the H/L ratio a good index of stress in birds and other vertebrates (Davis et al. 2008). For instance, 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). Changes in H/L ratio do not occur immediate after capture nevertheless. For example, Davis (2005) found that H/L ratios did not increase significantly within 1h of capture in House Finches and Cïrule et al. (2012) reported increased heterophil and decreased lymphocyte counts leading to increased H/L ratios between 60 and 120 minutes after capture of Great Tits. It is interesting to note that 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 minutes. On the other hand, we documented a clear decrease in the percentage and number of eosinophils that became significant after 90-150 min of capture. It is apparent that eosinophils, in addition to being indicators of macroparasite infections, can also serve as indicators of stress in some cases, manifesting their effect as decreased values (Davis et al. 2008). In fact, it has been argued that decreases in eosinophil numbers might be more related to a stress reaction than to a response to disease at least in some species (Jain 1986). 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 1h of capture of the birds. In summary, many relevant blood physiological parameters of health, nutrition, and immune function are not affected by handling times of up to 60 minutes (and in many cases longer periods). Packed-cell volume and blood nutritional parameters (except for glucose levels) seem to be less sensitive to handling stress than leukocyte profiles. Therefore, inclusion of blood physiological 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 1h of capture. This study provides the first report on physiological 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 non-breeding season and can serve as reference for continued monitoring of these patagonian populations and for comparison to other populations and shorebird species.

Acknowledgments:

We dedicate this work to one of the authors Allan Baker for their invaluable involvement in the study of shorebirds in Argentina. Now, he's flying around. We thank all people that helped us in the field, specially the Eco Huellas group, Mirta Carbajal, and Guardias Ambientales from Río Negro. We also thank the reviewers for their suggestions that helped improve our manuscript. For local arrangements and permits we thank Secretaría de Ambiente y Desarrollo Sustentable de Río Negro and Dirección de Flora y Fauna, and Subsecretaría de Conservación de Áreas Protegidas de Chubut. VLD, MB and MGP are members of CONICET. This contribution was supported by PICT-B 1053-2013 to V. D'Amico.

Literature Cited:

Apanius V. 1998. Stress and immune defense. *Adv Stud Behav* 27:133-153.

Averbeck C. 1992. Hematology and blood chemistry of healthy and clinically abnormal great blackbacked gulls (*Larus marinus*) and herring gulls (*Larus argentatus*). *Avian Pathol* 21:215-223.

Beldomenico PM, Telfer S, Gebert S, Lukomski L, Bennett M, Begon M. 2008. The dynamics of health in wild field vole populations: a haematological perspective. *J Anim Ecol* 77:984-997.

BirdLife International. 2012. *Charadrius falklandicus*. The IUCN Red List of Threatened Species 2012: e.T22693852A38771080. http://dx.doi.org/10.2305/IUCN.UK.2012-1.RLTS.T22693852A38771080.en. Assessed October 2015.

Buehler DM, Bhola N, Barjaktarov D, Goymann W, Schwabl I, Tieleman IB, Piersma T. 2008. Constitutive immune function responds more slowly to handling stress than corticosterone in a shorebird. *Physiol Biochem Zool* 81:673-681.

Campbell TW. 1995. *Avian Hematology and Cytology*. Iowa State University Press, Ames, Iowa, 104 pp.

Carey C. 2005. How physiological methods and concepts can be useful in conservation biology. *Int Comp Biol* 45:4–11

Carpenter FL. 1975. Bird hematocrits: effects of high altitude and strength of flight. *Comp Biochem Phys A* 50: 415-417.

Cïrule D, Krama T, Vrublevska J, Rantala MJ, Krams I. 2012. A rapid effect of handling on counts of white blood cells in a wintering passerine bird: a more practical measure of stress*? J Ornithol* 153:161–166

Cooke SJ, Sack L, Franklin CE, Farrell AP, Beardall J, Wikelski M, Chown SL. 2013. What is conservation physiology? Perspectives on an increasingly integrated and essential science. *Conserv Physiol* 1:cot001.

Corbel H, Geiger S, Groscolas R. 2010. Preparing to fledge: the adrenocortical and metabolic responses to stress in king penguin chicks. *Funct Ecol* 24:82-92.

D'Amico VL, Bertellotti M, Baker AJ, González PM. 2010. Hematological and plasma biochemistry values for endangered red knots (*Calidris canutus rufa*) at wintering and migratory sites in Argentina. *J Wildl Dis* 46:644-648.

