Physiological parameters of a plover during nonbreeding and breeding in Patagonia, Argentina

Journal:	<i>Canadian Journal of Zoology</i>
Manuscript ID	cjz-2021-0095.R2
Manuscript Type:	Article
Date Submitted by the Author:	25-Aug-2021
Complete List of Authors:	D'Amico, Verónica; National Scientific and Technical Research Council, Ecofisiología Aplicada al Manejo y Conservación de la Fauna Silvestre Hevia, Glenda D.; National Scientific and Technical Research Council, Ecofisiología Aplicada al Manejo y Conservación de la Fauna Silvestre Walker, Brian; Fairfield University, Biology González, Patricia ; Inalafquen Foundation and South American Shorebird Coordinator International Conservation Fund of Canada, Río Negro, Argentina Bertellotti, Marcelo; National Scientific and Technical Research Council, Ecofisiología Aplicada al Manejo y Conservación de la Fauna Silvestre
Is your manuscript invited for consideration in a Special Issue?:	Not applicable (regular submission)
Keyword:	blood parameters, neotropical shorebird, nonbreeding and breeding, Patagonian sites, Two-banded plovers, <i>Charadrius falklandicus</i>

SCHOLARONE™
Manuscripts

Physiological parameters of a plover during nonbreeding and breeding in Patagonia, Argentina

Running title: Physiological parameters of a Patagonian plover

Verónica L. D’Amico ¹, Glenda D. Hevia ¹, Brian Walker ², Patricia M. González ³, Marcelo Bertellotti ^{1,4}

¹ Laboratory of Ecophysiology Applied to Management and Conservation of Wildlife. Center of Studies of Marine Systems, National Scientific and Technical Research Council-Argentina, Puerto Madryn, Chubut, Argentina.

² Department of Biology, Fairfield University, Fairfield, CT, USA

³ Inalafquen Foundation and South American Shorebird Coordinator International Conservation Fund of Canada, Río Negro, Argentina.

⁴ Faculty of Production, Environment and Sustainable Development, University of Chubut, Puerto Madryn, Chubut, Argentina.

Correspondence to: damico@cenpat-conicet.gob.ar

21 Abstract

22 Migratory shorebirds are of the most threatened avian species due to effects of human
23 activities and climate change. Physiological parameters are useful to evaluate the health status
24 of free-living animals. Here we investigated lymphocytes, heterophils,
25 heterophils/lymphocytes, and glucose for female and male Two-banded plovers (TBPLs),
26 *Charadrius falklandicus* (Latham, 1790) during nonbreeding (body moult) and breeding
27 (incubation), in coastal areas of northern Patagonia Argentina. We also measured
28 corticosterone for first time for the species during breeding. Nonbreeding TBPLs were more
29 likely to have higher physiological measurements that were not dependent on body moult nor
30 body condition, being similar between sexes. Differences found in glucose are likely related
31 to recent feeding in nonbreeding birds compared with fasting during incubation. The
32 gregarious nonbreeding behaviour in comparison with the isolated breeding pairs, would
33 increase the risk of acquiring parasites and /or pathogens inducing a response of the immune
34 system reflected in higher values of lymphocytes. Baseline corticosterone during breeding
35 was similar between sexes and were lower compared with other values reported for breeding
36 plovers. TBPLs undergo energetically demanding physiological changes during the
37 nonbreeding stage, strengthening the idea that conservation of the environmental resources at
38 these locations may be more important than previously thought.

39
40
41 **Keywords:** blood parameters, neotropical shorebird, nonbreeding and breeding, Patagonian
42 sites, Two-banded plovers *Charadrius falklandicus*

44 Introduction

45 In recent decades, bird populations worldwide have experienced declines thought to
46 occur due to rapid, human-driven environmental change (Spooner *et al.* 2018). Examining
47 differences in physiological parameters in wildlife affected by human activities has become
48 increasing common, as such parameters are very useful indicators of the health status of free-
49 living animals (Cooke *et al.* 2013, Palacios *et al.* 2018, D'Amico and Bertellotti 2019).

50 Migratory shorebirds are significantly vulnerable species, mainly due to human
51 impacts that affect multiple stages in their life cycles (Wetlands International 2018). For wild
52 migratory shorebird species in South America, how physiological patterns are modified due to
53 human disturbances are relatively undescribed in the literature. Haematological
54 characteristics of wintering long distance migrants at Tierra del Fuego and at stopover sites
55 along their migratory flyway have been described in Red knots (*Calidris canutus rufa*,
56 Linnaeus, 1758) and White-rumped sandpipers (*Calidris fuscicollis*, Vieillot, 1819) (D'Amico
57 *et al.* 2010, 2016). However, there is limited information on endemic shorebird species,
58 especially during the breeding season.

59 The Two-banded plover (*Charadrius falklandicus*, Latham, 1790) (TBPL, hereafter) is
60 a neotropical shorebird species, endemic to southern South America. TBPLs breed mostly in
61 coastal areas, but also inland in Patagonia. During nonbreeding, it is known that they fly a
62 short northward migration, reaching as far north as Uruguay, Rio Grande do Sul, and
63 exceptionally Sao Paulo, Brazil (Wiersma *et al.* 2020). Among shorebird species using South
64 America sites, TBPLs can be easily found at northern Patagonian beaches throughout the
65 year, during both nonbreeding (from February-March to August) and breeding (mid-
66 September to early January) seasons.