Davies S, Rodriguez NS, Sweazea KL, Deviche P. 2013. The effect of acute stress and long-term corticosteroid administration on plasma metabolites in an urban and desert songbird. *Physiol Biochem Zool* 86:47-60.

Davis GS, Anderson KE, Carroll AS. 2000. The effects of long-term caging and molt of single comb white leghorn hens on heterophil to lymphocyte ratios, corticosterone and thyroid hormones. *Poultry Sci* 79:514-518.

Davis AK. 2005. Effects of handling time and repeated sampling on avian white blood cell counts. *J Field Ornithol* 76:334-338.

Davis AK, Maney DL, Maerz JC. 2008. The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. *Funct Ecol* 22:760-77.

Dietz MW, Jenni-Eiermann S, Piersma T. 2009. The use of plasma metabolites to predict weekly body-mass change in red knots. *Condor* 111:88-99.

Ellis RD, McWhorter TJ, Maron M. 2012. Integrating landscape ecology and conservation physiology. *Landsc Ecol* 27:1-12.

Fair J, Whitaker S, Pearson B. 2007. Sources of variation in haematocrit in birds. *Ibis* 149:535-552.

Fridolfsson AK, Ellegren H. 1999. A simple and universal method for molecular sexing of non-ratite birds. *J Avian Biol* 30: 116-121.

González PM. 2013. *Registros históricos de Aves Playeras en Bahía San Antonio y Río Grande, Patagonia Argentina. Informe Abril 2013*. Programa de Subsidios de la Ley para la Conservación de las Aves Migratorias Neotropicales. The Shorebird Recovery Project for Patagonia, South America 19 pp.

Hevia G. 2013. *Éxito reproductivo del Chorlo de Doble Collar (Charadrius falklandicus) y recomendaciones para el manejo de su población en dos áreas protegidas próximas a Puerto Madryn, (Chubut, Argentina)*. Magister Thesis, Manejo de Vida Silvestre, Centro de Zoología Aplicada, Universidad Nacional de Córdoba, Argentina, 82 pp.

Jain NC, Editor.1986. The hematopoietic system. In: *Schalm's Veterinary Hematology* 4th edition, Lea and Febiger, Philadelphia, p 350.

Jenni L, Müller S, Spina F, Kvist A, Lindström A. 2006. Effect of endurance flight on haematocrit in migrating birds. *J Ornithol* 147:531-542.

Kasprzyk M, Harrington B. 1989. Manual de campo para el estudio de playeros. - Manomet Bird Observatory, Massachusetts, USA.

Klaassen M, Hoye BJ, Nolet BA, Buttemer WA. 2012. Ecophysiology of avian migration in the face of current global hazard. *Proc R Soc B* 367:1719-1732.

Lindström KM, Hawley DM, Davis AK, Wikelski M. 2005. Stress responses and disease in three wintering house finch (*Carpodacus mexicanus*) populations along a latitudinal gradient. *Gen Comp Endocr* 143:231-239.

Lobato E, Moreno J, Merino S, Sanz JJ, Arriero E. 2005. Haematological variables are good predictors of recruitment in nestling pied flycatchers (*Ficedula hypoleuca*). *Ecoscience* 12:27-34.

Madliger C.L., Love OP. 2015. The power of physiology in changing landscapes: considerations for the continued integration of conservation and physiology. *Integr Comp Biol.* doi:10.1093/icb/icv001.

Martin LB. 2009. Stress and immunity in wild vertebrates: Timing is everything. *Gen Comp Endocr* 163:70-76.

McEwen BS, Biron CA, Brunson KW, et al. 1997. Neural-endocrine immune interactions: the role of adrenalcorticoids as modulators of immune function in health and disease. *Brain Res Rev* 23:79-133.

Narosky T, Yzurieta D. 2010. *Aves de Argentina y Uruguay: guía de identificación*, 16a edition, Vazquez Mazzini editor, Buenos Aires, 432 pp.

Nisbet ICT, Tseng FS, Fiorello CV, Apanius V. 2015. Changes in white blood cell parameters of Common Terns (*Sterna hirundo*) exposed to low levels of oil. *Waterbirds* 38:415-419.

Owen JC, Moore FR. 2006. Seasonal differences in immunological condition of three species of thrushes. *Condor* 108:389-398.