67 During the nonbreeding season, choosing habitat (either foraging or roosting),
68 obtaining food resources, and predation pressures are limiting factors for plovers, all of which

can greatly affect overwinter survival (Nol 2019). Moults are also important during nonbreeding, a process that is known to be a significant energetic drain (Newton 1968, review in Alerstam and Lindström 1990, Lindström *et al.* 1993, Murphy 1996). During breeding, significant energetic resources are also necessary to insure successful reproduction (Stenzel and Page 2019). For shorebirds nesting on open beaches, energy for reproduction can also be greatly affected by human activities, as evidenced by American Oystercatchers (*Haematopus palliatus*, Temminck, 1820) spending more time and energy on vigilance rather than incubating eggs on the nest in disturbed areas (e.g., Borneman *et al.* 2016). Even in cases where breeding birds do not show obvious behavioural signs of being disturbed by humans, there is evidence that physiology can still be affected, such as variation in immune function, nutritional condition and stress hormone levels (e.g., Brown 1996, Walker *et al.* 2006, Gibson *et al.* 2018). All these effects – obvious and less conspicuous – could result in reduced breeding success, and, ultimately, population declines (Pruner *et al.* 2011, Borneman *et al.* 2016).

Haematological parameters such as leucocyte counts (specifically lymphocytes (L) and heterophils (H)) provide a reliable tool to assess several functions and processes of the immune system (Davis *et al.* 2008). As such, they have frequently been used to assess the immunological impact of environmental stressors or investment in stressful activities (Davies and Maney 2018). More specifically, the H/L ratio is commonly used in a conservation and wildlife management perspective (Deikumah *et al.* 2015). During stress, these cells become mobilized and are shunted to different body compartments, depending on their function (Dhabhar *et al.* 1996). Shifts in circulating lymphocytes and heterophils occur after glucocorticoids peak (Davies and Maney 2018).

Lymphocytes are involved in immunological functions, for instance, as natural killer cells, taking part in cell-mediated innate immune defence against virally infected cells.

Lymphocytes also play a role in cell-mediated (T cells) and humoral (B cells) adaptive immunity, being responsible for recognizing specific pathogenic antigens (Davis *et al.* 2008). Heterophils are the main cells of innate cellular defence against pathogens, and they contribute to the inflammatory response, displaying killing mechanisms such as phagocytosis, cellular degranulation or oxidative burst (Genovese *et al.* 2013).

In addition to internal pathogens, both types of leukocytes also can respond to external stressors. It has been shown that in stressful environments such as severe climate change or habitat modifications, the number of heterophils increases, while the number of lymphocytes in peripheral circulating blood decreases (see review in Davis *et al.* 2008). Consequently, the H/L has been proposed as a measure of stress, with high ratios indicating stronger stress responses (examples in Davis *et al.* 2008, Davis and Maney 2018).

Adding to the immunological changes associated with stress, glucose levels in birds, which are directly related to energetic resources during a stressful process, may also be altered (Davies *et al.* 2013). Normally, glucose concentration reflects the actual level of carbohydrate ingestion and increases or decreases depending on the satiation of an individual. Therefore, glucose levels are regulated by pancreatic hormones and are maintained within a relatively narrow range to avoid hyper and hypoglycaemia (Whittow 2000). However, during stressors such as poor food conditions, habitats of low quality or inclement weather conditions, modified endocrine responses can affect glucose concentration in the blood stream (Remage-Healey and Romero 2001, Cockrem 2007).

Finally, in migrating birds, baseline levels of glucocorticoids (corticosterone in birds) play a role in adjusting physiology to meet general awareness to the potentially unpredictable environmental changes during migration, stopovers, and breeding (Piersma *et al.* 2000, Remage-Healey and Romero 2001, Landys-Ciannelli *et al.* 2002). Increased corticosterone reflects energy demands that could result in behaviour modifications such as changes in

feeding strategies (Wingfield *et al.* 1990), aggression (Kitaysky *et al.* 2003) and/or nest and parental care abandonment (Silverin 1998). When stressors are longer corticosterone is secreted to keep the blood glucose level constant to nourish the muscles, the heart and the brain, and a series of responses as those mediated by leukocytes, to ensure the renewal of reserves for the organism (Duval *et al.* 2010).

Since avian physiological investment during energetically costly periods are reflected in measurable changes in biological parameters, we investigated if values of lymphocytes, heterophils, their ratio (heterophils/lymphocytes - H/L), and glucose show different patterns during both nonbreeding and breeding seasons in TBPLs in Patagonia Argentina. We also added measures of corticosterone, but only during the breeding season. As breeding individuals must spend resources on vigilance, incubating eggs (they have a biparental care), and feeding, as well as being potentially subjected to significant impact of human activities since they nest on urban beaches, we expected physiological parameters indicative of stress to be elevated in breeding TBPLs, as compared to nonbreeding birds.

Sites used by shorebirds are suffering important modifications mainly as result of human activities, which could have potential impacts on physiology of birds with lasting effects on survival (Cooke *et al.* 2013, Gibson *et al.* 2018, Nol 2019). Therefore, these types of comparative evaluations, during both nonbreeding and breeding, can provide useful information for conservation management of sites and bird species.

Materials and Methods

Our sampling area was in northern Patagonia Argentina, between 40°- 42°S and 64°- 65°W, including beaches along Natural Protected Area Bahía de San Antonio (Río Negro province), Península Valdés and Puerto Madryn (Chubut province) (Figure 1). During the early nonbreeding stages (April 2015), we captured individuals using a cannon net launched

over a flock of birds resting at high tide on the beach. Captured individuals were kept in cages out of the sun and wind to avoid stress for overheating until blood samples were obtained (Kasprzyk and Harrington 1989). As physiological parameters can be dependent on moult condition (Moreno *et al.* 2001), prior to release, we registered body molt intensity (BMI) on breast feathers of birds, scoring from (no molting feathers); 1 (a few growing feathers); 2 (more than a few, less than a third); 3 (about a third); 4 (over half to 100% feathers in active molt) (Baker *et al.* 1998). The progress of primary moult (PM) was measured under the Newton (1966) system, summing scores from each feather giving a total moult score from 0 = all old feathers, to 50 = all new feathers. During breeding months (September to December 2016 and 2017) incubating adults (with up to three eggs, the maximum for this species) were captured on their nest using a rectangular trap of 40x20x20 cm. Because body condition can explain expression of physiological parameters as result of environmental changes in resources, stages of a shorebird annual cycle or illness, body mass (± 1 g), flattened wing cord (± 1 mm) and bill length (± 1 mm) were also registered during both seasons. A body condition index, calculated as the residuals of a linear regression of body mass on wing and bill measurements, was built to account for differences in structural size of the TBPL. Birds were immediately released at the site of capture after sampling. All individuals captured in this study showed no external signs of illness based on brightness of plumage, good flight capacity, and absence of feather damage from ectoparasites.

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 4°C until laboratory 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).

It is reported in this species during early stages of nonbreeding that significant alterations in physiological parameters were shown after 232 minutes of capture and handling (D'Amico *et al.* 2017). As such, blood samples were taken on average by 114 min ($SD = 58$, min = 10, max = 230) from cannon netting to bleeding. During breeding, samples were obtained on average at 2.4 min ($SD = 1$, min = 1, max = 5) after the time of trapping on the nest. However, elapsed time between capture and bleeding (handling time) was registered during both seasons to investigate the potential effect on the physiological parameters measured.

Corticosterone was only measured during breeding, as the capture technique during nonbreeding did not allow for the rapid collection of blood samples that is needed for accurate quantification of hormone levels before disturbance. Baseline corticosterone samples from breeding birds were all collected within five minutes of capture off of the nest (mean = 2, $SD = 0.5$, min = 1, max = 4) as it was reported that corticosterone does not modify values within the first three minutes after captured (Romero and Romero 2002, Romero and Reed 2005).

In the laboratory, microcapillary tubes were centrifuged at 12,000 g for 12 min (Cavour VT 1224, Argentina) and plasma and red blood cell portions were separated. Smears were examined under a light microscope scanning monolayer fields with similar densities of erythrocytes to obtain counts of heterophils and lymphocytes, after which the H/L ratio was calculated (Davis *et al.* 2008). Glucose concentration (mg/dL) was read using a spectrophotometer (Metrolab 1600 Plus, Argentina). Plasma levels of corticosterone were measured by a single radioimmunoassay following published procedures (Wingfield and Farner 1975). Briefly, corticosterone (ng/mL) was measured in duplicate from plasma after extraction with freshly re-distilled dichloromethane. We equilibrated all samples with approximately 2,000 cpm of tritiated corticosterone as an internal standard for determination of recovery following extraction, then added corticosterone antiserum to all samples. We

193 separated bound and free hormone by addition of dextran coated charcoal. Average recovery
194 value was 87.9%, while intra-assay variation was 11.9%, measured as the CV from standards
195 placed in all spins of the assay. Sensitivity of the assay was approximately 0.1 ng/mL.

196 TBPLs are not sexually dimorphic, therefore, sex was molecularly determined
197 following the protocol described for wader species by van der Velde *et al.* (2017). Briefly,
198 avian sex DNA marker amplification was performed using (PCR)-based methods (Bioer Life
199 Express Thermal Cycler, China) using specific oligonucleotide a primer set 2602F/2669R.
200 PCR products were compared to 100 bp DNA ladder. Males were identified as displaying a
201 single PCR band product (568 bp) while females showed two PCR band products (568 bp and
202 340 bp).

203 To investigate if handling time, body condition, BMI and active PM influenced the
204 physiological parameters measured, we performed a generalized linear model (GLZ) analysis.
205 Heterophils, lymphocytes, H/L ratio, and glucose were set as dependent variables, the main
206 effect of season (nonbreeding and breeding), sex (female and male), handling time, body
207 condition, BMI and active PM as covariates. We selected the distribution models and link
208 function with the best fit using the Akaike information criterion for small samples (AICc;
209 Akaike 1973, Hurvich and Tsai 1989). All analyses were performed using SPSS 15.0 and
210 significance is reported using an alpha of 0.05.

211

212 **Ethical consideration**

213 Given the ethical and social responsibility that involves the scientific and
214 technological activity, this study neither committed nor affected to human rights, nor did it
215 cause any harm to the environment, animals and / or future generations. The protocol for
216 capture and sampling birds were ethically reviewed and approved. Secretaría de Ambiente y

10

217 Desarrollo Sustentable de Río Negro (Provincia de Río Negro, Argentina): Exp N°011301-
218 COD-2008, Ley MN°2669, Res-219. Dirección de Flora y Fauna (Provincia de Chubut,
219 Argentina): Exp N°08/2016-DfyFs-MP Ley I N°566. Subsecretaría de Conservación de Áreas
220 Protegidas de Chubut (Provincia de Chubut, Argentina): Exp N°00396/2016-MT, Disp N°
221 020/16, Disp N°14/17 SsCyAP. Objectives, material and methods were examined and
222 approved by National Scientific and Technical Research Council, Argentina (CONICET): Res
223 N°2020-1910 APN-DIR#CONICET. This study was carried out considering the Nuremberg
224 Code, the Helsinki Declaration and its amendments, and Universal Declaration on Human
225 Genome and Human Rights adopted by the General Conference of UNESCO, 11/11/1997.
226 This study followed the guidelines on the care and use of wildlife (Canadian Council on
227 Animal Care, 2003 ISBN: 0-919087-39-6) and this study did not compromise any rare,
228 endangered or in extinction animal species. The authors confirm that the ethical policies of
229 the journal, have been adhered to.