Parga ML, Pendl H, Forbes NA. 2001. The effect of transport on hematologic parameters in trained and untrained Harris's Hawks (*Parabuteo unicinctus*) and Peregrine Falcons (*Falco peregrinus*). *J Avian Med Surg* 15:162-169.

Piersma T, Everaarts JM. 1996. Build-up of red blood cells in refuelling Bar-tailed godwits in relation to individual migratory quality. *Condor* 98:363-370.

Romero LM. 2004. Physiological stress in ecology: lessons from biomedical research. *Trends Ecol Evol* 19:249-255.

Romero LM, Reed JM. 2005. Collecting baseline corticosterone samples in the field: is under 3 min good enough? *Comp Biochem Phys A* 140:73-79.

Romero LM, Romero RC. 2002. Corticosterone responses in wild birds: the importance of rapid initial sampling. *Condor* 104:29-135.

Sahoo PK, Das Mahapatra K, Saha JN, Barat A, Sahoo M, Mohanty BR, Gjerde B, Ødegard J, Rye M, Salte R. 2008. Family association between immune parameters and resistance to *Aeromonas hydrophila* infection in the Indian major carp, *Labeo rohita*. *Fish Shellfish Immun* 25:163-169.

Scope A, Filip T, Gabler C, Resch F. 2002. The influence of stress from transport and handling on hematologic and clinical chemistry blood parameters of Racing Pigeons (*Columba livia domestica*). *Avian Dis* 46:224–229.

Sokal RR, Rohlf FJ. 2012. Biometry: the principles and practice of statistics in biological research, 4th edition, W. H. Freeman and Co., New York. 937 pp.

Thrall MA, Weiser G, Allison R, Campbell TW, editors. 2012.*Veterinary Hematology and Clinical Chemistry*, 2nd edition, Wiley-Blackwell, New York, 776 pp.

Wetlands International. 2015. *Waterbird population estimates*. Wetlands International, Wageningen, The Netherlands. Available from wpe.wetlands.org. Accessed October 2015.

Wiersma P, Kirwan GM, Boesman P. 2016. Two-banded Plover *(Charadrius falklandicus)*. In: *Handbook of the Birds of the World Alive*, del Hoyo J, Elliott A, Sargatal J, Christie DA, de Juana E editors. Lynx Editions, Barcelona

Wikelski M, Cooke SJ. 2006. Conservation physiology. *Trends Ecol Evol* 21:38–46.

Wingfield JC, Schwabl H, Mattocks PW Jr. 1990. Endocrine mechanisms of migration. In: *Bird migration: physiology and Ecophysiology*, Gwinner E, editor. Springer-Verlag, Berlin, pp. 232-256.

Wingfield JC, Kitaysky AS. 2002. Endocrine responses to unpredictable environmental events: stress or anti-stress hormones. *Integr Comp Biol* 42:600–609.

Woods RW, Woods A. 1997. Atlas of breeding birds of Falkland Islands. Anthony Nelson: Owestry, UK.

Table 1. Reference values for morphological and physiological parameters of adult Two-banded Plovers captured in northern Patagonia, Argentina during the non-breeding season. Sample sizes differ among measured parameters because blood volume was insufficient for all measurements in all captured birds.

	n	**Mean ± SE**	**Median (min-max)**
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)

H/L 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)

487

Table 2. Reference values reported for female and male Two-banded Plovers in northern Patagonia, Argentina during the non-breeding season. Note that sample sizes differ among measured parameters because blood volume was insufficient for all measurements in the subset of sexed individuals.

Parameter	**Females**			**Males**		
	n	**Mean ± SE**	**Median (min-max)**	**n**	**Mean ± SE**	**Median (min-max)**
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	14	28.6 ± 2	28.5 (17-40)	13	27.3 ± 2.9	27 (11-50)

cells

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)
H/L 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)

492

493 **Figure 1.** Location of study sites, Bahía San Antonio and Península Valdés, in northern
494 Patagonia, Argentina.
495
496 **Figure 2.** Physiological parameters of Two-banded Plovers that showed significant changes
497 as a function of handling times ranging between 10 and 232 minutes Timeline was divided
498 into 6 intervals of 30 minutes from capture. Graphs depict medians (dots) and interquartile
499 ranges (whiskers). Letters indicate results of *post-hoc* tests for multiple comparisons. Intervals
500 not sharing letters are significantly different from each other ($P < 0.05$). Sample sizes are
501 shown below each time interval for glucose levels (panel A) and for the four leukocyte
502 parameters (panel B).
503