230

231 Results

232 We captured 52 (25 females and 27 males) individuals during the nonbreeding season
233 and 51 (33 females and 18 males) individuals during the breeding season. During breeding,
234 none of the variables measured showed statistical differences between years (Kruskal-Wallis
235 test, $P > 0.05$) and thus, we pooled data to perform further analyses. Values of physiological
236 parameters obtained for female and male TBPLs during nonbreeding and breeding are
237 presented in table 1. The BMI registered for TBPLs during nonbreeding is also presented in
238 table 1. Size-corrected body mass was similar for both sexes and did not change significantly
239 between seasons (all $P > 0.05$) therefore, we showed body mass values instead residuals in
240 table 1.

241 The majority of TBPLs during nonbreeding were not active in primary feather
242 moult ($N = 46$). They had new primaries, but 5 individuals were finishing the moult of the
243 most external feather with $> 90\%$ of a total length of a new one (2 females, 3 males). Most
244 birds instead were in active pre-alternate body molt ($N = 45$), with only 6 non-moulting (4
245 females, 2 males).

246 Active body moult was similar in males and females, and was unrelated to
247 physiological parameters, including BMI and body mass or body condition index (all test $P >$
248 0.05). Breeding status and sex, as well as their interactions were non- significant factors on
249 body mass and body condition (all test $P > 0.05$).

250 Glucose values were significantly higher during nonbreeding ($W = 4.8$, $P = 0.02$),
251 regardless of the sex of the birds ($P = 0.44$) (Fig. 2). Lymphocytes also showed higher values
252 during nonbreeding ($W = 4.5$, $P = 0.03$), but they were not related to sex ($P = 0.50$) (Fig. 2).
253 No significant differences were observed for heterophils and H/L between both seasons and
254 between sexes (all $P > 0.05$).

255 The handling time did not affect any parameter studied neither between both seasons,
256 nor between sexes, nor the interaction between them (all test $P > 0.05$). No correlation
257 between corticosterone level and handling time was found (Spearman, $R = 0.125$, $P = 0.52$).

258 The baseline corticosterone levels obtained for breeding TBPLs ranged from 3.23
259 ng/mL to 13.1 ng/mL (Table 1). Values of corticosterone showed no differences between
260 sexes ($P = 0.77$). Additionally, corticosterone was not correlated with either glucose or H/L
261 (glucose $R = -0.114$, $P = 0.564$, H/L $R = 0.114$ $P = 0.565$).

262

263 Discussion

264 Values of physiological parameters were similar to those reported for many species of
265 birds (Campbell 1995). Glucose was in the range of values for birds in general (200-450

mg/dL, Sakas 2002) and for shorebirds in Patagonia (155-420 mg/dL, D'Amico *et al.* 2016). While we expected that TBPLs during breeding should have the highest stress-related values, the results tend to show higher stress levels among nonbreeding individuals, with significantly higher values in glucose and lymphocytes (Table 1) (Fig. 2). The latter is the most abundant leukocyte type in birds (Campbell 1995), including shorebirds (e.g., Buehler *et al.* 2008, D'Amico *et al.* 2010, 2017).

Differences in glucose levels must be interpreted cautiously as this parameter could be sometimes ambiguous as an indicator of stress (Lill 2011). Some authors reported that glucose represents an important fuel for muscle fibres used for birds to flight (Yap *et al.* 2017), others have shown that glucose decreased in response to exercise (Gerson and Guglielmo 2013) and still others have suggested that glucose levels have a limited applicability in studies of assessing physiological quality in waterbirds (Minias 2014).

Stored glucose (as glycogen) is accessed quickly, thus it is one of the most important carbohydrate energy sources during demanding activities such as moult (Minias 2014) when plasma concentrations can change significantly (Brown 1996, Lill 2011, Podlaszczuk *et al.* 2017). For instance, glucose of Red knots in the onset and middle of primary moult in Tierra del Fuego was higher than glucose of body moulting Red Knots in Bahía de San Antonio one month before migration (D'Amico *et al.* 2010). Nonbreeding TBPLs had finished flight feathers moult and were in moderate body moulting, but glucose was not dependent on BMI, nor in active body moult. Together with the fact that other TBPLs sampled in similar dates of previous years showed lower glucose than this study (mean 260 ± 8.2 mg/dl) (D'Amico *et al.* 2016), we concluded that body moulting is not explaining higher glucose values in nonbreeding TBPLs.

Since glucose is sensitive to recent feeding and nonbreeding TBPLs congregate at their high tide roosts after feeding, it is possible that we caught birds with higher glucose

values than fasting birds. However not all birds always behave in the same way, some begin to roost long before the tide covers the feeding areas, while others keep feeding until the intertidal is covered by the water (PMG, pers. obs.). This is reflected in their higher coefficient of variation relative to incubating TBPLs (Table 1). Although individuals could be facing stressors associated with feeding activity reducing body mass (e.g., predator activity, inclement weather or variation in food availability; Ricklefs and Wikelski 2002, Wingfield and Kitaysky 2002, Nol 2019), the body condition was similar during both seasons and for both sexes (Table 1). Therefore, differences found in glucose are likely related to recent feeding in nonbreeding birds compared with fasting during incubation.

Feeding and resting sites of nonbreeding TBPLs are usually shared with Nearctic shorebird species (i.e., White-rumped sandpipers and Red knots, D'Amico *et al.* 2004) and conspecific flocks (D'Amico *et al.* 2016). This gregarious behaviour in comparison with the isolated breeding pairs, increases the risk of acquiring parasites and /or pathogens due to the proximity of individuals (Côté and Poulin 1995, D'Amico and Barbosa 2011) thus inducing a response of the immune system reflected in the higher value of lymphocytes observed (Table 1 and Fig. 2).

The mean H/L was less than 1 during both nonbreeding and breeding for females and males (Table 1) suggesting that birds were not exhibiting high levels of physiological stress during either period. Other studies, for example in gulls, reported values of H/L about 0.6 in normal individuals compared to 2.9 in birds that were affected by stressors such as oiling, emaciation, injury, or parasite infection (Averbeck 1992).

Some migratory shorebird species have shown differences in haematological and biochemical parameters between sexes (e.g., Tsioura *et al.* 1999, Landy-Ciannelli *et al.* 2002, D'Amico *et al.* 2017, Valdebenito *et al.* 2021). However, for TBPLs results showed no differences between sexes neither during nonbreeding nor breeding (Fig. 2).

We were only able to measure corticosterone during the breeding season and found no differences between males and females in their baseline levels (Table 1). It may be hypothesized that breeding is more stressful for females, but as this species exhibits biparental care, perhaps differences between sexes should not be predicted at this stage of incubation. To our knowledge, these are the first measurements of corticosterone reported for this species, and for any shorebird species from northern Patagonia, Argentina. TBPLs showed an overall corticosterone range of 1.52-13.1 ng/mL, which is lower if compared, as an example, to another shorebird arriving on their breeding grounds (58 ng/mL - Red Knot *Calidris canutus islandica*, Linnaeus, 1758; Reneerkens *et al.* 2002). Additionally, the Kentish plover (*Charadrius alexandrinus*, Linnaeus, 1758) had slightly higher baseline corticosterone during chick rearing (~10 and 70 ng/mL - Kosztolányi *et al.* 2012).

As our data suggest that TBPLs undergo energetically demanding physiological changes during the nonbreeding stage, the idea that conservation of the environmental resources at northern Patagonia locations may be more important than previously thought. While disturbances at breeding areas is of concern, and often the focus of disturbance studies, nonbreeding habitats play a critical role for shorebirds, mainly considering that annual survival of plover populations is reported as lowest when degradation and/or human disturbance is high at nonbreeding sites (Gibson *et al.* 2018, Nol 2019).

335 **Acknowledgments:** We thank all people that helped us in the field, especially
336 Fundación Inalafquen and Guardias Ambientales of Río Negro during nonbreeding and L.
337 Silvetti, D. Jordan, N. Térorde, and members of Laboratory of the Group of Applied
338 Ecophysiology (GEA) of the Centre for Studies of Marine Systems (CESIMAR), during
339 breeding. We also thank Ignacio T. Moore, Virginia Tech University, for assistance with
340 hormone analyses.

341 **Competing interest statement:** The authors declare there are no competing interests.

342 **Contributors' statement:** VLD, PMG and MB conceived the idea and design. VLD
343 supervised research, formulated question and hypothesis. VLD, GDH, BW, PMG and MB
344 organized fieldwork, collected data and conducted the lab analyses. VLD wrote the paper and
345 BW substantially edited the paper, adding significant suggestions for improvement. VLD,
346 GDH, MB and PMG analysed the data. All authors contributed to revisions of the manuscript
347 drafts. VLD, BW, GDH, PMG and MB contributed substantial materials, resources, and
348 funding for field and laboratory work.

349 **Funding statement:** This contribution was supported by PIP 11220150100961 CO, PICT
350 2018-02178 to MB and VLD, IWSG 2016 to GDH and VD, IDEA Wild and Ornithological
351 Exchange to GDH.

352

References

- Akaike, H. 1973. Maximum likelihood identification of Gaussian autoregressive moving average models. *Biometrika* 60: 255-265.
- Alerstam, T. and Lindström, Å. 1990. Optimal Bird Migration: The Relative Importance of Time, Energy, and Safety. *In: Bird Migration*. Edited by Gwinner, E. Springer, Berlin, Heidelberg. pp 331-351.
- 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.
- Baker, A. J., González, P. M., Piersma, T., Minton, C. D. T., Wilson, J. R., Sitters, H., Graham, D., Jessop, R., Collins, P., de Goeij, P., Peck, M. K., Lini, R., Bala, L., Pagnoni, G., Vila, A., Bremer, E., Bastida, R., Ieno, E., Blanco, D., de Lima S. do Nascimento, I., Scherer, S. S., Schneider, M. P., Silva, A., and Rodrigues, A. A. E. 1998. Northbound migration of Red Knots *Calidris canutus rufa* in Argentina and Brazil: report on results obtained by an international expedition in March-April 1997. *Wader Study Group Bull.* **88**: 64-75.
- Bonerman, T. E, Rose, E. T. and Simons, T. R. 2016. Off-road vehicles affect nesting behaviour and reproductive success of American Oystercatchers *Haematopus palliatus*. *Ibis* **158**: 261-278.
- Brown, M. 1996. Assessing Body Condition in Birds. *In: Current Ornithology* 3rd chapter Vol 13. Edited by Nolan, V. and Ketterson, E. D. Plenum Press, New York. pp 67-135.
- Buehler, D. M., Bhola, N., Barjaktarov, D., Goymann, W., Schwabl, I., Tieleman, I. B., and Piersma, T. 2008. Constitutive immune function responds more slowly to handling Stress than corticosterone in a shorebird. *Physiol. Biochem. Zool.* **81**: 673-681.

- 377 Campbell, T. W. 1995. Avian Hematology and Cytology, 2nd edition. Iowa State
378 University Press, Ames, Iowa.
- 379 Carey, C. 2005. How physiological methods and concepts can be useful in conservation
380 biology. *Integr. Comp. Physiol.* **45**: 4-11.
- 381 Côté, I. M. and Poulin, R. 1995. Parasitism and group size in social animals: A meta-analysis.
382 *Behav. Ecol.* **6**: 159-165.
- 383 Cockrem, J. F. 2007. Stress, corticosterone responses and avian personalities. *J. Ornithol.*
384 **148**: S169–S178.
- 385 Cooke, S. J., Sack, L., Franklin, C. E., Farrell, A. P., Beardall, J., Wikelski, M., Chown, S. L.
386 2013. What is conservation physiology? Perspectives on an increasingly integrated and
387 essential science. *Conserv. Physiol.* **1**: cot001.
- 388 Dhabhar, F. S., Miller, A. H., McEwen, B. S., and Spencer, R. L. 1996. Stress- induced
389 changes in blood leukocyte distribution: Role of adrenal steroid hormones. *J. Immunol.*
390 **157**: 1638–1644.
- 391 D’Amico, V. L., Hernández, M. A. and Bala, L. O. 2004. Selección de presas en relación con
392 las estrategias de forrajeo de aves migratorias en Península Valdés, Argentina. *Ornitol.*
393 *Neotrop.* **15**: 357-364.
- 394 D’Amico, V. L., Bertellotti, M., Baker, A. J. and González P. M. 2010. Hematological and
395 plasma biochemistry values for endangered red knots (*Calidris canutus rufa*) at
396 wintering and migratory sites in Argentina. *J. Wildl. Dis.* **46**: 644-648.
- 397 D’Amico, V. L. and Barbosa, A. 2011. Flocking behaviour does not favour high chewing lice
398 load in shorebirds. *Ardeola* **58**: 49-56.
- 399 D’Amico, V. L., Hevia, G. D and Bertellotti, M. 2016. Blood parameters of migratory
400 shorebirds at Peninsula Valdes, Patagonia. *Wader Study* **123**: 190-194.

- 401 D'Amico, V. L., Palacios, M. G., Baker, A. J., González, P. M., Madrid, E. and Bertellotti,
402 M. 2017. Physiological parameters and their response to handling stress in a neotropical
403 migratory shorebird during the nonbreeding season. *J. Wildl. Dis.* **53**: 437-446.
- 404 Davies S., Rodriguez, N. S., Sweazea, K. L. and Deviche, P. 2013. The effect of acute stress
405 and long-term corticosteroid administration on plasma metabolites in an urban and
406 desert songbird. *Physiol. Biochem. Zool.* **86**: 47-60.
- 407 Davis A. K., and Maney, D. L. 2018. The use of glucocorticoid hormones or leucocyte
408 profiles to measure stress in vertebrates: What's the difference? *Methods Ecol. Evol.* **9**:
409 1556-1568.
- 410 Davis, A. K., Maney, D. L. and Maerz, J. C. 2008. The use of leukocyte profiles to measure
411 stress in vertebrates: a review for ecologists. *Funct. Ecol.* **22**: 760-77.
- 412 Deikumah, J. P., McAlpine, C. A. and Maron, M. 2015. Matrix intensification affects body
413 and physiological condition of tropical forest-dependent passerines. *PloS ONE* **10**:
414 e0128521.
- 415 Duval F., González F. and Rabia H. 2010. Neurobiology of stress. *Rev. Chil. Neuro-Psiquiatr.*
416 **48**: 307-318.
- 417 Genovese, K. J., He, H., Swaggerty, C. L. and Kogut, M. H. 2013. The avian heterophil. *Dev.*
418 *Comp. Immunol.* **41**: 334-340.
- 419 Gerson, A. R., & Guglielmo, C. G. 2013. Energetics and metabolite profiles during early
420 flight in American robins (*Turdus migratorius*). *J. Comp. Physiol. B* **183**: 983-991.
- 421 Gibson, D., Chaplin, M. K., Hunt, K. L. et al. 2018. Impacts of anthropogenic disturbance on
422 body condition, survival, and site fidelity of nonbreeding Piping Plovers. *The Condor*
423 **120**: 566-580.
- 424 Hurvich, C. M. and Tsai, C. L. 1989. Regression and time series model selection in small
425 samples. *Biometrika* **76**: 297-307.

- 426 Kasprzyk, M. and Harrington, B. 1989. Manual de campo para el estudio de playeros.
427 Manomet Bird Observatory, Massachusetts, USA.
- 428 Kitayskya, A. S., Kitaiskaia, E. V., Piatt, J. F. and Wingfield, J.C. 2002. Benefits and costs of
429 increased levels of corticosterone in seabird chicks. *Horm. Behav.* **43**: 140-149.
- 430 Kosztolányi, A., Küpper, C., Chastel, O., Parenteau, C., Yilmaz, K. T., Miklósi, A., Szekely,
431 T. and Lendvai, A. Z. 2012. Prolactin stress response does not predict brood desertion
432 in a polyandrous shorebird. *Horm. Behav.* **61**: 734-740.
- 433 Kunz T. H and Orrell, K. S. 2004. Energy Costs of Reproduction. Edited by Cutler J.
434 Cleveland Encyclopedia of Energy, Elsevier, ISBN 9780121764807,
435 <https://doi.org/10.1016/B0-12-176480-X/00061-9>. pp 423-442.
- 436 Landys-Ciannelli, M. M., Jukema, J. and Piersma, T. 2002. Blood parameter changes during
437 stopover in a long-distance migratory shorebird, the bar-tailed godwit *Limosa lapponica*
438 *taymyrensis*. *J. Avian Biol.* **33**: 451-455.
- 439 Lill, A. (2011). Sources of variation in blood glucose concentrations of free-living birds.
440 *Avian Biol. Res.* **4**: 78-86.
- 441 Lindström, A., Visser, G. H. and Daan, S. 1993. The energetic cost of feather synthesis in
442 proportional to basal metabolic rate. *Physiol. Zool.* **66**: 490-510.
- 443 Minias, P. 2014. High glucose concentrations are associated with symptoms of mild anaemia
444 in Whiskered Terns: consequences for assessing physiological quality in birds. *J.*
445 *Ornithol.* **155**: 1067-1070.
- 446 Moreno, J., Sanz, J., Merino, S. and Arriero, E. 2001. Daily energy expenditure and cell-
447 mediated immunity in pied flycatchers while feeding nestlings: interaction with moult.
448 *Oecologia* **129**: 492-497.
- 449 Murphy, M. E. 1996. Energetics and nutrition of molt. *In*: Avian energetics and nutritional
450 ecology. Edited by Carey C. New York: Plenum Press. pp 158–198.

- 451 Newton, I. (1966). The moult of the Bullfinch *Pyrrhula pyrrhula*. *Ibis* **108**: 41-67.
- 452 Newton, I. 1968. The temperatures, weights and body composition of moulting bullfinches.
- 453 *Condor* **70**: 323-332.
- 454 Nol, E. 2019. Nonbreeding Ecology. *In*: The Population Ecology and Conservation of
- 455 *Charadrius Plovers*. Edited by Colwell, M. A. and Haig, S. Press, Boca Raton, FL. pp:
- 456 185-215.
- 457 Palacios, M. G., D'Amico, V. L. and Bertellotti, M. 2018. Ecotourism effects on health and
- 458 immunity of Magellanic penguins at reproductive colonies with disparate touristic
- 459 regimes and population trends. *Conserv. Physiol.* **6**: 1-13.
- 460 Piersma, T., Reneerkens, J. and Ramenofsky, M. 2000. Baseline Corticosterone Peaks in
- 461 Shorebirds with Maximal Energy Stores for Migration: A General Preparatory
- 462 Mechanism for Rapid Behavioral and Metabolic Transitions? *Gen. Comp. Endocrinol.*
- 463 **120**: 118-126.
- 464 Podlasczuk, P., Włodarczyk, R., Janiszewski, T., Kaczmarek, K. and Minias, P. 2017. When
- 465 moult overlaps migration: moult-related changes in plasma biochemistry of migrating
- 466 common snipe. *PeerJ* **5**: e3057.
- 467 Pruner, R. A. Friel, M. J. and Zimmerman, J. A. 2011. Interpreting the influence of habitat
- 468 management actions on shorebird nesting activity at coastal state parks in the Florida
- 469 Panhandle. 4 2010-11 study final report. Florida Department of Environmental
- 470 Protection, Florida Park Service, Panama City, FL.
- 471 Reneerkens, J., Morrison, R. I. G., Ramenofsky, M., Piersma, T. and Windfield, J. C. 2002.
- 472 Baseline and stress-induced levels of corticosterone during different life cycle substages
- 473 in a shorebird on the High Arctic breeding grounds. *Physiol. Biochem. Zool.* **75**: 200-
- 474 208.

- 475 Ramage-Healey, L. and Romero, M. L. 2001. Corticosterone and insulin interact to
476 regulate glucose and triglyceride levels during stress in a bird. *Am. J. Physiol. Regul.*
477 *Integr. Comp. Physiol.* **281**: R994–R1003.
- 478 Ricklefs, R. E and Wikelski, M. 2002 The physiology/life-history nexus. *Trends Ecol Evol.*
479 **17**: 462-468.
- 480 Romero, L. M. and Romero, R. C. 2002. Corticosterone responses in wild birds: the
481 importance of rapid initial sampling. *Condor* **104**: 29-135.
- 482 Romero, L. M. and Reed, J. M. 2005. Collecting baseline corticosterone samples in the field:
483 is under 3 min good enough? *Comp. Biochem. Physiol. A* **140**: 73-79.
- 484 Sakas, P. S. 2002. *Essentials of Avian Medicine: A Practitioner's Guide* 2nd edition. AAHA
485 Press.
- 486 Silverin, B. 1998. Behavioural and hormonal responses of the pied flycatcher to
487 environmental stressors. *Anim. Behav.* **55**: 1411-1420.
- 488 Spooner, F. E. B., Pearson, R. G. and Freeman, R. 2018. Rapid warming is associated with
489 population decline among terrestrial birds and mammals globally. *Glob. Change Biol.*
490 **24**: 2521-2531.
- 491 Stenzel, L. E. and Page, G. W. 2019. Breeding Biology of Charadrius Plovers. *In*: The
492 Population Ecology and Conservation of Charadrius Plovers. Edited by Colwell, M. A.
493 and Haig, S. Press, Boca Raton, FL. pp: 91-125.
- 494 Tsipoura, N., Scanes, C. G. and Burger, J. 1999. Corticosterone and growth hormone levels in
495 shorebirds during spring and fall migration stopover. *J. Exp. Zool.* **284**: 645-651.
- 496 Valdebenito, J.O., Halimubieke, N. and Lendvai, A. Z., et al. 2021. Seasonal variation in sex
497 specific immunity in wild birds. *Sci. Reps.* 11:1349
- 498 van der Velde M., Haddrath, O., Verkuil, Y., Baker, A. J. and Piersma, T. 2017. New primers
499 for molecular sex identification of waders. *Wader Study* **124**: 147-151.

- 500 Walker, B. G., Boersma, P. D. and Wingfield, J. C. 2006. Habituation of adult Magellanic
501 penguins to human visitation as expressed through behavior and corticosterone
502 secretion. *Conserv. Biol.* **20**: 146-154.
- 503 Wetlands International. 2018. Waterbird population estimates. Available from
504 wpe.wetlands.org. Accessed October 2018.
- 505 Whittow, G. C. 2000. Sturkie's avian physiology. Academic Press, San Diego
- 506 Wingfield, J. C. and Kitaysky, A. S. 2002. Endocrine responses to unpredictable
507 environmental events: stress or anti-stress hormones. *Integr. Comp. Biol.* **42**: 600-609.
- 508 Wingfield, J. C., Schwabl, H. and Mattocks, P. W. Jr. 1990. Endocrine mechanisms of
509 migration. *In*: Bird migration: physiology and ecophysiology. Edited by Gwinner, E.
510 Springer-Verlag, Berlin. pp 232-256.
- 511 Wingfield, J. C., and Farner, D. S. 1975. The determination of five steroids in avian plasma
512 by radioimmunoassay and competitive protein-binding. *Steroids* **26**: 311-327.
- 513 Yap, K. N., Kim, O. R., Harris, K. C. and Williams, T. D. P. 2017. Physiological effects of
514 increased foraging effort in a small passerine. *J. Exp. Biol.* **220**: 4282-4291.
- 515 Wiersma, P., Kirwan, G. M. and Boesman, P. F. D. 2020. Two-banded Plover (*Charadrius*
516 *falklandicus*), version 1.0. *In*: Birds of the World. Edited by del Hoyo, J., Elliott, A.,
517 Sargatal, J., Christie, D. A. and de Juana E. Cornell Lab of Ornithology, Ithaca, NY,
518 USA. <https://doi.org/10.2173/bow.twbplo1.01>
519

Table 1. Values of glucose (GLU), heterophils (H), lymphocytes (L), heterophil/lymphocyte (H/L) measured for females and males Two-banded plovers (*Charadrius falklandicus*) during both nonbreeding (A) and breeding (B). Body mass, wing and bill lengths and the percentage of coefficient of variation (CV) for all measures are also showed. For individuals during nonbreeding, the body moult intensity (BMI) is presented. For individuals during breeding values of corticosterone is presented.

A- Nonbreeding														
Female (N = 25)								Male (N = 27)						
	Mean	SD	Median	SE	Min	Max	CV	Mean	SD	Median	SE	Min	Max	CV
GLU (mg/dL)	294	62	294	12	201	461	21	311	53	311	10	216	422	17
H %	33	10	32	2.1	19	57	31	30	12	33	2.3	6.9	46	39
L %	50	9.1	50	1.8	36	66	18	55	12	54	2.2	26	73	21
H/L	0.71	0.32	0.6	0.06	0.29	1.6	46	0.61	0.33	0.6	0.06	0.14	1.5	55
Body mass (g)	63	4.5	62	0.9	53	73	7.1	63	3.5	64	0.67	54	69	5.5
Wing (mm)	125	3.2	125	3.2	120	130	2.5	127	3.5	127	0.67	116	133	2.7
Bill (mm)	18	1	18	0.21	16	20	5.7	19	1	19	0.2	15	20	5.6
BMI	1.5	0.92	2	0.18	0	3	62	1.5	0.75	2	0.14	0	3	50

24

B- Breeding														
Female (N = 33)								Male (N = 18)						
	Mean	SD	Median	SE	Min	Max	CV	Mean	SD	Median	SE	Min	Max	CV
GLU (mg/dL)	252	29	254	5	189	310	11	252.5	30.4	248	7.18	218	343	12.1
H %	17	9.8	16	1.7	1.9	35	58	14.7	10.6	12.7	2.5	1.9	42.0	72.2
L %	44	15	44	2.6	14	75	34	45.1	16.7	41.8	3.9	20	71	36.9
H/L	0.4	0.4	0.3	0.1	0.0	1.9	86	0.4	0.5	0.2	0.1	0.0	1.8	114.2
CORT (ng/mL)	6.8*	3.4	6.1	0.79	1.5	13	49	6.6**	3.4	5.4	1.1	3.2	13.1	52.3
Body mass (g)	64	2.5	65	0.4	58	70	3.8	62.2	4	63	0.9	50	67	6.4
Wing (mm)	128	3.6	129	0.6	116	134	2.8	127.5	3.1	128.5	0.7	122	133	2.4
Bill (mm)	18	0.9	18	0.1	17	20	4.9	18.4	0.8	18.3	0.2	17	19.7	4.6

528 *N = 18

529 **N = 10

530

Figure captions

Figure 1. Sampling area at northern Patagonia (Argentina). Filled dots belong to the nonbreeding site Area Natural Protegida Bahía de San Antonio (BSA, Río Negro, province), and empty dots belong to breeding sites: Playa Blanca (PB, Península Valdés, Chubut province) and Playa Paraná (PP, Puerto Madryn, Chubut province). Base map extracted from the Argentine National Cartographic Agency of the National Geographic Institute (IGN).

Figure 2. Median, quartiles and extreme values of the physiological parameters measured: glucose (mg/dL, GLU), lymphocytes (% , L), heterophils (% , H) and H/L. The asterisk denotes significant differences between breeding (B) and non-breeding (NB) seasons.

Draft

542 **Figure 1**

543

544

545 **Figure 2**

546

547

548

549